

1     **A systematic cell size screen uncovers coupling of growth to division by the**  
2                     **p38/HOG network in *Candida albicans***

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25

26 **Abstract**

27 Cell size is a complex trait that responds to developmental and environmental cues. Quantitative  
28 analysis of the size phenome in the pathogenic yeast *Candida albicans* uncovered 195 genes that  
29 markedly altered cell size, few of which overlapped with known size genes in other yeast species.  
30 A potent size regulator specific to *C. albicans* was the conserved p38/HOG MAPK module that  
31 mediates the osmotic stress response. Basal HOG activity inhibited the SBF G1/S transcription  
32 factor complex in a stress-independent fashion to delay the G1/S transition. The HOG network  
33 also governed ribosome biogenesis through the master transcriptional regulator Sfp1. Hog1 bound  
34 to the promoters and cognate transcription factors for both the G1/S and ribosome biogenesis  
35 regulons and thereby directly linked cell growth and division. These results illuminate the  
36 evolutionary plasticity of size control and identify the HOG module as a nexus of cell cycle and  
37 growth regulation.

38

## 39 **Introduction**

40 A central and longstanding problem in cell biology is how cells maintain a uniform cell size,  
41 whether in single celled organisms or in the multitude of tissues of metazoans (1, 2). In most  
42 eukaryotes, attainment of a critical cell size appears to be necessary for commitment to cell division  
43 in late G1 phase, called Start in yeast and the Restriction Point in metazoans. This critical cell size  
44 threshold coordinates cell growth with cell division to establish a homeostatic cell size (1). The  
45 dynamic control of cell size facilitates adaptation to changing environmental conditions in  
46 microorganisms and therefore is essential to maximize fitness (3, 4). In the budding yeast  
47 *Saccharomyces cerevisiae*, the size threshold is dynamically modulated by nutrients. Pre-Start G1  
48 phase cells grown in the optimal carbon source glucose pass Start at a smaller size if shifted to  
49 glycerol, whereas cells shifted from a poor to rich nutrient source pass Start at a larger size (1, 5).  
50 Nutrient conditions similarly dictate cell size in the fission yeast *Schizosaccharomyces pombe*,  
51 although control is primarily exerted at the G2/M transition (5). In metazoans, size control is  
52 important for tissue-specific functions and organ or organism size (6). Cell size is often perturbed  
53 in human disease, for example in diabetes, tuberous sclerosis, mitochondrial disorders, aneuploid  
54 syndromes, cancer and aging (1, 7). Notably, a loss of cell size homeostasis, termed pleomorphism,  
55 correlates with poor cancer prognosis (8).

56  
57 Cell size is fundamentally dictated by the balance between cell growth and division. The analysis  
58 of small-sized mutants in yeast led to key insights into the cell cycle machinery (9-13). In all  
59 eukaryotes, cell division is controlled by the cyclin dependent kinases (CDKs), which serve to  
60 coordinate the replication and segregation of the genome (14). In *S. cerevisiae*, the G1 cyclins  
61 Cln1, Cln2 and Cln3 trigger Start, whereas the B-type cyclins Clb1-Clb6 catalyze replication and  
62 mitosis, all via activation of the same Cdc28 kinase catalytic subunit. The expression of ~200  
63 genes at the end of G1 phase, most vitally *CLN1/2*, is controlled by transcription factor complexes  
64 composed of Swi4 and Swi6 (SBF), and Mbp1 and Swi6 (MBF). Activation of SBF/MBF depends  
65 primarily on the Cln3-Cdc28 kinase, the key target of which is Whi5, an inhibitor of SBF/MBF-  
66 dependent transcription (15, 16). Another transcriptional inhibitor called Nrm1 specifically  
67 inhibits MBF after Start but does not cause a marked size phenotype under conditions of nutrient  
68 sufficiency (16). Size control in *S. pombe* is exerted through inhibition of G2/M phase CDK  
69 activity by the Wee1 kinase, which is encoded by the first size control gene discovered (9, 11).

70 Size is also partially regulated at Start in *S. pombe* through an SBF/MBF- like G1/S transcription  
71 factor complex and the Nrm1 inhibitor (17). The CDK-dependent control of G1/S transcription in  
72 metazoans is analogously mediated by the cyclin D-Rb-E2F axis (15, 18, 19).

73

74 Cell growth depends on the coordinated synthesis of protein, RNA, DNA and other  
75 macromolecules (1, 20, 21). The production of ribosomes consumes a large fraction of cellular  
76 resources and depends on an elaborate ribosome biogenesis machinery (1) that is controlled in part  
77 by the conserved TOR (Target Of Rapamycin) nutrient sensing network (6). Systematic size  
78 analysis in yeast uncovered many ribosome biogenesis (*Ribi*) genes as small size mutants, and  
79 revealed two master regulators of *Ribi* gene expression, the transcription factor Sfp1 and the AGC  
80 kinase Sch9, as the smallest mutants (22). These observations lead to the hypothesis that the rate  
81 of ribosome biogenesis is one metric that dictates cell size (23). Sfp1 and Sch9 are critical effectors  
82 of the TOR pathway and form part of a dynamic, nutrient-responsive network that controls the  
83 expression of *Ribi* and ribosomal protein (*RP*) genes (23). Sfp1 activity is controlled through its  
84 TOR-dependent nuclear localization (22-25) and is physically linked to the secretory system by its  
85 interaction with the Rab escort factor Mrs6 (24, 26). Sch9 is phosphorylated and activated by TOR,  
86 and in turn inactivates a cohort of repressors of *RP* genes called Dot6, Tod6 and Stb3 (27). The  
87 TOR network also controls size in *S. pombe* and metazoans (28).

88

89 Systematic genetic analysis has uncovered hundreds of genes that directly or indirectly affect cell  
90 size. Direct size analysis of all strains in the *S. cerevisiae* gene deletion collection uncovered a  
91 number of potent size regulators, including Whi5, Sfp1 and Sch9 (22, 29, 30), and revealed inputs  
92 into size control from ribosome biogenesis, mitochondrial function and the secretory system (23-  
93 26). Subsequent analyses of many of these size mutants at a single cell level have suggested that  
94 the critical cell size at Start may depend on growth rate in G1 phase and/or on cell size at birth (31,  
95 32). Visual screens of *S. pombe* haploid and heterozygous deletion collections for size phenotypes  
96 also revealed dozens of novel size regulators, many of which altered size in a genetically additive  
97 fashion (33, 34). A large number of genes appear to influence size in metazoan species. A large-  
98 scale RNAi screen in *Drosophila melanogaster* tissue culture cells revealed hundreds of genes as  
99 candidate size regulators, including known cell cycle regulatory proteins (35). Despite overall  
100 conservation of the central processes that control cell growth and division, many functionally

101 equivalent size regulators appear not to be conserved at the sequence level. For example, the G1/S  
102 transcriptional regulators SBF/MBF and Whi5 bear no similarity to the metazoan counterparts E2F  
103 and Rb, respectively (1). A number of TOR effectors are also poorly conserved at the sequence  
104 level, including the ribosome biogenesis transcription factors Sfp1 in yeast and Myc in metazoans  
105 (1).

106

107 *Candida albicans* is a diploid ascomycete yeast that is a prevalent commensal and opportunistic  
108 pathogen in humans. *C. albicans* is a component of the normal human flora, colonizing primarily  
109 mucosal surfaces, gastrointestinal and genitourinary tracts, and skin (36). Although most *C.*  
110 *albicans* infections entail non-life-threatening colonization of surface mucosal membranes,  
111 immunosuppressed patients can fall prey to serious infections, such as oropharyngeal candidiasis  
112 in HIV patients and newborns, and lethal systemic infections known as candidemia (37). Interest  
113 in *C. albicans* is not limited to understanding its function as a disease-causing organism, as it has  
114 an ecological niche that is obviously distinct from the classic model ascomycete *S. cerevisiae*. In  
115 this regard, *C. albicans* has served as an important evolutionary milepost with which to assess  
116 conservation of biological mechanisms, and recent evidence suggests a surprising extent of  
117 rewiring of central signalling and transcriptional networks as compared to *S. cerevisiae* (38-42).

118

119 In order to probe the conservation of the extensive size homeostasis network, we performed a  
120 quantitative genome-wide analysis of a systematic collection of gene deletion strains in *C.*  
121 *albicans*. Our results revealed an unexpected high degree of divergence between genes that affect  
122 size in *C. albicans* versus *S. cerevisiae* and uncovered previously undocumented regulatory  
123 circuits that govern critical cell size at Start in *C. albicans*. In particular, we delineate a novel  
124 stress-independent function of the p38/HOG MAPK network in coupling cell growth to cell  
125 division. Our genetic and biochemical analysis suggests that the HOG module directly interacts  
126 with central components of both the cell growth and cell division machineries in *C. albicans*. This  
127 systematic analysis thus provides insights into the general architecture and evolvability of the  
128 eukaryotic cell size control machinery.

129

## 130 **Results**

### 131 **Analysis of the cell size phenome in *C. albicans***

132 The diploid asexual lifestyle of *C. albicans* complicates the discovery of loss-of-function  
133 phenotypes because both alleles must be inactivated (43). To uncover genes required for cell size  
134 homeostasis in *C. albicans*, we therefore directly screened four systematic collections of  
135 homozygous diploid gene deletion strains that encompassed 426 transcriptional regulators (41,  
136 44), 81 kinases (40) and a general collection of 666 functionally diverse genes (45). We also  
137 screened a large set of 2360 strains from the GRACE (Gene Replacement and Conditional  
138 Expression) collection, which bear a gene deletion at one locus and an integrated tetracycline-  
139 regulated allele at the other locus (46). In total, 2630 viable mutant strains (2478 unique mutants)  
140 representing ~ 40 % of all predicted genes in *C. albicans* were individually assessed for their size  
141 distribution under conditions of exponential growth in rich medium. We additionally examined  
142 selected deletion strains of *C. albicans* orthologs of known size genes in *S. cerevisiae* (*sch9*, *pop2*,  
143 *ccr4*, *nrm1* and *CLN3/cln3*) that were not present in extant deletion collections (**Supplementary**  
144 **file 1**). Clustering of size distributions across the cumulative datasets revealed distinct subsets of  
145 both large and small mutants, relative to the majority of mutants that exhibited size distributions  
146 comparable to those of wild-type (wt) control strains (**Figure 1A-D**). Mean, median and mode cell  
147 size were estimated for each mutant strain, and mutants were classified as large or small on the  
148 basis of a stringent cut-off of a 20 % increase or decrease in size as compared to the parental strain  
149 background. This cut-off value was determined based on a benchmark set of conserved small  
150 (*sch9*, *sfp1*) and large (*swi4*, *pop2*, *ccr4*) sized mutants for which size was reduced or increased at  
151 least 20 % as compared to parental strains. Based on this criterion, we identified 195 mutants that  
152 exhibited a size defect compared to their parental strain, comprised of 104 small sized (*whi*) and  
153 91 large sized (*lge*) mutants (**Supplementary file 2 and 3**).

154  
155 Gene Ontology (GO) enrichment analysis revealed that size mutants were predominantly defective  
156 in functions related to signalling, metabolism, transcriptional regulation, growth and cell cycle  
157 control (**Figure 1–figure supplement 1A**). For example, disruption of the HOG MAPK pathway  
158 (*hog1*, *pbs2*, *ssk2*), regulators of the G2/M phase transition (*swe1*, *gin4*, *hsl1*, *dbp11*), and protein  
159 localization to cell cortex (*mlc1*, *iqg1*, *gin4*) resulted in a small cell size phenotype. Conversely,  
160 mutants defective in functions related to the G1/S phase transition (*cln3*, *swi4*, *grr1*) and  
161 cytokinesis (*kin2*, *kin3*, *sec3*, *eng1*, *sun41*) caused a large cell size phenotype. As in *S. cerevisiae*,  
162 disruption of the central SBF (Swi4-Swi6) G1/S transcription factor complex increased cell size

163 (Figure 1–figure supplement 2E), whereas mutation of the ribosome biogenesis regulators Sch9  
164 and Sfp1 reduced cell size, as did inactivation of Cbf1, the major transcriptional regulator of  
165 ribosomal protein genes in *C. albicans* and other ascomycetes (47, 48).

166

167 Disruption of many other genes also perturbed size homeostasis in *C. albicans*, including genes  
168 implicated in cell wall structure and integrity (*gsc1*, *chs1*, *crh12*, *sun41*, *kre1*, *cbk1*), amino acid  
169 biosynthesis (*ssy1*, *car2*, *aro80*, *leu3*, *stp1*, *acol*), and cellular respiration (*nuo1*, *mci4*, *cox19*,  
170 *hap2*, *hap43*), amongst many other processes (Supplementary file 3). Interestingly, 57 of the 195  
171 size mutants identified by our screen have been shown previously to be required for pathogenesis  
172 ( $p$ -value=1.23e-10). This set of genes included those with functions in transcriptional control of  
173 biofilm and invasive filament formation (*ndt80*, *efg1*, *nrg1*, *ace2*, *zcf27*) as well as known adhesion  
174 genes (*ahr1*, *efg1*). This overlap suggested that cell size homeostasis may be elemental to *C.*  
175 *albicans* fitness inside the host (Figure 1E, Figure 1–figure supplement 1A and Supplementary  
176 file 7).

177

### 178 **Limited overlap of the fungal cell size phenome across species**

179 *C. albicans* and *S. cerevisiae* represent yeast genera separated by ~70M years of evolution (49)  
180 and share the morphological trait of budding, as well as core cell cycle and growth regulatory  
181 mechanisms (50, 51). However, recent evidence has uncovered an extensive degree of rewiring of  
182 both cis-transcriptional regulatory circuits and signalling pathways across many cellular and  
183 metabolic processes between the two yeasts (38, 40, 48, 52). To assess the extent of conservation  
184 and plasticity of the size phenome between the two species, genes that affected cell size in *C.*  
185 *albicans* were compared to their corresponding orthologs in *S. cerevisiae*. Surprisingly, we found  
186 minimal overlap between homozygous size mutants in both species (Figure 2A-B). Only four  
187 small size mutants we tested were common between *C. albicans* and *S. cerevisiae*, namely the  
188 master ribosome biogenesis regulators Sfp1 and Sch9, the CDK-inhibitory kinase Swe1 and the  
189 G1/S transcriptional repressor Nrm1. Similarly, only four large size mutants were shared, namely  
190 the SBF subunits Swi4 and Swi6 and two components of the CCR-NOT transcriptional complex,  
191 Ccr4 and Pop2 (Figure 2B). Heterozygous loss of the G1 cyclin Cln3 also caused a large size  
192 phenotype (53), but this gene was not included in the deletion collections we screened. It is likely  
193 that a number of other size regulators not represented in our collections may also be shared between

194 *C. albicans* and *S. cerevisiae*, but nonetheless the limited degree of overlap in size regulators was  
195 strikingly limited considering that our collections spanned ~40 % of all *C. albicans* genes.  
196 Unexpectedly, six *C. albicans* small sized mutants (*gis2*, *ctk3*, *ssn6*, *hsl1*, *mdm20*, *sst2*) exhibited  
197 a large phenotype in *S. cerevisiae*, and conversely two *C. albicans* large mutants (*alg13* and *tif6*)  
198 caused a small cell size in *S. cerevisiae*. To confirm these disparities in the size control network  
199 independently of the particular cut-offs used to define large and small mutants in different studies,  
200 we examined the relationship between mode size of all screened *C. albicans* mutants and their  
201 counterparts in *S. cerevisiae*. Overall, no correlation was observed between the size phenomes of  
202 the two species across any of the *C. albicans* deletion collections (**Figure 2C-G**).

203

204 We also compared size genes in *C. albicans* to those previously identified in a systematic visual  
205 screen for size mutants in the *S. pombe* haploid deletion strain collection (33). For the 18 validated  
206 size regulators in this *S. pombe* screen, a total of 10 had obvious *C. albicans* orthologs in our  
207 datasets. Comparison of size phenotypes for these 10 genes revealed that only the CDK-inhibitory  
208 kinase *swe1* (*wee1* in *S. pombe*) caused an analogous small size phenotype in both species. Overall,  
209 these comparative analyses demonstrated that relatively few specific gene functions required for  
210 cell size homeostasis were conserved between *C. albicans*, *S. pombe* and *S. cerevisiae*.

211

### 212 **Novel Start regulators in *C. albicans***

213 Previous work has shown that disruption of cell growth rate is often accompanied by a small cell  
214 size phenotype, for example by mutations in *RP* or *Ribi* genes (22, 54). To identify *bona fide*  
215 negative Start regulators, as opposed to mere growth rate-associated effects, doubling times were  
216 determined for the 104 homozygous small size mutants identified in our screens (**Supplementary**  
217 **file 3**). Mutants that exhibited a greater than 10 % increase in doubling time as compared to the wt  
218 controls were enriched in functions associated with macromolecular synthesis and were removed  
219 from subsequent consideration for this study. As expected, amongst the 63 remaining candidates  
220 predicted to more directly couple growth to division (**Supplementary file 4**), we recovered three  
221 known conserved repressors of Start, namely Sfp1, Sch9 and Swe1. Strikingly, the candidate set  
222 of Start regulators in *C. albicans* contained many conserved genes that do not affect size in *S.*  
223 *cerevisiae*, including transcription factors (Hmo1, Dot6), the kinase Sok1, and components of the  
224 HOG MAPK pathway (Ssk2, Pbs2 and Hog1). Other novel potent candidate regulators of Start

225 that were unique to *C. albicans* included genes with functions related to nitrogen metabolism  
226 (Aro80, Dur35), fatty acid biosynthesis (Ino4, Asg1) and iron metabolism (Hap2, Hap43). We also  
227 observed that loss of the F-box protein Grr1 resulted in a small size, consistent with the fact that  
228 this SCF ubiquitin ligase subunit eliminates the G1 cyclin Cln3 in *C. albicans* (55). While Grr1-  
229 mediated elimination of Cln3 is conserved in *S. cerevisiae*, the *grr1* deletion does not have a small  
230 size phenotype, in part because Cln3 is redundantly targeted by the F-box protein Cdc4 in this  
231 yeast (56).

232  
233 We demonstrated the effect of six *C. albicans* size regulators on the timing of Start by assessing  
234 the correlation between size and bud emergence in a synchronous early G1 phase population of  
235 cells obtained by centrifugal elutriation. We used this assay to determine the effect of three potent  
236 novel size control mutants that conferred a small size phenotype (*ahr1*, *hog1*, *hmo1*) and, as a  
237 control, disruption of a conserved known regulator of Start (*sfp1*). Additionally, the Start transition  
238 in *C. albicans* was characterized for two large size mutants (*swi6* and *cln3*). The critical cell size  
239 of the four small sized mutants *ahr1*, *hog1*, *hmo1* and *sfp1* was markedly reduced as compared to  
240 the wt parental strain (**Figure 2H**). Conversely, as expected Start was delayed in the large sized  
241 mutants *CLN3/cln3* and *swi6*. These results demonstrate that the transcription factors Ahr1 and  
242 Hmo1, and the MAPK Hog1 are novel *bona fide* repressors of Start in *C. albicans*, and that aspects  
243 of the Start machinery appear to have diverged between *C. albicans* and *S. cerevisiae*.

244

#### 245 **Basal activity of the HOG MAPK pathway delays Start**

246 A *hog1* mutant strain had a median size that was 30 % smaller than its congeneric parental strain, at  
247 50.2 and 71.7 fL respectively (**Figure 3A** and **3C**). To ascertain that this effect was mediated at  
248 Start, we evaluated two hallmarks of Start, namely bud emergence and the onset of SBF-dependent  
249 transcription as a function of cell size in synchronous G1 phase cells obtained by elutriation. As  
250 assessed by mode size of cultures for which 25 % of cells had a visible bud, the *hog1* mutant passed  
251 Start after growth to 41 fL, whereas a parental wt control culture passed Start at a much larger size  
252 of 55 fL (**Figure 3F**). Importantly, in the same experiment, the onset of G1/S transcription was  
253 accelerated in the *hog1* strain as judged by the peak in expression of the two representative G1  
254 transcripts, *RNR1* and *PCL2* (**Figure 3G-H**). These results demonstrated that the Hog1 kinase  
255 normally acts to delay the onset of Start.

256 Other main elements of the HOG pathway, namely the MAPKK Pbs2, the MAPKKK Ssk2, the  
257 phospho-relay mediator Ypd1 and the two-component transducer Sln1, were also required for  
258 normal cell size homeostasis (**Figure 3A-C**). Disruption of the upstream negative regulators Ypd1  
259 and Sln1 caused a large size whereas mutation of the core MAPK module (Ssk2, Pbs2 and Hog1)  
260 caused a small size phenotype. As the cultures for these experiments were grown in constant  
261 normo-osmotic conditions, we inferred that the effect of the HOG module on cell size was  
262 unrelated to its canonical role in the osmotic stress response. Consistent with this interpretation,  
263 mutation of the known osmotic stress effectors of the HOG pathway in *C. albicans*, namely the  
264 glycerol biosynthetic genes *GPD1*, *GPD2* and *RHR2* (57, 58), did not cause a cell size defect  
265 (**Figure 3–figure supplement 1 and Supplementary file 1**). To address whether basal activity of  
266 the HOG MAPK module might be required for size control, we tested the effect of phosphorylation  
267 site mutants known to block signal transmission. Mutation of the activating phosphorylation sites  
268 on either Hog1 (Thr174 and Tyr176) or Pbs2 (Ser355-and Thr359) to non-phosphorylatable  
269 residues phenocopied the small size of *hog1* and *pbs2*, respectively (**Figure 3D**). This result  
270 demonstrated that a basal level of Hog1 and Pbs2 activity was required for Start repression under  
271 non-stress conditions. To examine the possible role of the HOG pathway in communicating  
272 nutrient status to the Start machinery, the effects of different carbon sources on cell size were  
273 assessed in *hog1* and wt strains. Cell size was reduced on poor carbon sources in the *hog1* strain  
274 to the same extent as the wt strain, suggesting that the HOG module was not required for carbon-  
275 source regulation of cell size (**Figure 3–figure supplement 2**). These results demonstrate that the  
276 HOG module relays a stress- and carbon source-independent signal for size control to the Start  
277 machinery in *C. albicans*.

278  
279 Previous genome-wide screens in *S. cerevisiae* failed to uncover a role for the HOG pathway in  
280 size control (22, 29-31). To confirm these results, cell size distributions of HOG pathway mutants  
281 in *S. cerevisiae* (*hog1*, *pbs2*, *ssk1*, *ssk2*, *opy2* and *sho1* strains) were assessed in rich medium. None  
282 of the *S. cerevisiae* mutants had any discernable size defect as compared to a parental wt strain  
283 (**Figure 3–figure supplement 3**).

284

285 **Hog1 acts upstream of the SBF transcription factor complex**

286 Cln3-dependent activation of the Swi4-Swi6 transcriptional complex drives G1/S progression in  
287 both *S. cerevisiae* and *C. albicans* (51, 53, 59, 60) such that *CLN3/cln3*, *swi6* and *swi4* mutants all  
288 exhibited large size and a G1 phase delay (**Figure 1–figure supplement 2E, Figure 2H and 4A**).  
289 To examine the functional relationship between the HOG pathway and these canonical Start  
290 regulators, we characterized their genetic interactions by size epistasis. We observed that the small  
291 size of a *hog1* mutant strain was partially epistatic to the large size of the heterozygous *CLN3/cln3*  
292 mutant (**Figure 4A**), suggesting that the HOG pathway may function in parallel to Cln3. Deletion  
293 of *SWI4* in the *hog1* mutant strain resulted in large size comparable to that of *swi4* mutant,  
294 suggesting that Hog1 acts upstream of Swi4 to inhibit Start (**Figure 4B**). In support of this finding,  
295 co-immunoprecipitation assays revealed that Hog1 physically interacted with Swi4 in a  
296 rapamycin-sensitive manner and that the Hog1-Swi4 interaction was insensitive to osmotic stress  
297 (**Figure 4C**). In *C. albicans*, the Nrm1 inhibitor is known to interact with the SBF complex to  
298 repress the G1/S transition (61), and consistently a *nrm1* mutant exhibited a reduced cell size  
299 (**Figure 4D**). We found that a *nrm1 hog1* double mutant had a smaller size than either of the *nrm1*  
300 or *hog1* single mutants, suggesting that Nrm1 and Hog1 act in parallel pathways to inhibit G1/S  
301 transcription (**Figure 4D**). Collectively, these genetic and biochemical results identified Hog1 as  
302 a new regulator of SBF in *C. albicans*, and suggested that Hog1 may transmit signals from the  
303 TOR growth control network to the G1/S machinery.

304

### 305 **The Ptc1 and Ptc2 phosphatases control Start via basal Hog1 activity**

306 MAPK activity is antagonized by the action of serine/threonine- (Ser/Thr) phosphatases, tyrosine  
307 (Tyr) phosphatases, and dual specificity phosphatases that are able to dephosphorylate both  
308 Ser/Thr and Tyr residues (62). In *S. cerevisiae*, after adaptation to osmotic stress, components of  
309 the HOG pathway are dephosphorylated by both protein Tyr phosphatases and type 2C Ser/Thr  
310 phosphatases (62, 63). In *C. albicans*, recent work has identified the two Tyr phosphatases Ptp2  
311 and Ptp3 as modulators of the basal activity of Hog1 (64). A prediction of the HOG-dependent  
312 size control model is that disruption of the phosphatases that modulate Hog1 basal activity should  
313 cause a large cell size. However, none of the Tyr-phosphatase single mutants *ptp1*, *ptp2* or *ptp3*,  
314 nor a *ptp2 ptp3* double mutant exhibited a noticeable cell size defect (**Figure 5B** and  
315 **Supplementary file 1**). In contrast, deletion of the type 2C Ser/Thr phosphatase Ptc2 conferred a  
316 median size of 84.9 fL, which was 24 % larger than the parental wt control size of 68 fL, while a

317 *ptc1 ptc2* double mutant strain had an even larger size of 90.5 fL (**Figure 5A**). To confirm that the  
318 large size phenotype of the *ptc* mutants was mediated directly via effects on Start, we evaluated  
319 the critical cell size of both *ptc2* and *ptc1 ptc2* mutants in elutriated G1 cells. Whereas wt control  
320 cells passed Start at 49 fL, the critical cell size of the *ptc2* and *ptc1 ptc2* mutant strains was  
321 increased by 59 % to 78 fL and 87 % to 92 fL, respectively (**Figure 5C**). To determine whether  
322 Hog1 is an effector of Ptc1 and Ptc2 at Start, we examined the epistatic relationship between the  
323 *hog1* and *ptc1 ptc2* mutations. The size of the *hog1 ptc1 ptc2* triple mutant was identical to that of  
324 *hog1* single mutant, indicating that Hog1 functions downstream of Ptc1 and Ptc2 for the control  
325 of cell size (**Figure 5D**). These data suggested that Ptc1 and Ptc2 phosphatases may modulate the  
326 basal phosphorylation state of Hog1 to govern the timing of Start onset and critical cell size.

327

### 328 **Hog1 activates ribosome biosynthetic gene transcription and inhibits G1/S transcription**

329 To explore the role of Hog1 at Start, we assessed genome-wide transcriptional profiles using  
330 custom microarrays. G1 phase cells for *hog1* mutant and wt strains were collected by centrifugal  
331 elutriation, followed by microarray analysis of extracted total RNA. Gene set enrichment analysis  
332 of transcriptional profiles (65, 66) revealed that the *hog1* strain was defective in expression of  
333 genes that function in protein translation, including members of the 48S/43S translation initiation  
334 complex, structural components of the small and large subunits of the ribosome, and tRNA-  
335 charging components (**Figure 6A** and **Supplementary file 5**). Transcription of genes that function  
336 in mitochondrial transport, the tricarboxylic acid cycle, protein degradation by the 26S proteasome  
337 and respiration were also downregulated in a *hog1* deletion. Conversely, the G1/S transcriptional  
338 program (51) was hyperactivated in a *hog1* mutant, consistent with the above results for *RNR1* and  
339 *PCL2*. These results suggested that Hog1 activates multiple processes that underpin cellular  
340 growth in addition to its role as a negative regulator of the G1/S transcriptional program.

341

342 It has been previously reported that Hog1 in *S. cerevisiae* and its ortholog p38 in humans directly  
343 bind and activate downstream transcriptional target genes (67-72). In *S. cerevisiae*, Hog1 thus  
344 associates with DNA at stress-responsive genes and is required for recruitment of general  
345 transcription factors, chromatin modifying activities and RNA Pol II (68, 71, 73, 74). However,  
346 although mechanisms of Hog1-dependent transcription have been investigated under osmotic  
347 stress conditions, the function of this kinase in normal growth conditions in the absence of stress

348 has not been explored. In order to assess whether Hog1 might directly regulate gene expression  
349 relevant to cell size control in *C. albicans*, we profiled the genome-wide localization of Hog1 in  
350 G1 phase cells obtained by centrifugal elutriation from TAP-tagged Hog1 and untagged control  
351 strains. Hog1 binding sites in the genome were determined in duplicate by chromatin  
352 immunoprecipitation and microarray analysis (ChIP-chip). These experiments revealed that  
353 Hog1<sup>TAP</sup> was significantly enriched at 276 intergenic regions and 300 ORFs when compared to  
354 the untagged control (**Supplementary file 6**). The ORF and promoter targets of Hog1 were  
355 strongly represented for translation and *Ribi* genes (**Figure 6B**), in accord with the above  
356 expression profiles. These data suggested that Hog1 may directly activate expression of the *Ribi*  
357 regulon and other translation-associated genes. The strong enrichment for Hog1 at translation and  
358 *Ribi* loci suggested that Hog1 may be required for maximal translational capacity as G1 phase cells  
359 approach Start. Consistently, we observed that a *hog1* mutant exhibited increased sensitivity to the  
360 protein translation inhibitor cycloheximide as compared to a wt strain (**Figure 6C**). These results  
361 suggested that Hog1 may directly activate ribosome biogenesis and protein translation as cells  
362 approach Start.

363

#### 364 **Hog1 is required for Sfp1-dependent gene expression and recruitment to target promoters**

365 Based on the conserved role of the Sfp1 transcription factor and the kinase Sch9 in ribosome  
366 biogenesis and cell size control in *C. albicans*, we examined genetic interactions between these  
367 factors and the HOG pathway. To identify potential epistatic interactions, we overexpressed *SCH9*  
368 or *SFP1* in a *hog1* strain. The overexpression of *SFP1* but not *SCH9* restored the *hog1* strain to a  
369 near wt cell size distribution (**Figure 7A**). In contrast, overexpression of *HOG1* in either an *sfp1*  
370 or *sch9* had no effect on the small size of either strain (data not shown). These results suggested  
371 that Sfp1 might act downstream of Hog1. Consistent with this interpretation, we found that the  
372 gene expression defects of six *Ribi* and translation genes (*RPS12*, *RPS28B*, *RPS32*, *EIF4E* and  
373 *TIF6*) in a *hog1* strain were rescued by the overexpression of *SFP1* (**Figure 7B**).

374

375 Given the apparent genetic relationship between Hog1 and Sfp1, we examined whether the two  
376 proteins physically interacted. We evaluated the interaction at endogenous levels using a  
377 chromosomal HA-tagged Sfp1 allele and polyclonal antibodies that recognize Pbs2 and Hog1.  
378 Capture of Sfp1<sup>HA</sup> from cell lysates followed by antibody detection revealed that Sfp1 interacted

379 with both Pbs2 and Hog1 (**Figure 7C**). Notably, the Sfp1 interaction with both Hog1 and Pbs2  
380 was abolished by either osmotic stress or rapamycin (**Figure 7C**). These results suggested that the  
381 timing of Start may be governed in part by modulation of the Hog1-Sfp1 interaction by stress and  
382 nutrient signals.

383

384 We then examined whether Sfp1 played an analogous role in Start control in *C. albicans* as in *S.*  
385 *cerevisiae*. As described above, an *sfp1* deletion strain was extremely small and passed Start at  
386 only 42 % of wt size (**Figure 2H and Figure 2-figure supplement 1A**). Consistently,  
387 transcriptional profiles of a strain bearing a tetracycline-regulated allele of *SFP1* demonstrated that  
388 expression of the *Ribi* regulon was partially Sfp1-dependent (**Figure 7-figure supplement 1A**).  
389 We also found that an *sfp1* strain was as sensitive to the protein translation inhibitor cycloheximide  
390 as a *hog1* strain (**Figure 7-figure supplement 1B**). These data demonstrated that Sfp1 is a  
391 transcriptional activator of *Ribi* genes and a negative regulator of Start in *C. albicans*.

392

393 The finding that both Hog1 and Sfp1 controlled the expression of *Ribi* genes, together with the  
394 finding that Hog1 acted upstream of Sfp1, led us to hypothesize that Hog1 might be required for  
395 the recruitment of Sfp1 to its target genes. To test this hypothesis, we used ChIP-qPCR to measure  
396 *in vivo* promoter occupancy of Sfp1<sup>HA</sup> at eight representative *Ribi* and RP genes that were also  
397 bound by Hog1. While Sfp1 was detected at each of these promoters in a wt strain the ChIP signals  
398 were abrogated in the *hog1* mutant strain (**Figure 7D**). From these data, we concluded that Sfp1  
399 regulates the *Ribi* regulon in a Hog1-dependent manner, and that the HOG module lies at the  
400 interface of the G1/S transcription and growth control machineries in *C. albicans*

401

## 402 **Discussion**

403 This systematic genetic analysis of size control in *C. albicans* represents the first genome-wide  
404 characterization of the mechanisms underlying regulation of growth and division in a pathogenic  
405 fungus. As is the case for other species that have been examined to date, cell size in *C. albicans* is  
406 a complex trait that depends on diverse biological processes and many hundreds of genes (22, 29-  
407 31, 33, 35). Of particular note, our screen and subsequent molecular genetic analysis uncovered a  
408 novel function for the Hog1 as a critical nexus of the growth and division machineries. The HOG  
409 module thus represents a long-sought direct linkage between cell growth and division (**Figure 8**).

## 410 **Conservation and divergence of cell size control mechanisms**

411 Inactivation of many genes that control ribosome biogenesis and protein translation in *C. albicans*  
412 resulted in a small cell size, consistent with the notion that the rate of ribosome biogenesis is a  
413 component of the critical size threshold (1, 23). Mutation of the key conserved *Ribi* regulators  
414 Sch9 and Sfp1, as well as other *Ribi* factors, thus reduced cell size in *C. albicans*. Previous studies  
415 have shown that several RP and *Ribi* trans-regulatory factors have been evolutionarily rewired in  
416 *C. albicans* compared to *S. cerevisiae* (47). Consistently, we found that deletion of *CBF1*, which  
417 encodes a master transcriptional regulator of RP genes in *C. albicans* but not *S. cerevisiae*, also  
418 resulted in a *whi* phenotype. Our analysis also unexpectedly reveals that size regulators may switch  
419 between positive and negative functions between the two yeasts. For example, mutation of the  
420 conserved transcription factor Dot6 that controls rRNA and *Ribi* expression caused a strong *whi*  
421 phenotype in *C. albicans*, in contrast to the large size phenotype conferred in *S. cerevisiae* (27).  
422 These results illustrate the evolutionary plasticity of size control mechanisms at the transcriptional  
423 level.

424  
425 In *C. albicans*, the G1/S phase cell cycle machinery remains only partially characterized but  
426 nevertheless appears to exhibit disparities compared to *S. cerevisiae*. For instance, despite  
427 conservation of SBF and Cln3 function (53, 75), the G1/S repressor Whi5 (15, 18) and the G1/S  
428 activator Bck2 (76) appear to have been lost in *C. albicans*. In *S. cerevisiae*, cells lacking *cln3* are  
429 viable and able to pass Start due to the redundant role of Bck2 (76), whereas in *C. albicans* Cln3  
430 is essential, presumably due to the absence of a Bck2 equivalent (77). Nrm1 also appears to have  
431 replaced Whi5 as it interacts physically with the SBF complex and acts genetically as a repressor  
432 of the G1/S transition in *C. albicans* (61). Consistently, we observe that *nrm1* mutant exhibits a  
433 reduced cell size as a consequence of accelerated passage through Start. In addition, the promoters  
434 of genes that display a peak of expression during the G1/S transition lack the SCB cis-regulatory  
435 element recognized by the SBF complex in *S. cerevisiae* and are instead enriched in MCB-like  
436 motifs (51).

437

## 438 **Control of Start by the HOG network**

439 Our systematic size screen uncovered a new stress-independent role of the HOG signaling network  
440 in coordinating cell growth and division. Hog1 and its metazoan counterparts, the p38 MAPK

441 family, respond to various stresses in fungi (78) and metazoans (79). In contrast to these stress-  
442 dependent functions, our data suggests that the basal level activity of the module is required to  
443 delay the G1/S transition under non-stressed homeostatic growth conditions. This function of the  
444 HOG module appears specific to *C. albicans* as compared to *S. cerevisiae*. Recent work has shown  
445 that loss of the metazoan ortholog p38 $\beta$  causes small cell and organism size in *D. melanogaster*  
446 (80). In mice, inactivation of the two Hog1 paralogs p38 $\gamma$  and p38 $\delta$  alters both cell and organ size,  
447 including in the heart and the liver (81, 82). Consistently, we have also determined that CRISPR-  
448 mediated disruption of p38 $\alpha$  or p38 $\beta$  in human cells causes a small size phenotype (data not  
449 shown). These observations suggest the role of Hog1 in size control may be more widely conserved  
450 and that *C. albicans* may be a suitable yeast model to dissect the mechanisms whereby Hog1 links  
451 the growth machinery with cell cycle commitment decision. Here, we show that the entire HOG  
452 module is required for cell size control in *C. albicans*, and demonstrate a unique role for the type  
453 2C phosphatases Ptc1 and Ptc2 in size control. In contrast, modulation of the basal activity of Hog1  
454 by the tyrosine phosphatases Ptp2 and Ptp3 in response to a reduction of TOR activity is required  
455 for the separate response of hyphal elongation (64). The mechanisms whereby the same MAPK  
456 module can specifically respond to stress, nutrient and cell size remains to be resolved.

457  
458 The signals sensed by the HOG network that couple growth to division also remain to be  
459 elucidated. Deletion of the upstream negative regulators of the HOG module, Ypd1 and Sln1,  
460 caused an increase in cell size, consistent with the negative regulation of Start by the entire HOG  
461 network. Previous studies have suggested that Sln1 histidine phosphotransferase activity is  
462 required for cell wall biogenesis in both *S. cerevisiae* and *C. albicans* (83, 84). Interestingly, we  
463 also found that disruption of the beta-1,3-glucan synthase subunit Gsc1 also caused a reduced size  
464 in *C. albicans*. Based on this result, we speculate that accumulation of cell wall materials, such as  
465 glucans, and/or cell wall mechanical properties may be sensed through basal activity of the HOG  
466 module in order to link growth rate to division. This model is analogous to that postulated in  
467 bacteria, whereby the enzymes that synthesize cell wall peptidoglycan help establish cell size  
468 control by maintaining cell width (85). In support of this notion, perturbation of the cell wall leads  
469 to a G1 phase cell cycle arrest in *S. cerevisiae* via the PKC/Slt2 signalling network (31, 86, 87).

470

471 **The HOG network lies at the nexus of growth and cell cycle control**

472 The nature of the linkage between growth to division represents a longstanding general problem  
473 in cell biology. The complex genetics of size control, reflected in the hundreds of genes that  
474 directly or indirectly affect size, confounds the notion of a simple model of size control (2). Our  
475 analysis of Hog1 interactions with the known growth and division machineries nevertheless  
476 suggests that the HOG module may directly link growth and division to establish the size threshold  
477 at Start. We demonstrate that the HOG module acts genetically upstream of Sfp1 to activate *Ribi*  
478 and translation-related genes, and specifically that Hog1 is required for the expression of many  
479 genes implicated in ribosome biogenesis and the recruitment of Sfp1 to the relevant promoters.  
480 We also demonstrate that Hog1 and its upstream kinase Pbs2 both physically interact with Sfp1,  
481 and that Hog1 localizes to many ribosome biogenesis promoters, consistent with a direct regulatory  
482 mechanism. These data suggest that basal activity of the HOG module determines ribosome  
483 biogenesis and protein synthesis rates. Strikingly, the HOG module also exhibits strong genetic  
484 interactions with the SBF transcriptional machinery. The large cell size caused by loss of SBF  
485 function is thus epistatic to the small size caused by HOG module mutations. Since Hog1  
486 physically interacts with SBF, the HOG module is ideally positioned to communicate the activity  
487 of the growth machinery to the cell cycle machinery. We speculate that under conditions of rapid  
488 growth, Hog1 and/or other components of the HOG module may be sequestered away from SBF,  
489 thereby delaying the onset of G1/S transcription. In the absence of Hog1 basal activity, this balance  
490 is set to a default state, in which SBF is activated prematurely for a given rate of growth. Taken  
491 together, these observations suggest a model whereby the HOG module directly links growth to  
492 cell cycle commitment (**Figure 8**). The control of SBF by the HOG module appears to operate in  
493 parallel to Cln3, Nrm1 and nutrient conditions, suggesting that multiple signals are integrated at  
494 the level of SBF, in order to optimize adaptation to different conditions (2). Further analysis of the  
495 functional relationships between the HOG module and the numerous other genes that affect size  
496 in *C. albicans* should provide further insights into the linkage between growth and division.

497

### 498 **Plasticity of the global size control network and organism fitness**

499 It has been argued that optimization of organism size is a dominant evolutionary force because  
500 fitness depends exquisitely on adaptation to a particular size niche (88). The strong link between  
501 size and fitness has been elegantly demonstrated through the artificial evolution of *E. coli* strains  
502 adapted to different growth rates (3). Comparison of the size phenomes of the opportunistic

503 pathogen *C. albicans* and the saprophytic yeasts *S. cerevisiae* and *S. pombe* reveals many  
504 variations in the growth and cell cycle machineries that presumably reflect the different lifestyles  
505 of these yeasts. Intriguingly, 30 % of the size regulators identified in our *C. albicans* screen have  
506 been previously identified as virulence determinants for this pathogen. This striking correlation  
507 suggests that cell size may be an important virulence trait. It is known that other fungal pathogens  
508 including *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Cryptococcus neoformans* and  
509 *Mucor circinelloides* adjust their cell size either to access specific niches in the host or to escape  
510 from host immune cells (89). For example, the novel gray cell type recently identified in *C.*  
511 *albicans* is characterized by a small size, a propensity to cause cutaneous infections, and reduced  
512 colonization of internal organs (90, 91). Conversely, the host immune system appears to be able  
513 to sense *C. albicans* size to modulate the immune response and thereby mitigate tissue damage at  
514 the site of infection (92). These lines of evidence underscore the complex relationship between  
515 cell size and pathogen fitness. The evident scope and plasticity of the global size control network  
516 provides fertile ground for adaptive mechanisms to optimize organism size and fitness.

517

518

## 519 **Materials and methods**

### 520 **Strains, mutant collections and growth conditions**

521 *C. albicans* strains were cultured at 30°C in yeast-peptone-dextrose (YPD) medium supplemented  
522 with uridine (2 % Bacto peptone, 1 % yeast extract, 2 % w/v dextrose, and 50 mg/ml uridine).  
523 Alternative carbon sources (glycerol and ethanol) were used at 2 % w/v. Wt and mutant strains  
524 used in this study together with diagnostic PCR primers are listed in **Supplementary file 8**. The  
525 kinase (40), the TF (41) and generalist (45) mutant collections used for cell size screens were  
526 acquired from the genetic stock center (<http://www.fgsc.net>). The GRACE (46) collection was  
527 purchased from the National Research Council of Canada research center (NRC Royalmount,  
528 Montreal). The transcriptional regulator (44) mutant collection was kindly provided by Dr.  
529 Dominique Sanglard (University of Lausanne). Growth assay curves were performed in triplicate  
530 in 96-well plate format using a Sunrise™ plate-reader (Tecan) at 30°C under constant agitation  
531 with OD<sub>595</sub> readings taken every 10 min for 24h. TAP and HA tags were introduced into genomic  
532 loci as previously described (93). Overexpression constructs were generated with the Clp-Act-cyc  
533 plasmid which was linearized with the *StuI* restriction enzyme for integrative transformation (94).  
534 The tetracycline repressible mutants *sfp1/pTET-SFPI* and *gpd1/pTET-GPDI* were from the

535 GRACE collection.

536

### 537 **Cell size determination**

538 Cell size distributions were analyzed on a Z2-Coulter Counter (Beckman). *C. albicans* cells were  
539 grown overnight in YPD at 30°C, diluted 1000-fold into fresh YPD and grown for 5h at 30°C to a  
540 an early log phase density of  $5 \times 10^6$  -  $10^7$  cells/ml. For the GRACE collection, all strains and the wt  
541 parental strain CAI-4 were grown overnight in YPD supplemented with the antibiotic doxycycline  
542 (40µg/ml) to achieve transcriptional repression. Deletion mutant strains were also grown to early  
543 log phase in the presence of doxycycline (40 µg/ml) to allow direct comparisons across the  
544 different strain collections. We note that high concentration of doxycycline (100 µg/ml) cause a  
545 modest small size phenotype in *C. albicans* but the screen concentration of 40 µg/ml doxycycline  
546 did not cause an alteration in cell size. 100 µl of log phase (or 10 µl of stationary phase) culture  
547 was diluted in 10 ml of Isoton II electrolyte solution, sonicated three times for 10s and the  
548 distribution measured at least 3 times on a Z2-Coulter Counter. Size distributions were normalized  
549 to cell counts in each of 256 size bins and size reported as the peak mode value for the distribution.  
550 Data analysis and clustering of size distributions were performed using custom R scripts that are  
551 available on request.

552

### 553 **Centrifugal elutriation**

554 The critical cell size at Start was determined by plotting budding index as a function of size in  
555 synchronous G1 phase fractions obtained using a JE-5.0 elutriation rotor with 40 ml chamber in a  
556 J6-Mi centrifuge (Beckman, Fullerton, CA) as described previously (95). *C. albicans* G1 phase  
557 cells were released in fresh YPD medium and fractions were harvested at an interval of 10 min to  
558 monitor bud index. For the *hog1* mutant strain, additional size fractions were collected to assess  
559 transcript levels of the *RNRI*, *PCL2* and *ACT1* as cells progressed through G1 phase at  
560 progressively larger sizes.

561

### 562 **Gene expression profiles**

563 Overnight cultures of *hog1* mutant and wt strains were diluted to an OD<sub>595</sub> of 0.1 in 1 L fresh YPD-  
564 uridine media, grown at 30°C to an OD<sub>595</sub> of 0.8 and separated into size fractions by elutriation at  
565 16°C. A total of  $10^8$  G1 phase cells were harvested, released into fresh YPD medium and grown

566 for 15 min prior to harvesting by centrifugation and stored at -80°C. Total RNA was extracted  
567 using an RNAeasy purification kit (Qiagen) and glass bead lysis in a Biospec Mini 24 bead-beater.  
568 Total RNA was eluted, assessed for integrity on an Agilent 2100 Bioanalyzer prior to cDNA  
569 labeling, microarray hybridization and analysis (96). The GSEA Pre-Ranked tool  
570 (<http://www.broadinstitute.org/gsea/>) was used to determine statistical significance of correlations  
571 between the transcriptome of the *hog1* mutant with a ranked gene list (97) or GO biological process  
572 terms as described by Sellam *et al.* (97). Data were visualized using the Cytoscape (98) and  
573 EnrichmentMap plugin (99).

574

### 575 **Promoter localization by ChIP-chip and ChIP-qPCR**

576 ChIP analyses were performed as described using a custom Agilent microarray containing 14400  
577 (8300 intergenic and 6100 intragenic) 60-mer oligonucleotides that covered all intergenic regions,  
578 ORFs and different categories of non-coding RNAs (tRNAs, snoRNAs, snRNAs and rRNA (93).  
579 A total of  $10^7$  G1 phase cells were harvested from log phase cultures by centrifugal elutriation and  
580 released into fresh YPD medium for 15 min. Arrays were scanned with a GenePix 4000B Axon  
581 scanner, and GenePix Pro software 4.1 was used for quantification of spot intensities and  
582 normalization. Hog1 genomic occupancy was determined in duplicate ChIP-chip experiments,  
583 which were averaged and thresholded using a cutoff of two standard deviations (SDs) above the  
584 mean of log ratios (giving a 2-fold enrichment cutoff). For ChIP analysis of HA-tagged Sfp1,  
585 qPCR was performed using an iQ™ 96-well PCR system for 40 amplification cycles and  
586 QuantiTect SYBR Green PCR master mix (Qiagen) using 1 ng of captured DNA and total genomic  
587 DNA extracted from the whole cell extract. The coding sequence of the *C. albicans ACT1* gene  
588 was used as a reference for background in all experiments. Values were calculated as the mean of  
589 triplicate experiments.

590

### 591 **Protein immunoprecipitation and immunoblot**

592 Cultures of epitope-tagged strains were grown to OD<sub>595</sub> of 1.0–1.5 in YPD and either treated or  
593 not with rapamycin (0.2 µg/ml) or NaCl (0.5 M) for 30 min. Cells were harvested by centrifugation  
594 and lysed by glass beads in IP150 buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM MgCl<sub>2</sub>,  
595 0.1 % Nonidet P-40) supplemented with Complete Mini protease inhibitor cocktail tablet (Roche  
596 Applied Science) and 1 mM phenylmethylsulfonyl fluoride (PMSF). 1 mg of total protein from

597 clarified lysates was incubated with 50 µl of monoclonal mouse anti-HA (12CA5) antibody (Roche  
598 Applied Science), or 20 µl anti-Pbs2 rabbit polyclonal antibody or 20µl anti-Hog1 rabbit  
599 polyclonal antibody (Santa Cruz) and captured on 40 µl Protein A-Sepharose beads (GE) at 4°C  
600 overnight. Beads were washed three times with IP150 buffer, boiled in SDS-PAGE buffer, and  
601 resolved by 4–20 % gradient SDS-PAGE. Proteins were transferred onto activated polyvinylidene  
602 difluoride (PVDF) membrane and detected by rabbit anti-HA (1:1000) antibody (QED  
603 Biosciences) and IRDye680 secondary antibody (LI-COR).

604

605

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619

620

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## 886 **Figure Legends**

887 **Figure 1. The cell size phenome of *C. albicans*.** Clustergrams of size profiles of four different  
888 systematic mutant collections of *C. albicans*: (A) a set of 81 kinases (40); (B) a set of 166  
889 transcription factors (41); (C) a general collection of 666 diverse genes (45); (D) the GRACE  
890 conditional collection of 2360 genes (46). Size distributions of each mutant (rows, represented as  
891 a heatmap) were normalized as percentage of total counts, smoothed by averaging over a seven-  
892 bin sliding window, and hierarchically clustered using a custom R script. (E) Overlap between *C.*  
893 *albicans* size and virulence phenotypes. Avirulent mutant phenotypes were obtained from CGD  
894 based on decreased competitive fitness in mice and/or reduced invasion and damage to host cells.  
895

896 **Figure 2. Comparative analysis of two systematic fungal cell size phenomes.** Overlap between  
897 small (A) and large sizes strains (B) for *C. albicans* (Ca; this study) and *S. cerevisiae* (Sc) (22).  
898 (C-G) Scatter plots of mean sizes of *S. cerevisiae* deletion strains versus their counterparts in *C.*  
899 *albicans* in different mutant collections: (C) GRACE (46); (D) general (45); (E) kinases (40); (F)  
900 transcriptional regulators (44); (G) transcription factors (41). Dashed lines indicate standard  
901 deviation. (H) Size regulators in *C. albicans* act at Start. Early G1-phase cells of different potent  
902 size mutants and wt (SN250 (45)) were isolated by centrifugal elutriation, released into fresh YPD  
903 medium and monitored for bud emergence and cell size at 10 min intervals until the entire  
904 population was composed of budded cells.

905  
906 **Figure 3. Basal activity of HOG pathway is required for normal Start onset and cell size**  
907 **homeostasis.** (A-B) Size distributions of different mutant strains for the HOG pathway in *C.*  
908 *albicans*. (C) Schematic of the canonical HOG pathway in *C. albicans* and summary of mode size  
909 for each mutant strain. The *ssk1* strain exhibited constitutive filamentation that precluded size  
910 determination (ND = not determined). (D) Mutation of the two activating phosphorylation sites on  
911 Hog1 (T174A and Y176F, termed AF) and Pbs2 (S355D and T359D, termed DD) confers a small  
912 size phenotype. (E-H) Acceleration of Start in a *hog1* strain. (E) Elutriated G1 phase daughter  
913 cells were released into fresh media and assessed for size as a function of time, (F) bud emergence  
914 as a function of size and (G-H) G1/S transcription. *RNR1* and *PCL2* transcript levels were assessed  
915 by quantitative real-time PCR and normalized to *ACT1* levels.

916

917 **Figure 4. Genetic interactions between the HOG pathway and the G1/S transcriptional**  
918 **machinery. (A)** Additive effect of *hog1* and *Cln3/cln3* mutations on cell size. The wt strain was  
919 the SN148-Arg<sup>+</sup> parental background. **(B)** A *swi4* mutation is epistatic to a *hog1* mutation for cell  
920 size. The wt was the SN250. **(C)** Co-immunoprecipitation assays for Hog1 and Swi4. Cultures  
921 were treated as indicated with rapamycin at 0.5 µg/ml and NaCl at 0.5 M for 30 min. **(D)** Additive  
922 effect of *hog1* and *nrm1* mutations on cell size. The wt strain was the SN250.

923  
924 **Figure 5. Ptc1 and Ptc2 control Start via Hog1. (A)** Size distributions of a wt strain (SN250)  
925 and *ptc1*, *ptc2* and *ptc1 ptc2* deletion mutants. **(B)** Size distributions of a wt strain (SN250), a *ptp2*  
926 single mutant, and a *ptp2 ptp3* double mutant. **(C)** Start is delayed in *ptc* mutants. Elutriated G1  
927 phase daughter cells were released into fresh media and monitored for bud emergence as a function  
928 of size. **(D)** The small cell size of a *hog1* mutant is epistatic to the large size of a *ptc1 ptc2* double  
929 mutant.

930  
931 **Figure 6. A Hog1-dependent transcriptional program in G1 phase cells. (A)** GSEA analysis  
932 of differentially expressed genes in a *hog1* mutant relative to a congenic wt strain (SN250). Cells  
933 were synchronized in G1 phase by centrifugal elutriation and released in fresh YPD medium for  
934 15 min and analyzed for gene expression profiles by DNA microarrays. Up-regulated (red circles)  
935 and down-regulated (blue circles) transcripts are shown for the indicated processes. The diameter  
936 of the circle reflects the number of modulated gene transcripts in each gene set. Known functional  
937 connections between related processes are indicated (green lines). Images were generated in  
938 Cytoscape with the Enrichment Map plug-in. **(B)** Genome-wide promoter occupancy of Hog1 in  
939 G1 phase cells. Gene categories bound by Hog1 were determined by GO term enrichment. *p*-values  
940 were calculated using hypergeometric distribution. **(C)** Growth rate and cycloheximide (CHX 200  
941 µg/ml) sensitivity of wt and *hog1* mutant strains.

942  
943 **Figure 7. Hog1-dependent recruitment of Sfp1 to promoter DNA. (A)** Size distributions of wt  
944 (wt-pAct), *hog1* (*hog1/hog1*), *SFP1*-overexpression (wt/pAct-SFP1) and *hog1 SFP1*-  
945 overexpression (*hog1/hog1/pAct-SFP1*) strains. **(B)** Increased *SFP1* dosage restores expression of  
946 representative *Ribi* and RP transcripts in a *hog1* mutant strain. Relative expression levels of the six  
947 transcripts were assessed by real-time qPCR as normalized to *ACT1*. Values are the mean from

948 two independent experiments. (C) Sfp1 interactions with Pbs2 and Hog1. Anti-HA  
949 immunoprecipitates from a strain bearing an integrated SFP1<sup>HA</sup> allele grown in the absence or  
950 presence of NaCl (0.5 M) or rapamycin (0.5 µg/ml) were probed with anti-HA, anti-Hog1 or anti-  
951 Pbs2 antibodies. (D) Reduced Sfp1 localization to *Ribi* gene promoters in a *hog1* mutant strain.  
952 Values are the mean from three independent ChIP-qPCR experiments for each indicated promoter.  
953

954 **Figure 8. Architecture of the Start machinery in *C. albicans*.** Hog1 inhibits the SBF G1/S  
955 transcription factor complex and in parallel controls Sfp1 occupancy of *Ribi* gene promoters, and  
956 thereby directly links growth and division. Basal activity of Hog1 is modulated by the  
957 phosphatases Ptc1 and Ptc2 to govern the timing of Start onset. Parallel Start pathways revealed  
958 by genetic interactions with Hog1, as well as other prominent size control genes in *C. albicans*  
959 revealed by size screens, are also indicated. Size regulators for which gene inactivation led to small  
960 and large size phenotypes are indicated in red and green, respectively.

961

## 962 **Supplementary figure legends**

963 **Figure 1. Figure supplement 1. Biological functions of size control genes in *C. albicans*.** (A)  
964 GO biological process term enrichment of all 195 size mutants identified in this study. (B) GO  
965 term enrichment of small sized mutants. (C) GO term enrichment of large sized mutants. *p*-values  
966 were calculated based on a hypergeometric distribution (see [http://go.princeton.edu/cgi-](http://go.princeton.edu/cgi-bin/GOTermFinder)  
967 [bin/GOTermFinder](http://go.princeton.edu/cgi-bin/GOTermFinder)).

968

969 **Figure 1. Figure supplement 2. Size distributions of different *C. albicans* size mutants.** The  
970 indicated mutant strains and congenic wt control strains were grown to early log phase in rich YPD  
971 medium and sized on a Z2 coulter channelizer.

972

973 **Figure 3. Figure supplement 1. Size distributions of mutants defective in glycerol**  
974 **biosynthesis.** (A) *gpd2* mutant and wt (SN250) strains were grown to early log phase in rich YPD  
975 medium prior to cell size determination. (B) The indicated wt (CAI4), *gpd1* and *rhr2* strains were  
976 grown to early log phase in rich YPD medium in the presence of doxycycline and sized on a Z2  
977 coulter channelizer.

978

979 **Figure 3. Figure supplement 2. A *hog1* deletion mutant strain adjusts cell size in response to**  
980 **different carbon sources.** Size distribution of log-phase cultures of the indicated wt and *hog1*  
981 strains (SN250 background) grown in synthetic glucose (red curve), glycerol (blue) and ethanol  
982 (black) medium.

983

984 **Figure 3. Figure supplement 3. Disruption of central components of *S. cerevisiae* HOG**  
985 **network under non-stressed normo-osmotic conditions.** Cultures of the indicated strains were  
986 grown to early log phase in rich YPD medium and sized on a Z2 coulter channelizer. Wt (BY4741)  
987 and *sfp1Δ* strains were included as controls.

988

989 **Figure 7. Figure supplement 1.** Conservation of Sfp1 function in *C. albicans* as a transcriptional  
990 activator of *Ribi* genes. (A) Network visualization of transcriptional changes in a tet-*SFP1/sfp1*  
991 conditional mutant strain. Genes expressed at reduced (blue) or elevated (red) levels after Sfp1  
992 repression were organized into functionally connected networks (green lines) based on Gene  
993 Ontology biological process terms. Node size indicates the magnitude of change. Data were  
994 visualized using Cytoscape and the Enrichment Map plug-in. (B) A pTET-*SFP1/sfp1* conditional  
995 mutant exhibited increased sensitivity to the protein translation inhibitor cycloheximide (CHX).  
996 Cells were grown in YPD at 30°C, and OD<sub>595</sub> readings were taken every 10 min.

997

## 998 **Additional files**

999 **Supplementary file 1.** Experimental size data of individual mutant strains from the four different  
1000 *C. albicans* gene deletion collections used in this study. Mean, median and mode size of each strain  
1001 are indicated.

1002

1003 **Supplementary file 2.** List of 195 size mutants in *C. albicans* that had a greater than 20 % increase  
1004 or decrease in size compared wt control strains.

1005

1006 **Supplementary file 3.** List of 195 smallest and largest deletion and conditional mutants in *C.*  
1007 *albicans* grouped according to GO biological process terms.

1008

1009 **Supplementary file 4.** Doubling-times for small size mutants in *C. albicans*.

1010 **Supplementary file 5.** Gene Set Enrichment Analysis (GSEA) for expression profiles in G1 phase  
1011 cells determined in a *hog1* strain.

1012

1013 **Supplementary file 6.** Genome-wide promoter occupancy profile of Hog1 in G1 phase cells.

1014

1015 **Supplementary file 7.** Size mutants that exhibited a known virulence defect. Data were extracted  
1016 from CGD database and reference (100).

1017

1018 **Supplementary file 8.** List of *C. albicans* strains and primers used in this study.

Figure 1

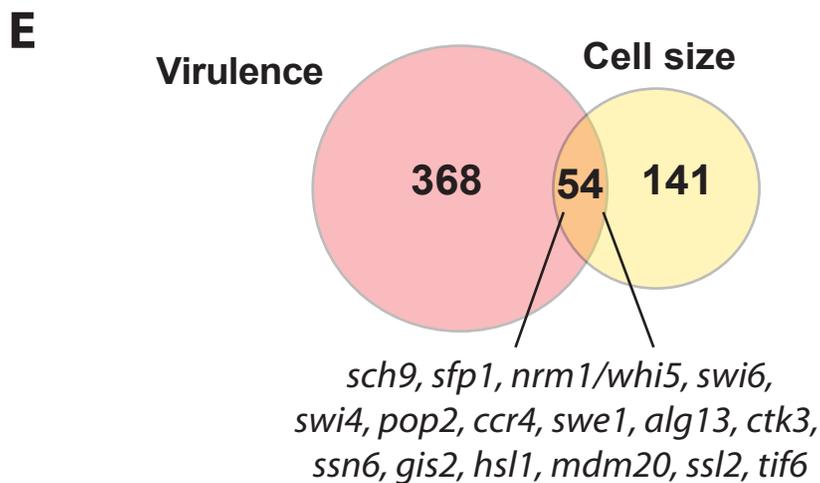
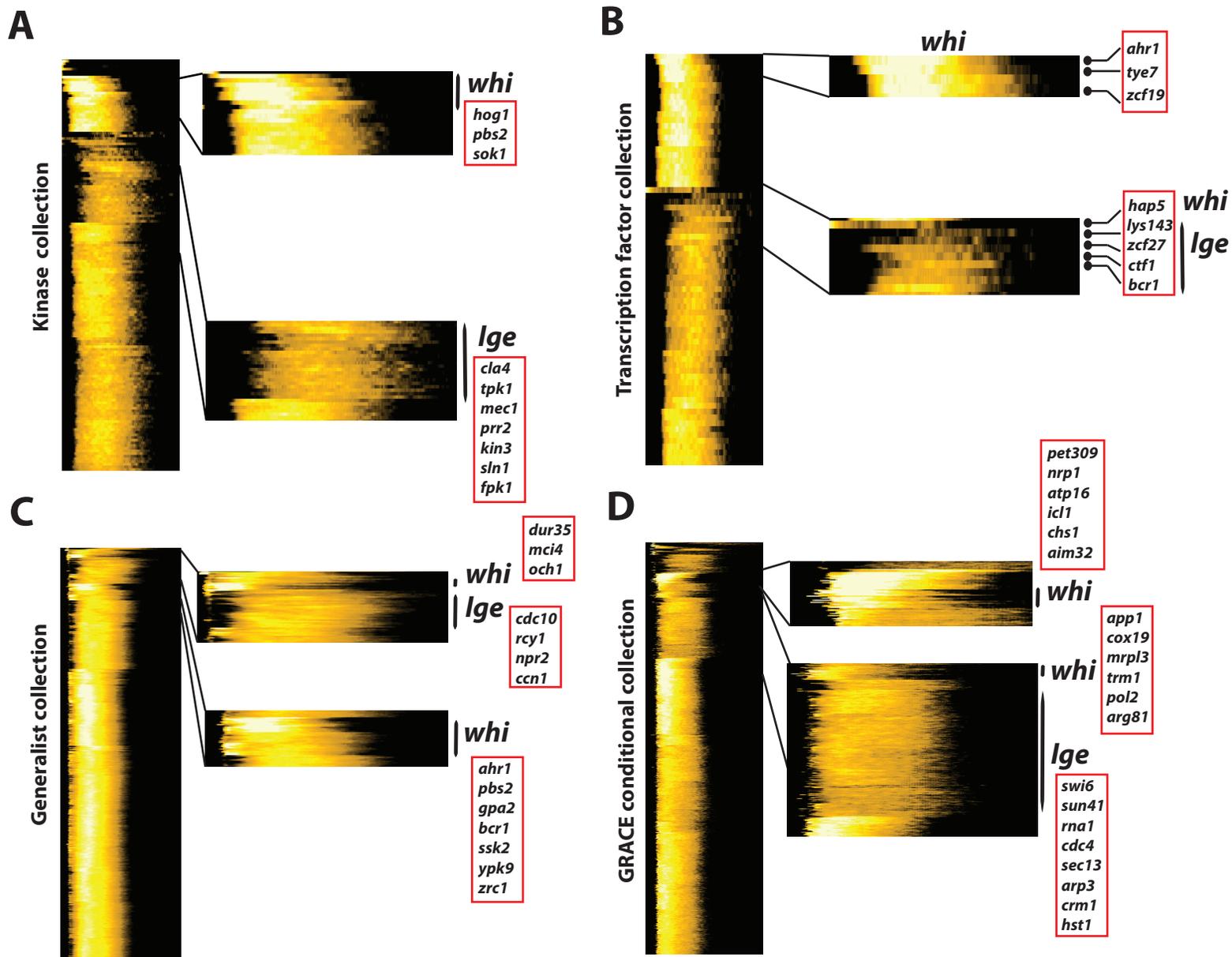


Figure 2

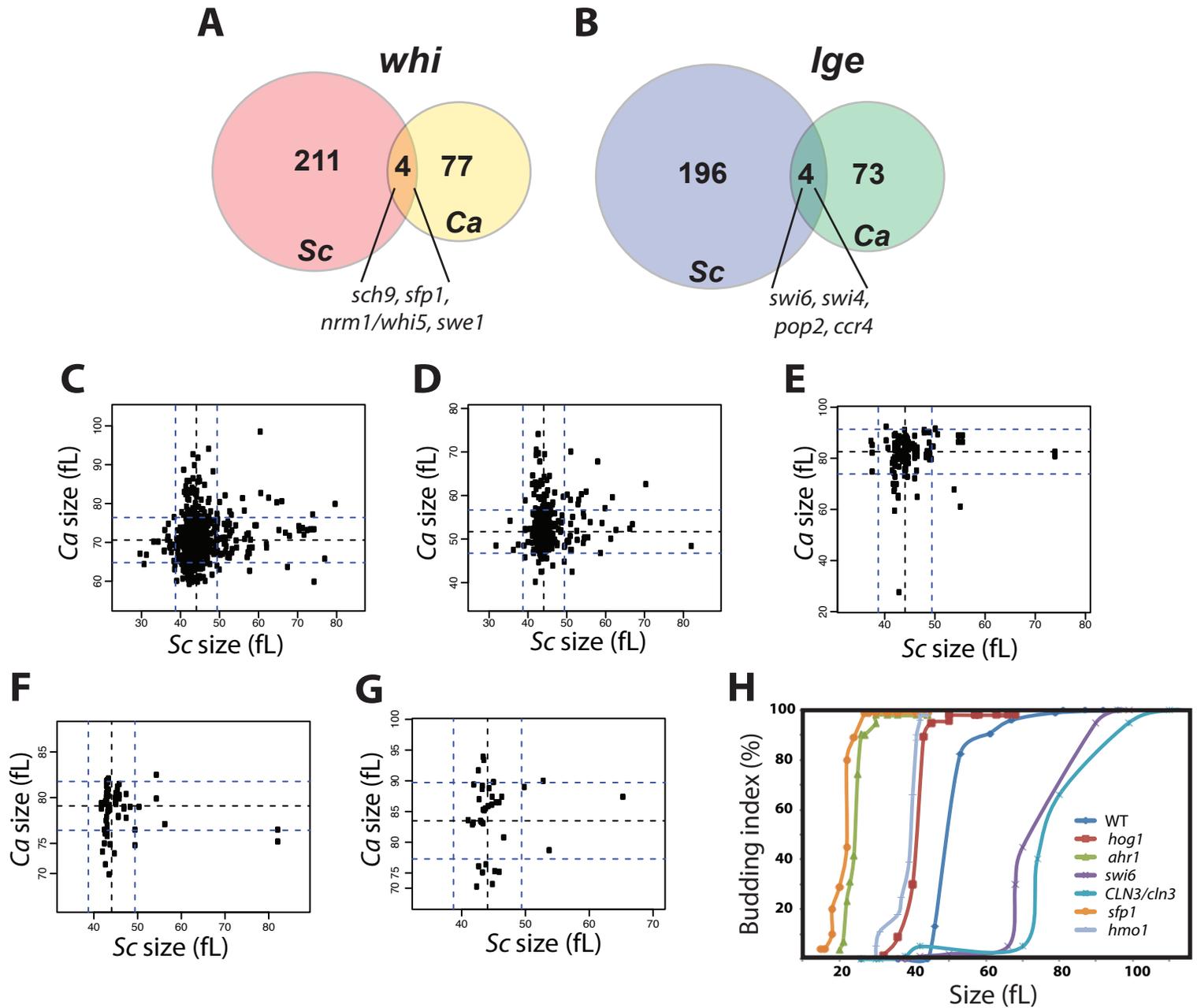


Figure 3

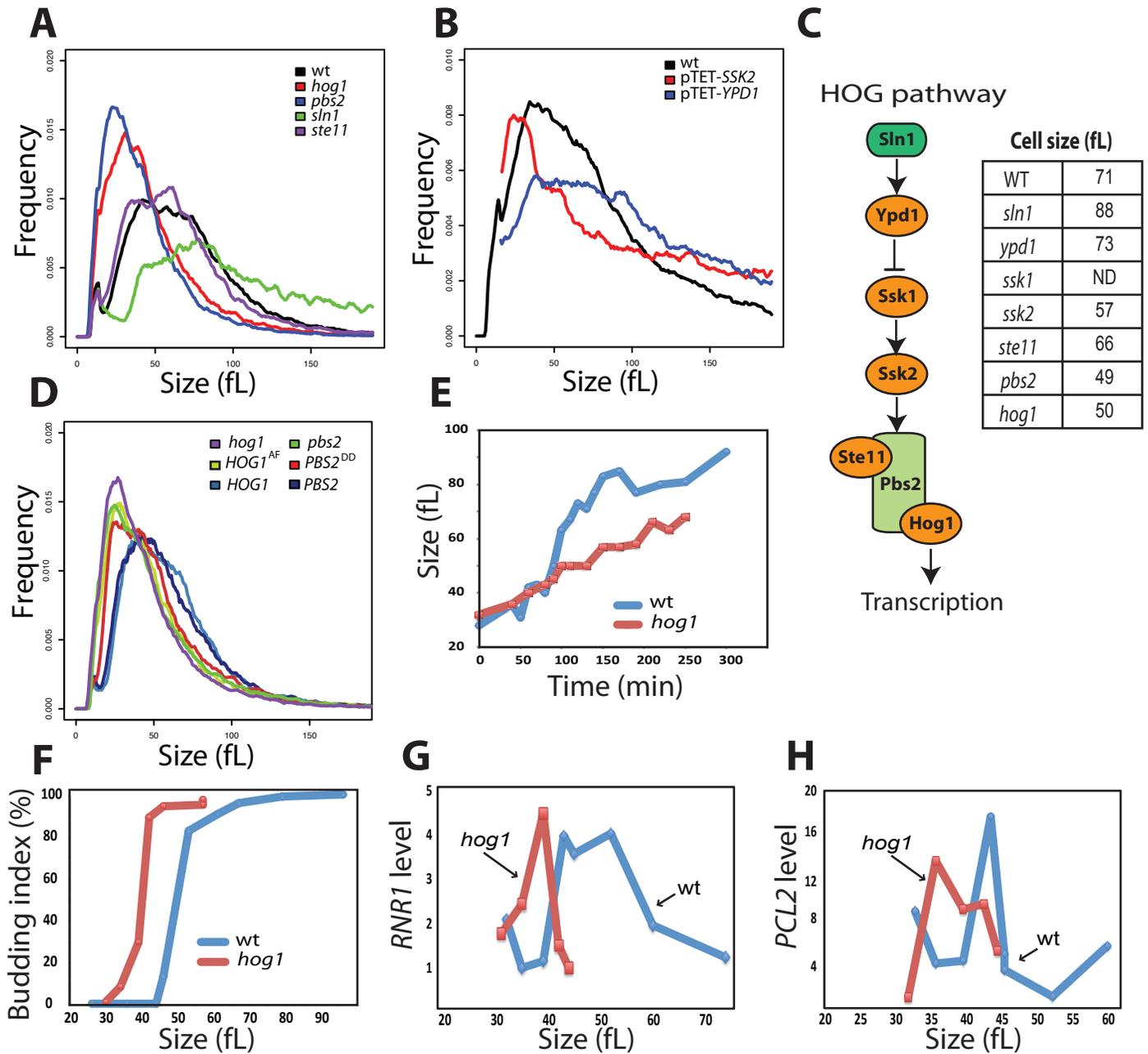


Figure 4

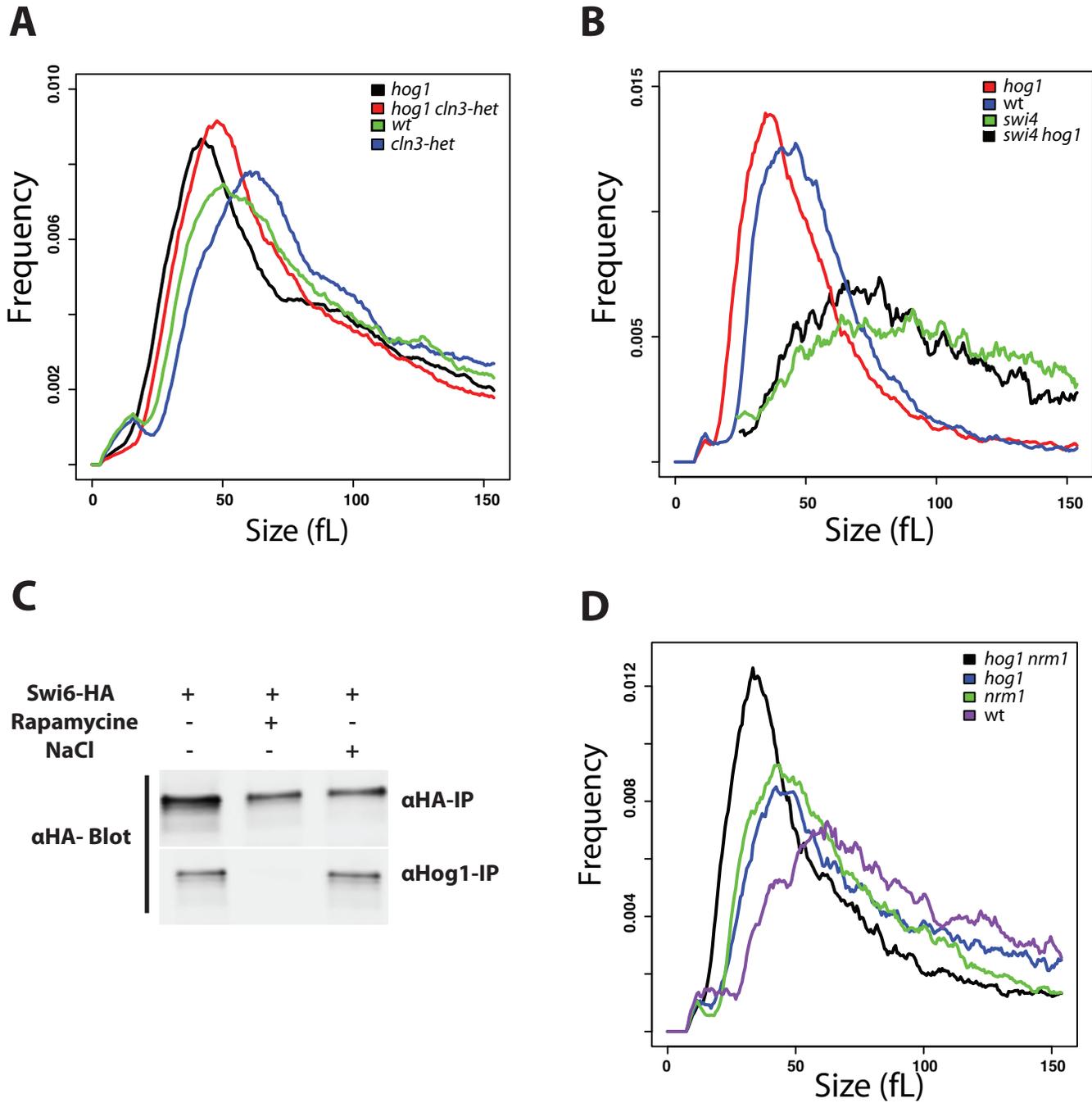


Figure 5

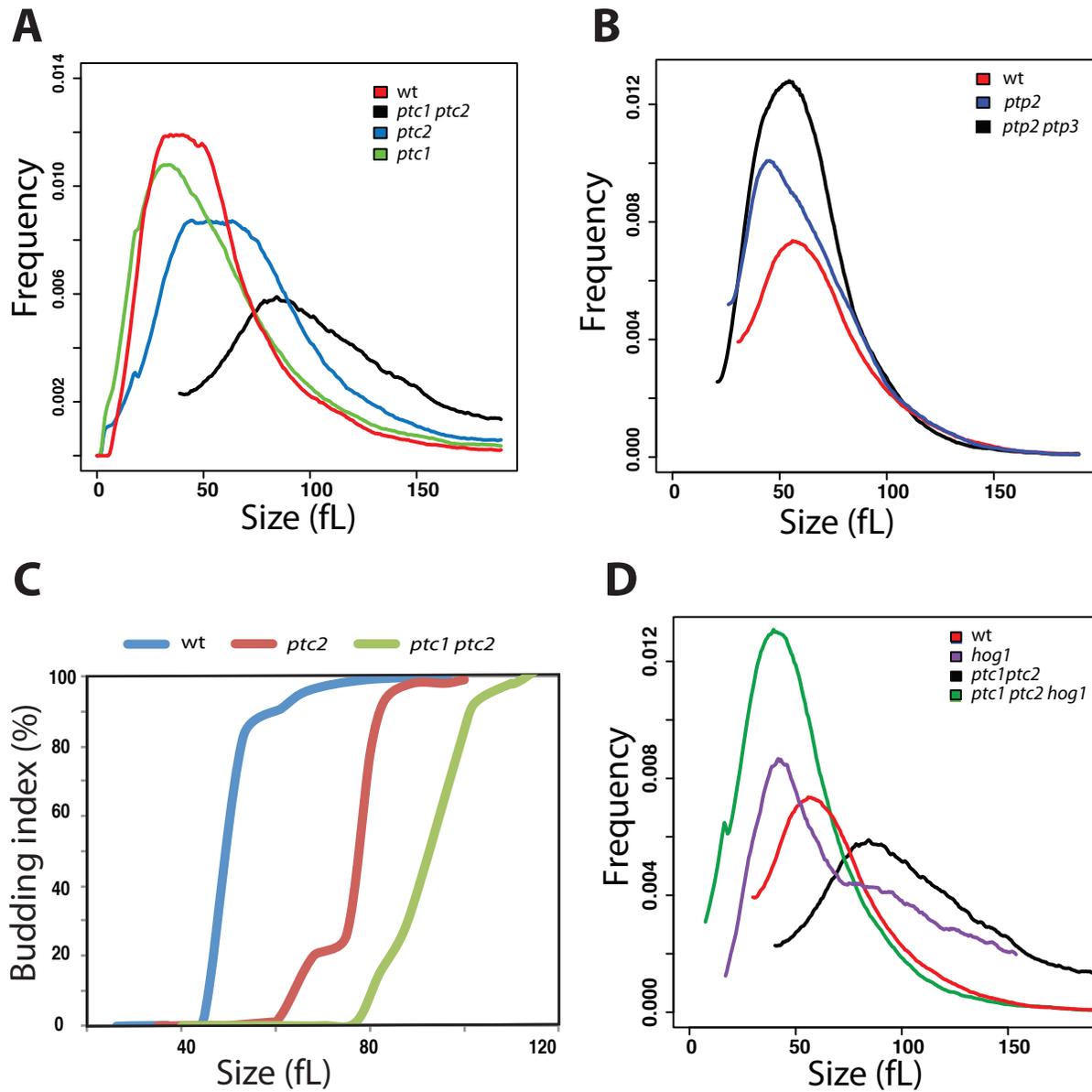
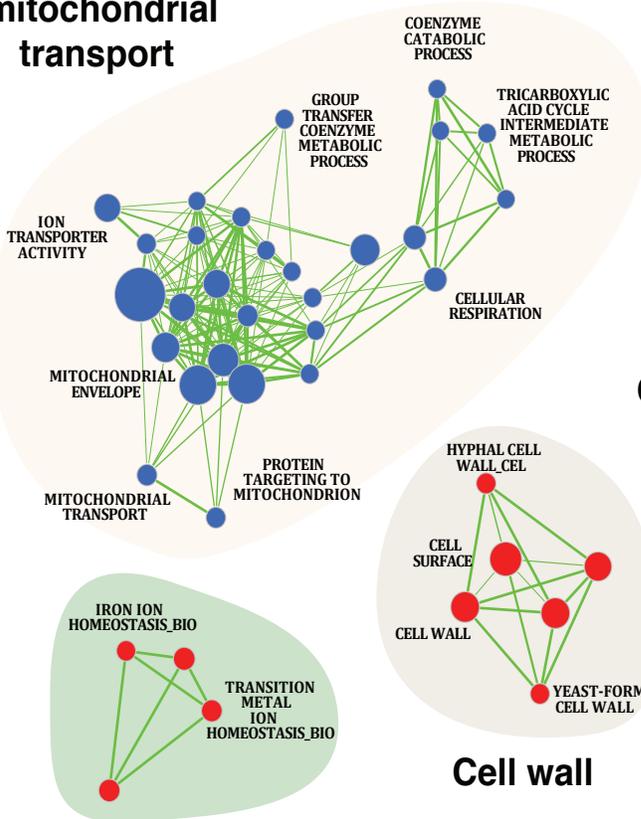


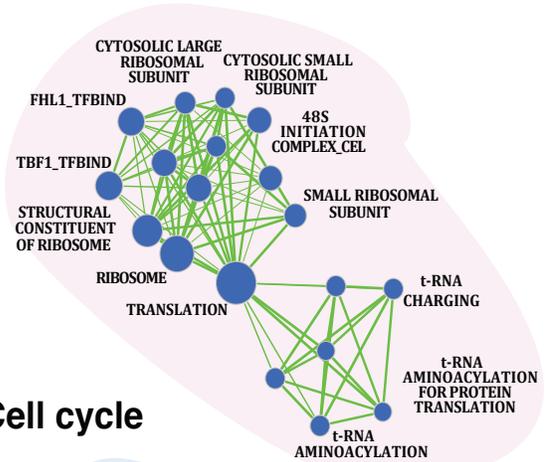
Figure 6

**A**

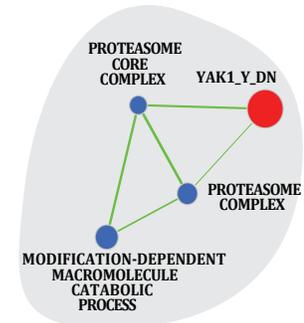
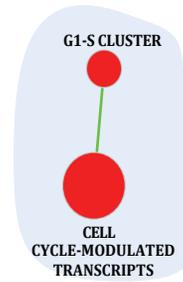
**Respiration & mitochondrial transport**



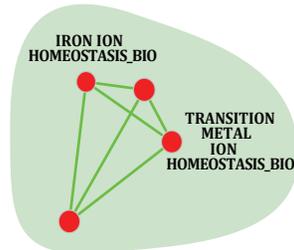
**Translation & ribosome biogenesis**



**Cell cycle**

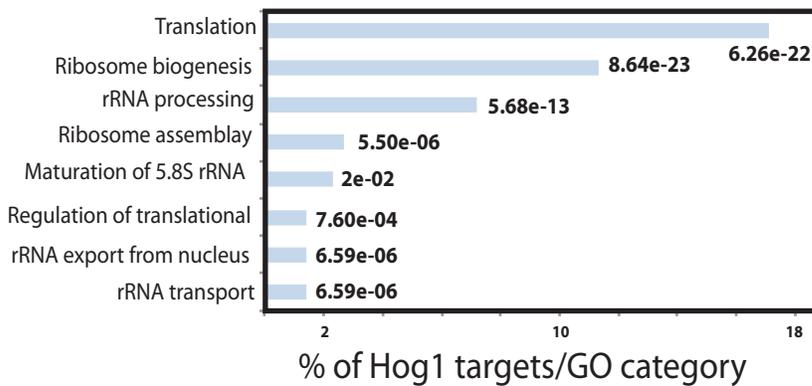


**Iron homeostasis**



**Proteasome**

**B**



**C**

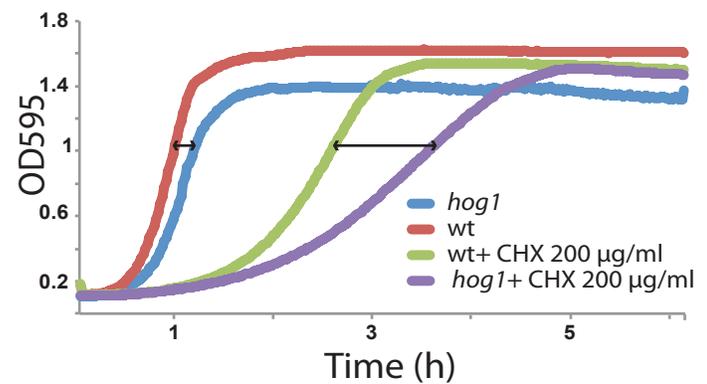


Figure 7

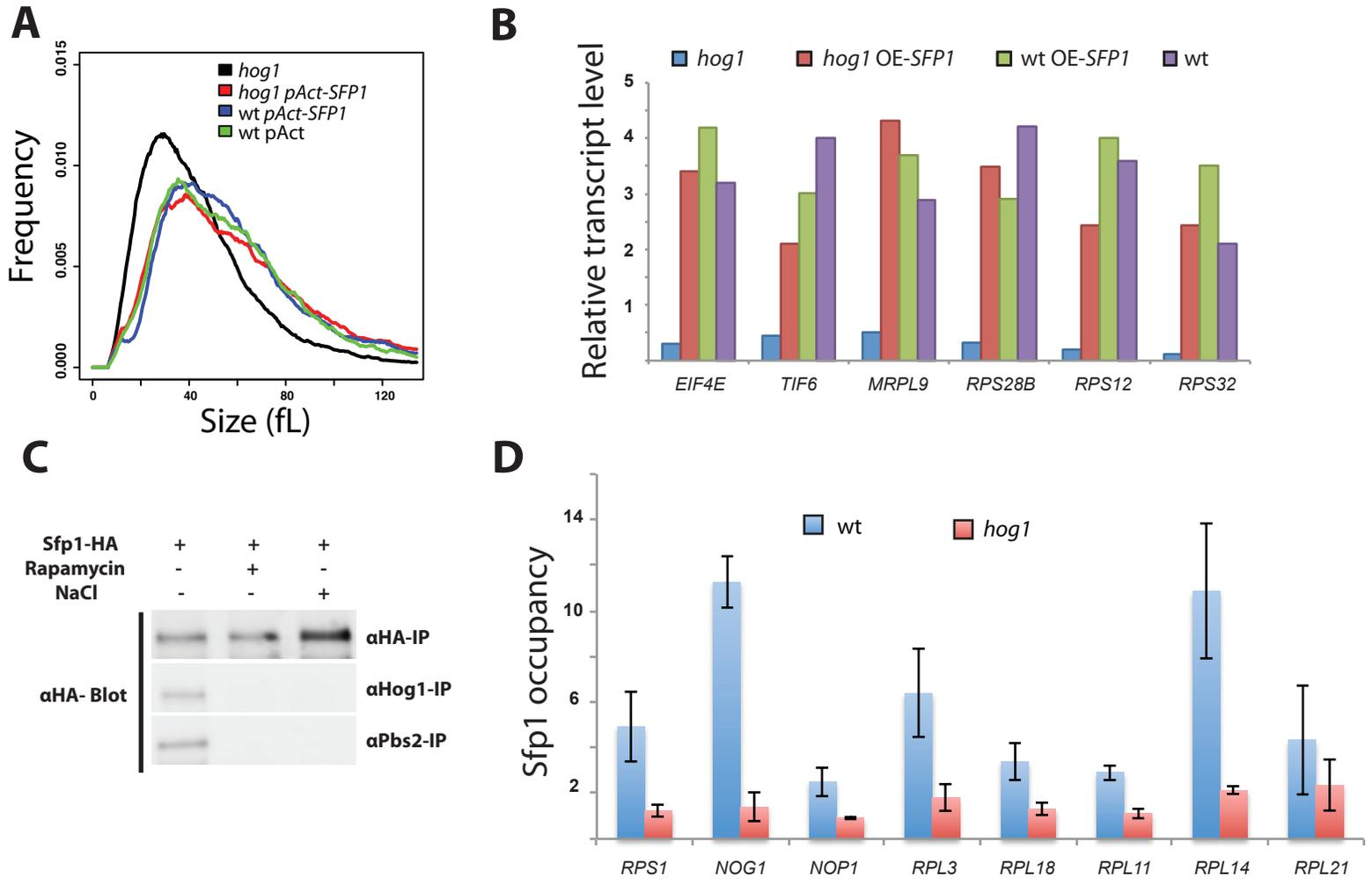
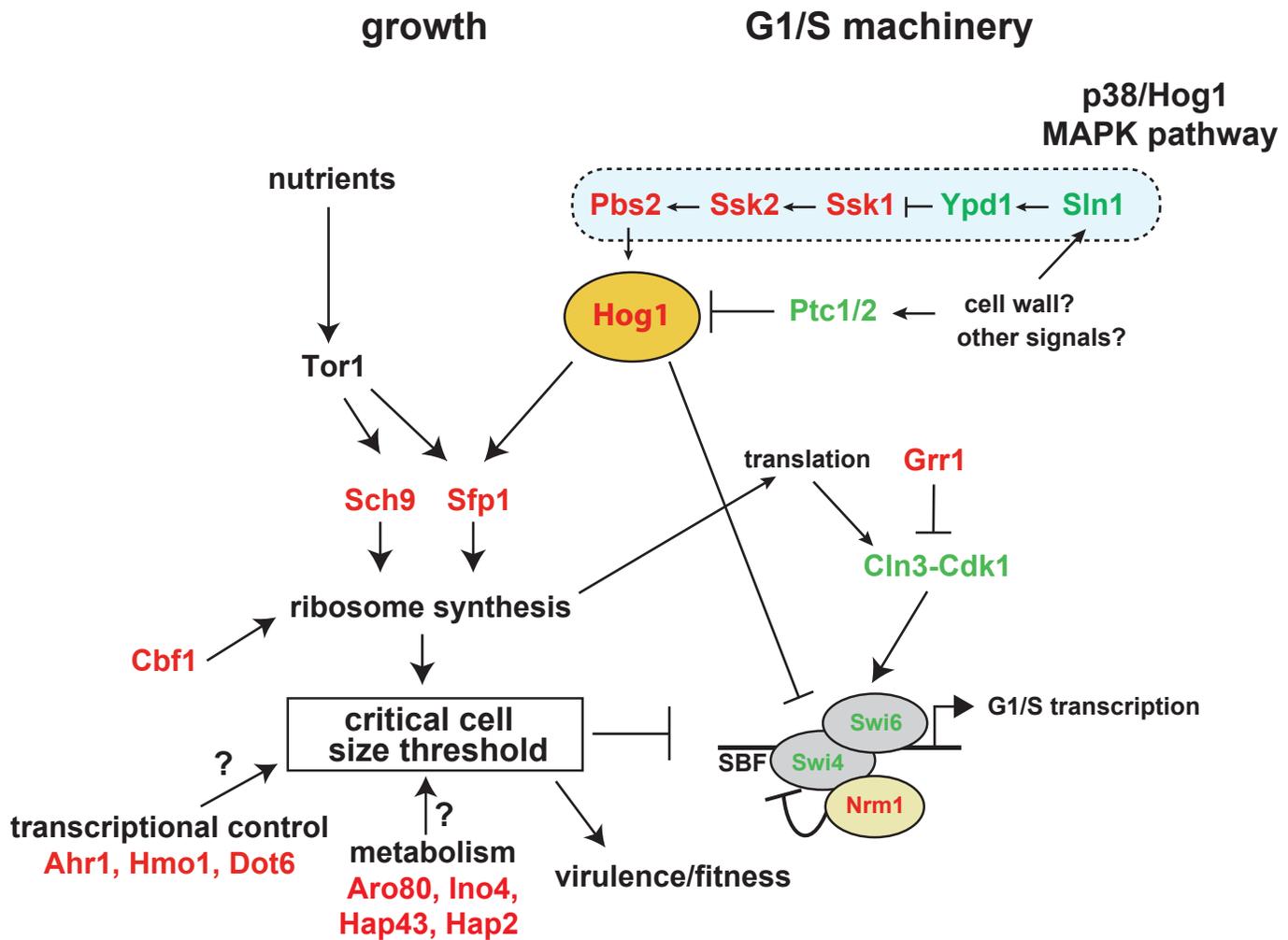
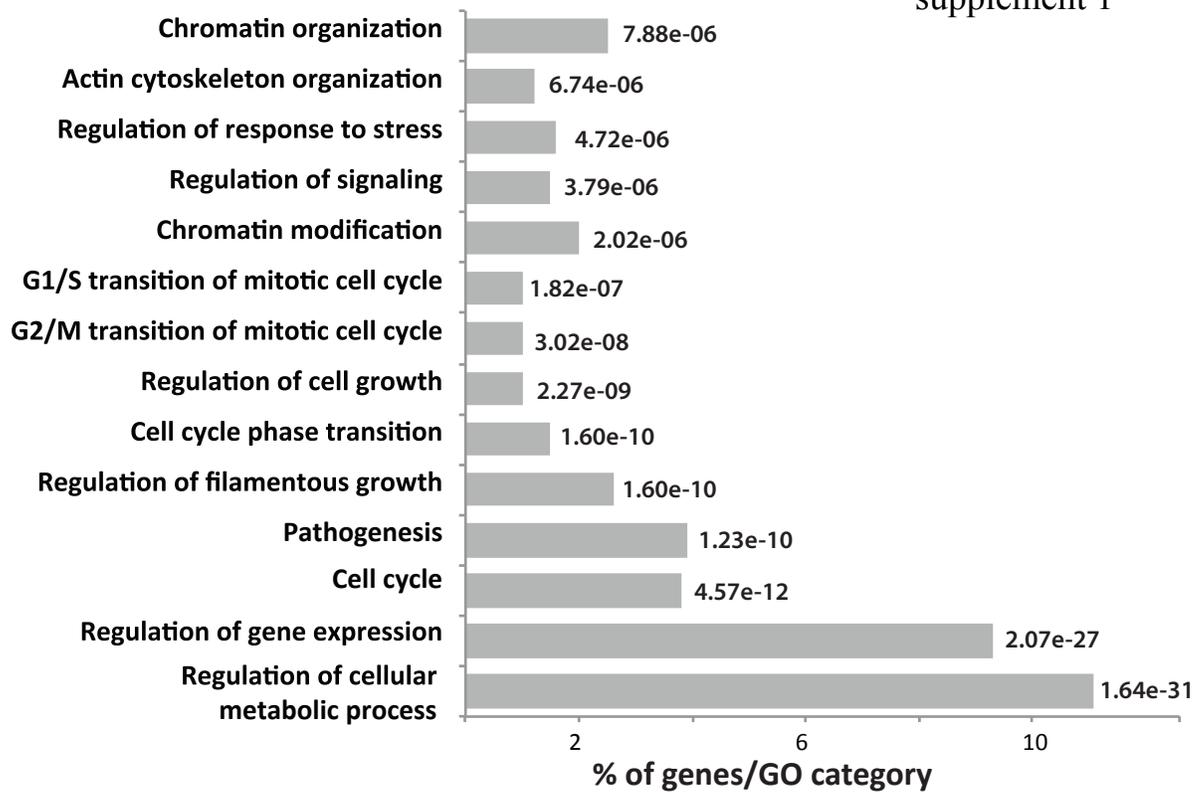


Figure 8

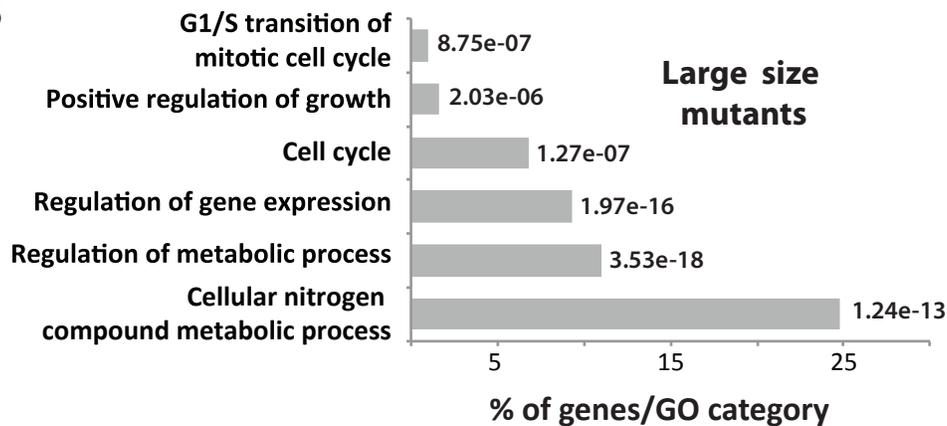


**A**

Figure 1–figure supplement 1



**B**



**C**

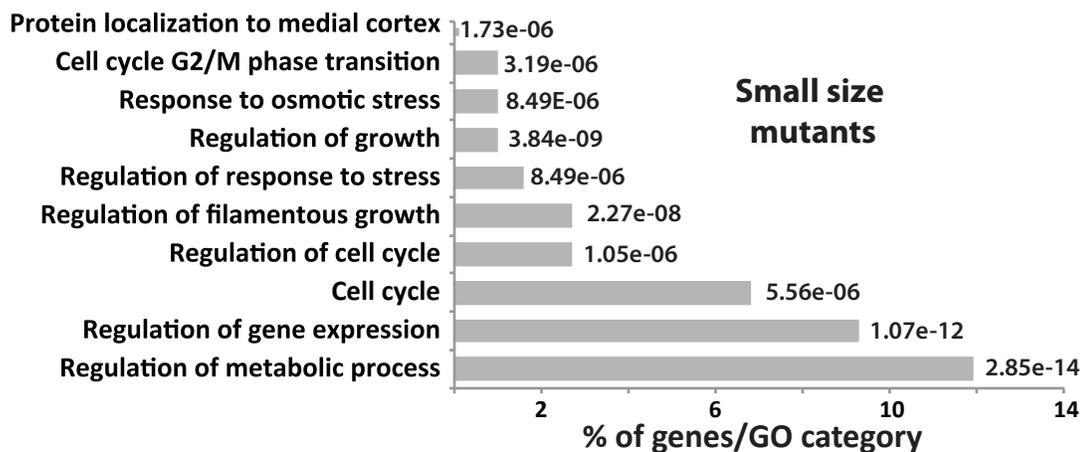


Figure 1–  
figure  
supplement 2

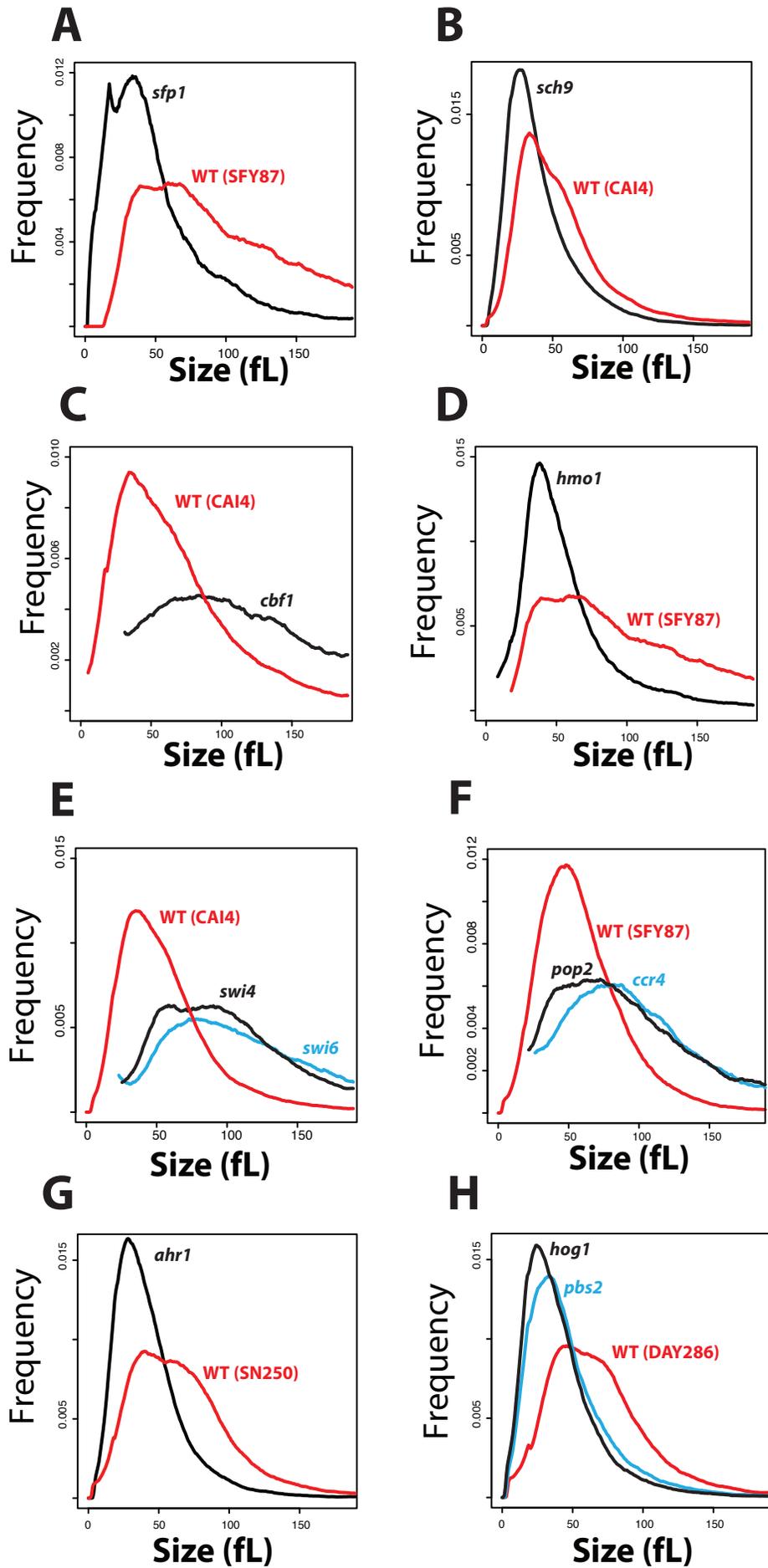


Figure 3–figure supplement 1

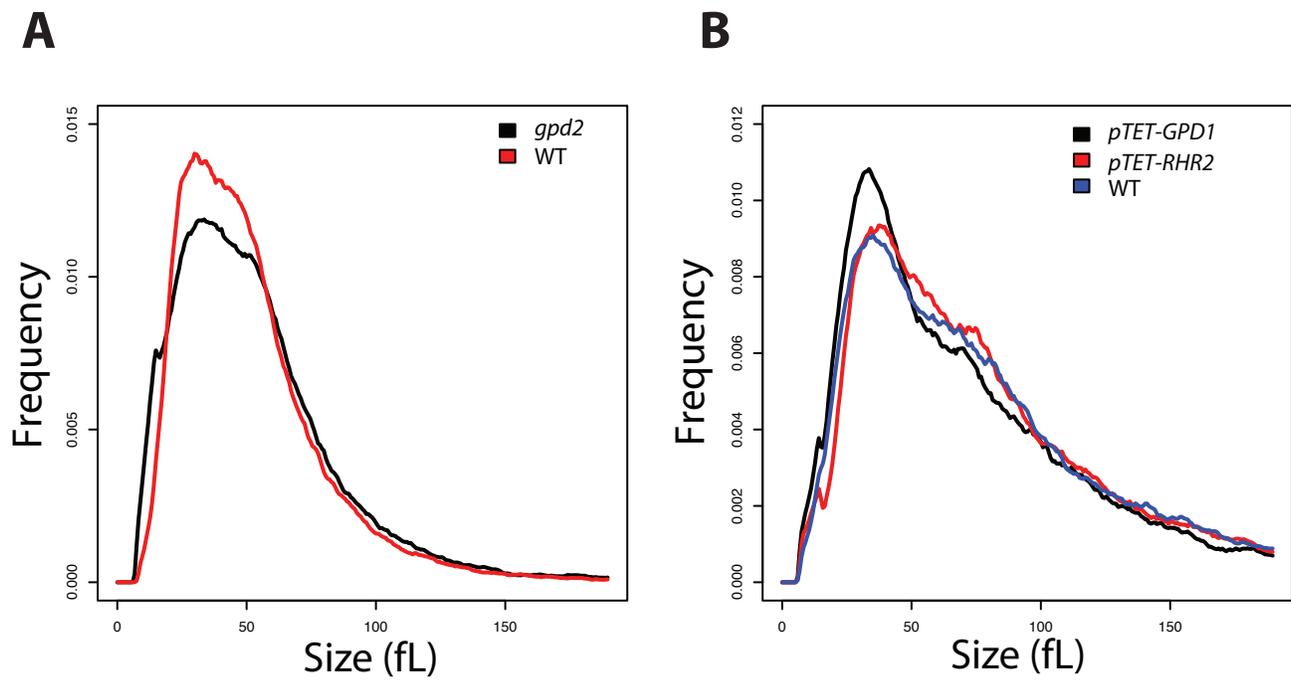


Figure 3—figure supplement 2

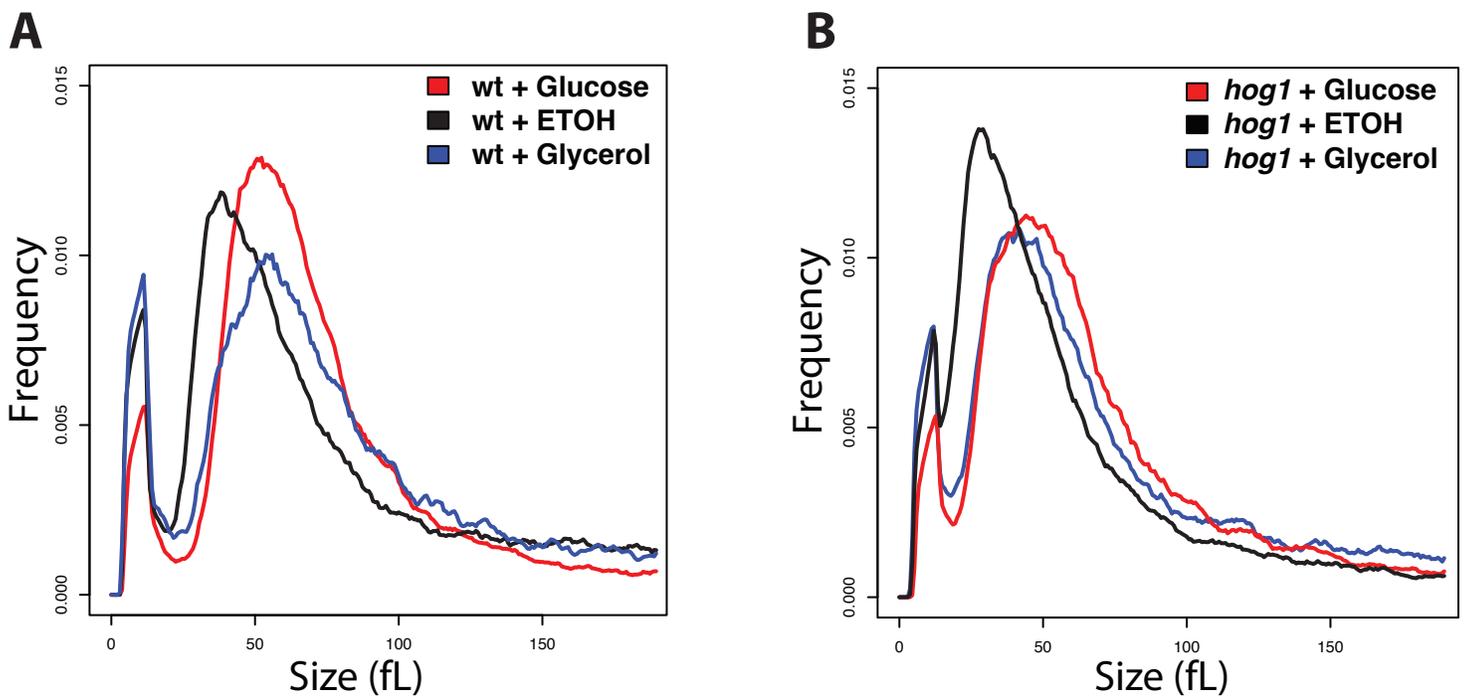


Figure 3—figure supplement 3

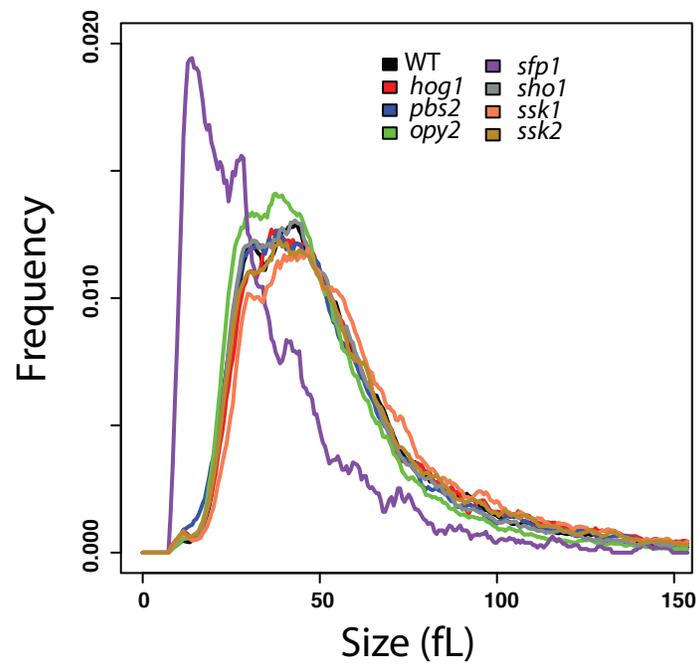


Figure 7–figure supplement 1

