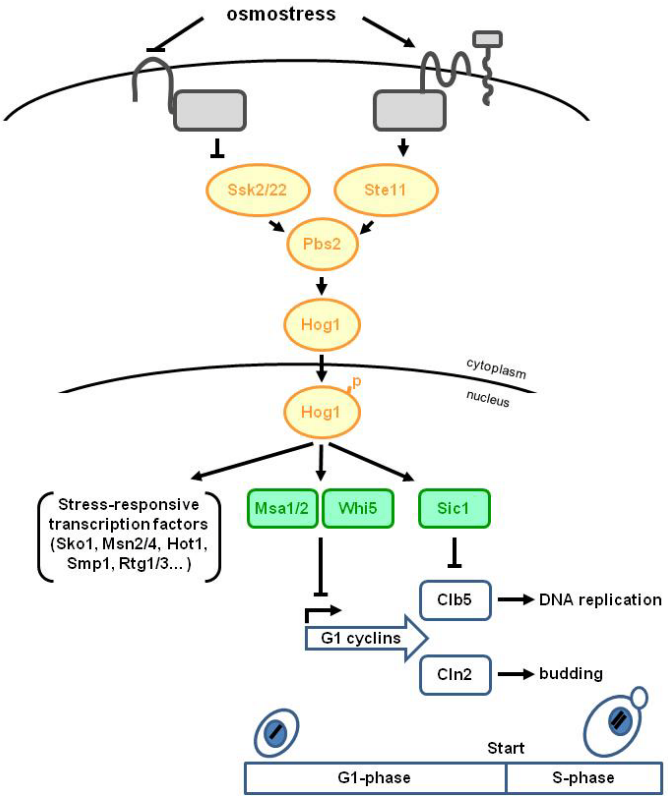
SUPPLEMENTAL MATERIAL

Yeast strains employed in this study are:

S. cerevisiae strains derived from W303 (*MATa, his3 leu2 trp1ura3 ade2 can1*) obtained in this work are: YAG163 (*bar1Δ::NAT*); YAG115 (*pCLN2-dVenus-LEU2*); YAG56 (*pCLB5-qVenus-LEU2*); YAG106 (*bar1Δ::LEU2, SWI4-HA-hph*); YAG127 (*bar1Δ::LEU2, hog1Δ::NAT, SWI4-HA-hph*); YMZ33 (*HOG1-HA-HIS*); YPC455 (*whi5Δ::LEU2*); YAG227 *(bar1Δ::HIS1; Msa1-HA-NAT*); YAG234 (*bar1Δ::HIS1; hog1Δ::URA3, MSA1-HA-NAT*); YAG228 (*bar1Δ::HIS1, msa1^{9A}-HA-NAT*); YAG1 (*msa1Δ::LEU2*); YAG162 (*bar1Δ::HIS1, whi5Δ::URA3, msa1Δ::LEU2, msa2Δ::hph*); YAG243 (*bar1Δ::NAT, ENO1-GFP-KAN*); YAG246 (*bar1Δ::HIS1, whi5Δ::URA3, msa1Δ::LEU2, msa2Δ::hph, ENO1-mCherry-KAN*); YAG230 (*bar1Δ::HIS1, whi5^{3A}-KAN, msa1^{9A}-NAT, msa2Δ::hph*); YPC459 (W303 *WHI5-HA-KAN*); YAG248 (W303 *bar1Δ::TRP1, whi5^{3A}-NAT, msa1^{9A}-KAN, msa2Δ::hph, pCLN2-dVenus-LEU2*); YAG240 (W303 *bar1Δ::TRP1, whi5^{3A}-NAT, msa1^{9A}-KAN, msa2Δ::hph, pCLB5-dVenus-LEU2*)

For genetic activation of the HOG pathway, the following derivatives from strain TM141 (*MATa ura3 leu2 trp1 his3*) were generated in this study: YAG57 (*sln1^{ts}, bar1Δ::TRP1*); YAG109 (*sln1^{ts}, bar1Δ::LEU2, SWI4-HA-hph*); YAG110 (*sln1^{ts}, bar1Δ::LEU2, hog1Δ::NAT, SWI4-HA-hph*); YAG62 (*sln1^{ts}, bar1Δ::TRP1, whi5Δ::URA3, msa1Δ::LEU2, msa2Δ::hph*); YAG254 (*sln1^{ts}, bar1Δ::NAT, hog1Δ::URA3*); YAG256 (*sln1^{ts}, bar1Δ::TRP1, whi5^{3A}-NAT, msa1^{9A}-KAN, msa2Δ::hph*); YAG235 (*sln1^{ts}, bar1Δ::TRP1, pCLN2-dVenus-LEU2*); YAG236 (*sln1^{ts}, bar1Δ::TRP1, pCLB5-dVenus-LEU2*); YAG237 (*sln1^{ts}, bar1Δ::TRP1, whi5^{3A}-NAT, msa1^{9A}-KAN, msa2Δ::hph, pCLN2-dVenus-LEU2*); YAG238 (*sln1^{ts}, bar1Δ::TRP1, whi5^{3A}-NAT, msa1^{9A}-KAN, msa2Δ::hph, pCLB5-dVenus-LEU2*); YAG239 (*sln1^{ts}, bar1Δ::NAT, hog1Δ::URA3, pCLN2-dVenus-LEU2); YAG247 (sln1^{ts}, bar1Δ::NAT, hog1Δ::URA3, pCLB5-dVenus-LEU2*); YAG214 (*sln1^{ts}, bar1Δ::TRP1, WHI5-HA-KAN*); YAG215 (*sln1^{ts}, bar1Δ::TRP1, hog1Δ::URA3, WHI5-HA-KAN*); YAG253 (*sln1^{ts}, bar1Δ::NAT*, *MSA1-HA-KAN*); YAG255 (sln1^{ts}, *bar1Δ::NAT, hog1Δ::URA3, MSA1-HA-KAN*).

Plasmids used in this study were pGEX4T-Hog1 and pGEX4T-Pbs2^{EE} (*PBS2* with Ser514-Glu and Thr518-Glu mutations). *WHI5*, *whi5^{3A}* (with mutations Ser88-Ala, Thr143-Ala, Thr215-Ala), *MSA1* and *msa1^{9A}* (bearing residues Thr95, Thr99, Ser121, Ser128, Thr397, Thr406, Ser415, Ser420 and Thr544 mutated to Ala) were cloned into pGEX-6P-1. GST or GST-Hog1 used in co-precipitation assays were expressed in yeast cells under the control of a constitutive promoter from plasmid pRS426.


osmostress
Ssk2/22
Ste11
Pbs2
Hog1
cytoplasm
nucleus
P
Hog1
Stress-responsive transcription factors (Sko1, Msn2/4, Hot1, Smp1, Rtg1/3...)
Msa1/2
Whi5
Sic1
Clb5
DNA replication
G1 cyclins
Cln2
budding
Start
G1-phase
S-phase

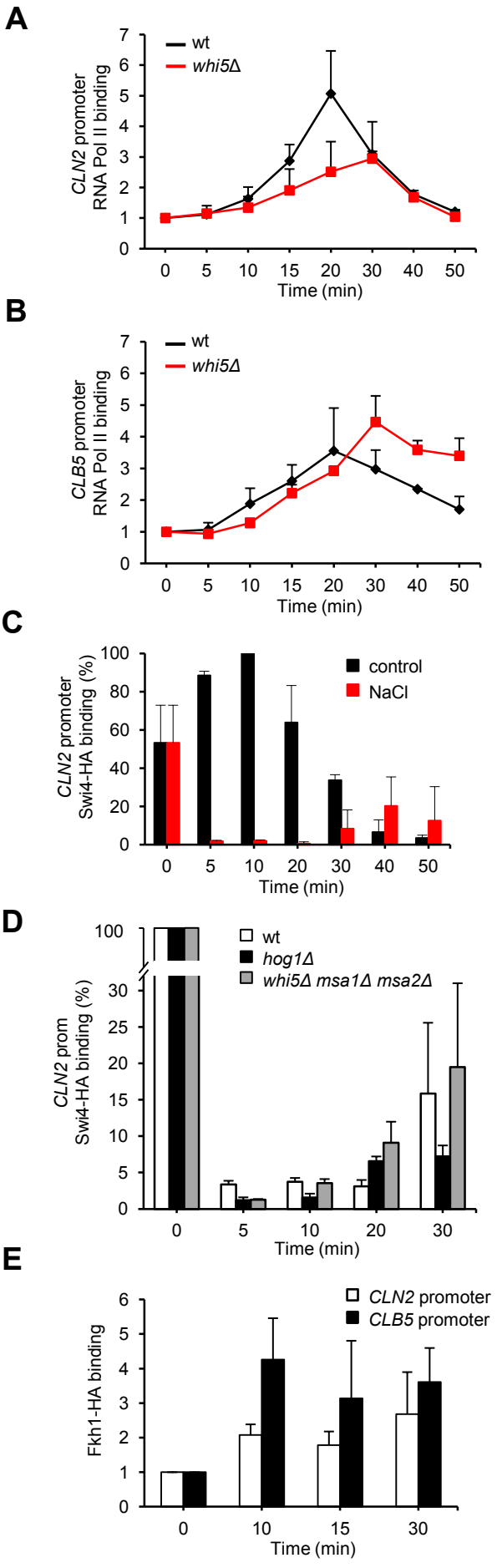

A
wt
whi5Δ
CLN2 promoter
RNA Pol II binding
Time (min)
B
wt
whi5Δ
CLB5 promoter
RNA Pol II binding
Time (min)
C
control
NaCl
CLN2 promoter
Swi4-HA binding (%)
Time (min)
D
wt
hog1Δ
whi5Δ msa1Δ msa2Δ
CLN2 prom
Swi4-HA binding (%)
Time (min)
E
CLN2 promoter
CLB5 promoter
Fkh1-HA binding
Time (min)

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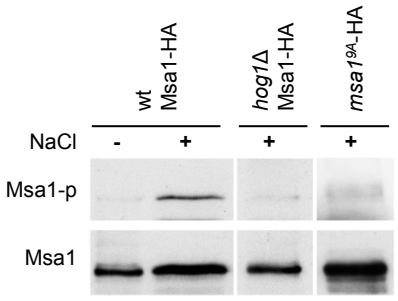
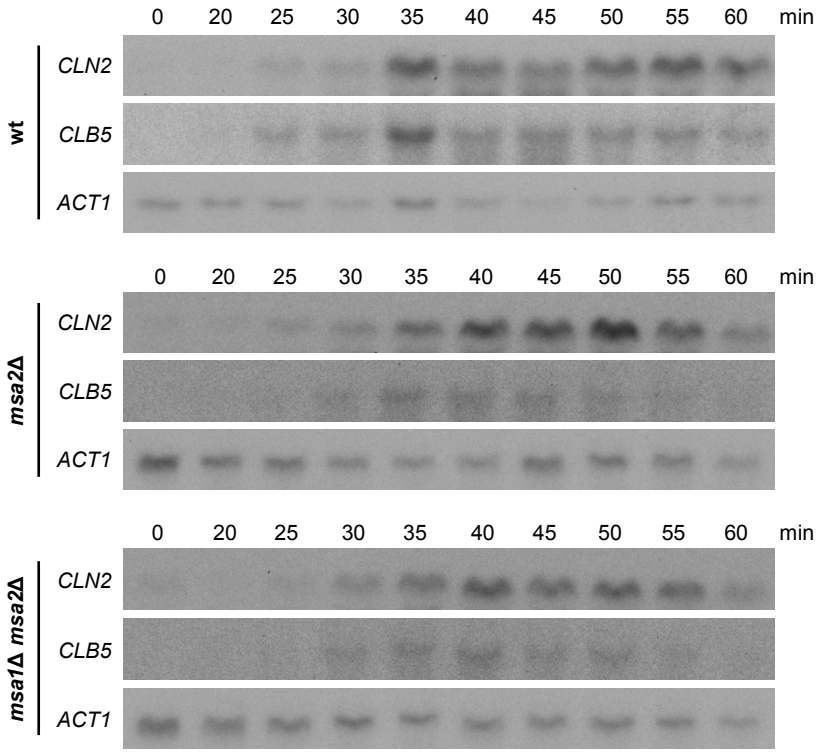
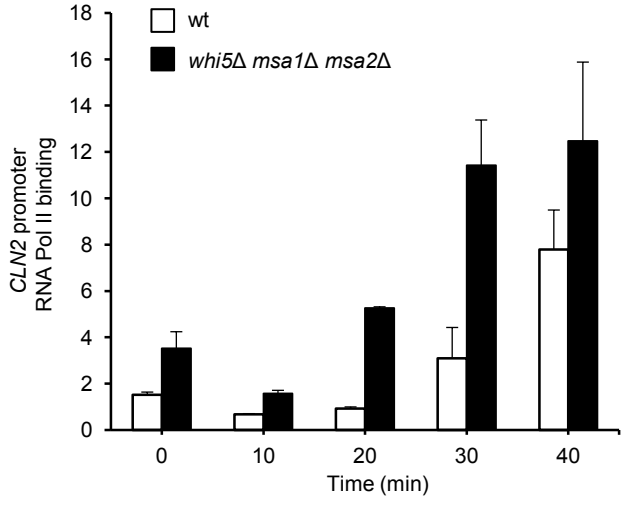
Figure S3

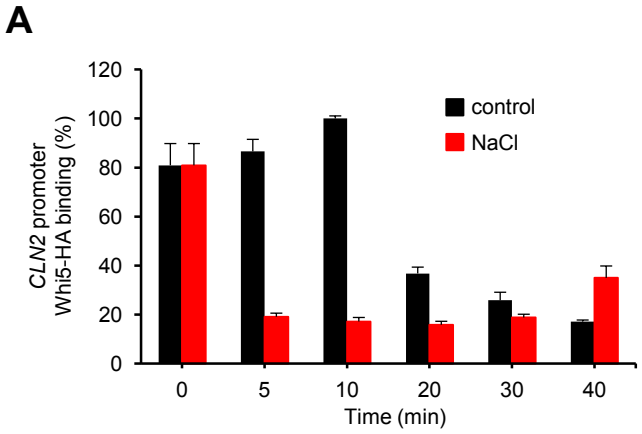


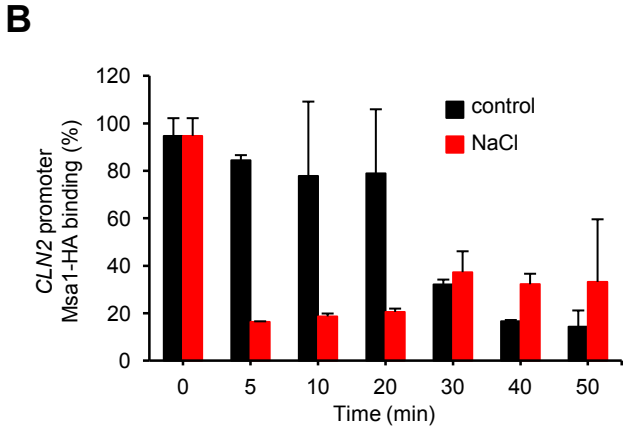
A

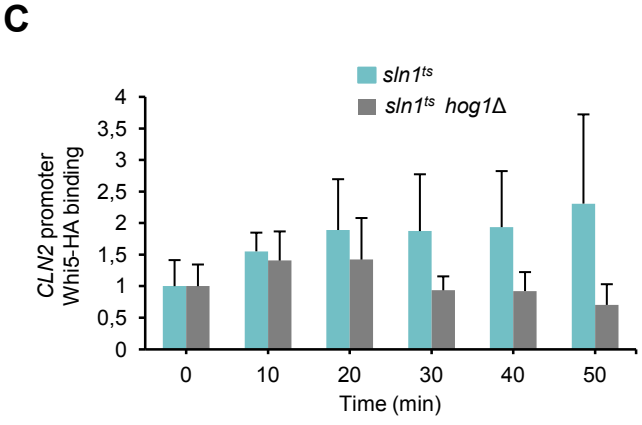

0 20 25 30 35 40 45 50 55 60 min
wt
CLN2
CLB5
ACT1
0 20 25 30 35 40 45 50 55 60 min
msa2Δ
CLN2
CLB5
ACT1
0 20 25 30 35 40 45 50 55 60 min
msa1Δ msa2Δ
CLN2
CLB5
ACT1

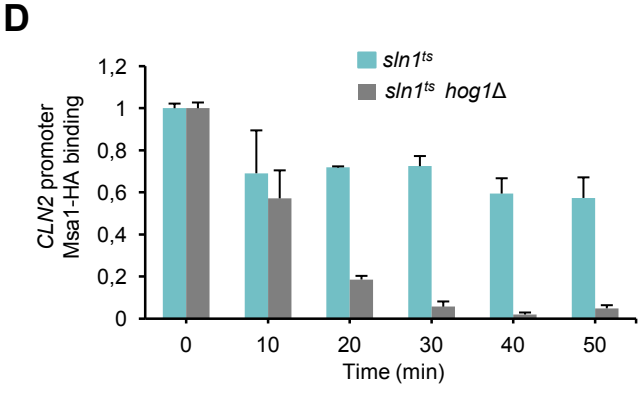
B


wt
whi5Δ msa1Δ msa2Δ
CLN2 promoter
RNA Pol II binding
0
2
4
6
8
10
12
14
16
18
0
10
20
30
40
Time (min)


A
120
100
80
60
40
20
0
CLN2 promoter
Whi5-HA binding (%)
control
NaCl
0
5
10
20
30
40
Time (min)


B
120
100
80
60
40
20
0
CLN2 promoter
Msa1-HA binding (%)
control
NaCl
0
5
10
20
30
40
50
Time (min)


C
sln1ts
sln1ts hog1Δ
4
3,5
3
2,5
2
1,5
1
0,5
0
CLN2 promoter
Whi5-HA binding
0
10
20
30
40
50
Time (min)


D
sln1ts
sln1ts hog1Δ
1,2
1
0,8
0,6
0,4
0,2
0
CLN2 promoter
Msa1-HA binding
0
10
20
30
40
50
Time (min)

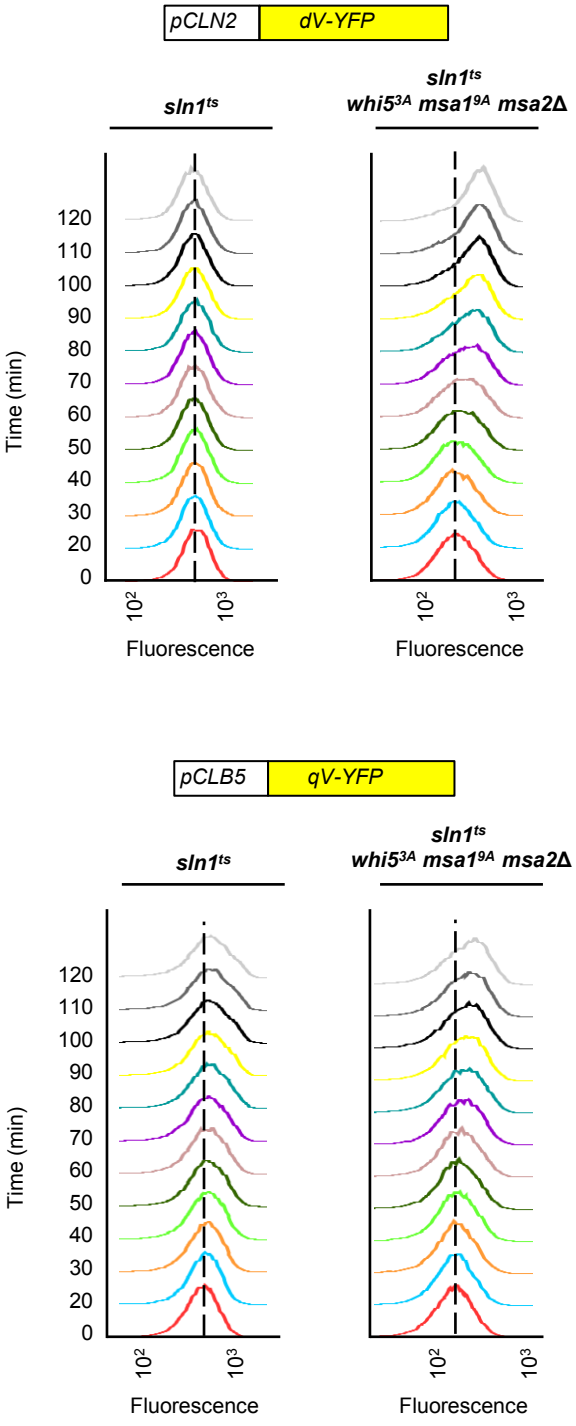

pCLN2
dV-YFP
sln1ts
sln1ts whi53A msa19A msa2Δ
Time (min)
120
110
100
90
80
70
60
50
40
30
20
0
10²
10³
Fluorescence
pCLB5
qV-YFP
sln1ts
sln1ts whi53A msa19A msa2Δ
Time (min)
Fluorescence

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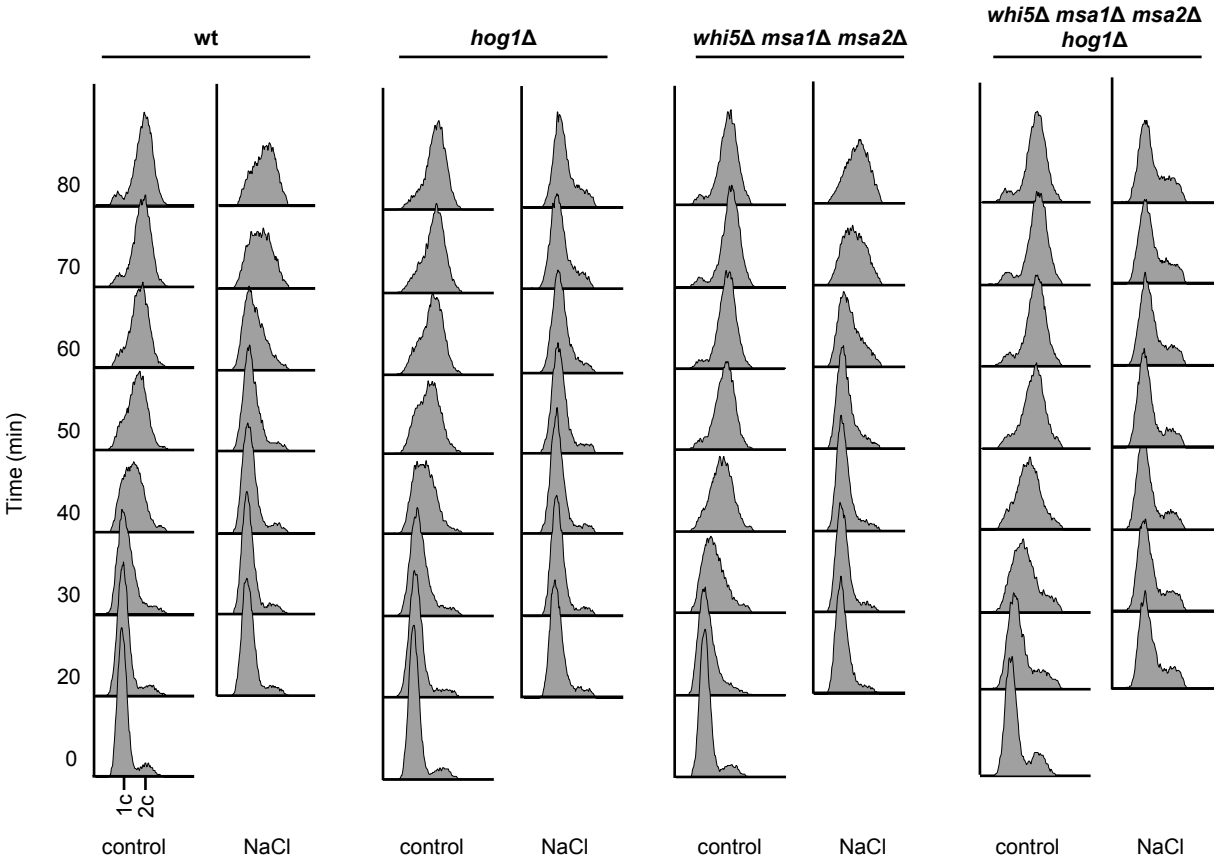
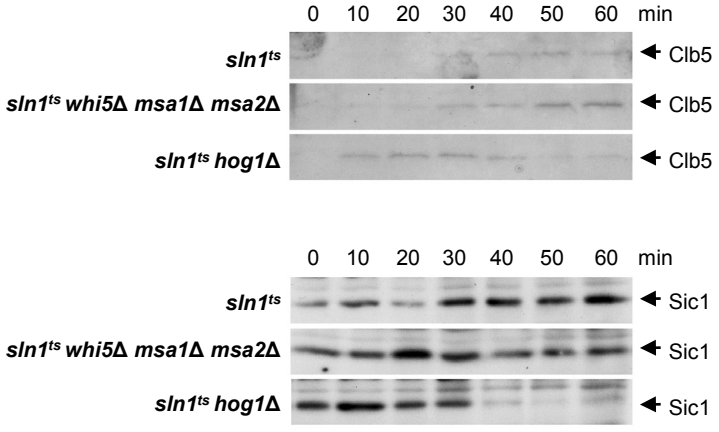
Figure S7




0 10 20 30 40 50 60 min
sln1ts
sln1ts whi5Δ msa1Δ msa2Δ
sln1ts hog1Δ
Clb5
Clb5
Clb5
0 10 20 30 40 50 60 min
sln1ts
sln1ts whi5Δ msa1Δ msa2Δ
sln1ts hog1Δ
Sic1
Sic1
Sic1

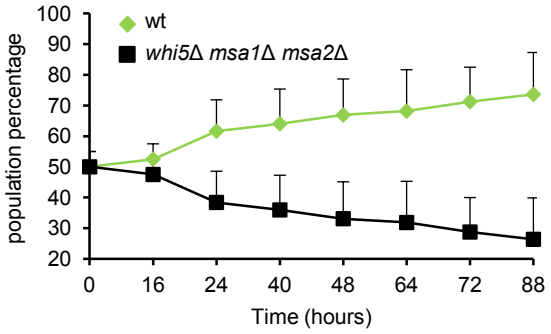

wt
whi5Δ msa1Δ msa2Δ
population percentage
100
90
80
70
60
50
40
30
20
0
16
24
40
48
64
72
88
Time (hours)

Figure S1. Hog1 regulates G1-S transition through a dual mechanism

In response to osmostress, two independent upstream osmosensing mechanisms lead to the activation of specific MAPKKKs (Ssk2/22 and Ste11) that converge on the common Pbs2 MAPKK. Activated Pbs2 phosphorylates the Hog1 MAPK. Activated Hog1 goes into the nucleus and activates a transcriptional inhibitory mechanism involving at least Whi5, Msa1 and Msa2, resulting in the downregulation of *CLN2* and *CLB5* transcription. In addition, Hog1 is able to phosphorylate and stabilize the Clb-CDK inhibitor Sic1. Both mechanisms work together to efficiently delay entry into S-phase upon osmostress.

Figure S2. Binding of RNA Pol II and transcription factors to G1-cyclin promoters upon osmostress

(A, B) Whi5 alters efficient binding of RNA Pol II to G1-cyclin promoters. Wild type (black lines) and *whi5*Δ (red lines) cells were synchronized in G1 with alpha-factor, and released into YPD in the presence of 0.4M NaCl. Chromatin-bound RNA Pol II was immunoprecipitated at the indicated times using monoclonal anti-Rpb1 antibodies (8WG16, Covance). The precipitate was analyzed by real-time PCR using specific primers for the *CLN2* (A) or the *CLB5* (B) promoter regions. Data are the average ± SD of two independent experiments and were normalized to a telomeric region used as an internal control.

(C) Swi4 transcription factor binding to the *CLN2* promoter is abolished upon osmostress. Cells with chromosomally tagged Swi4-HA were synchronized with alpha-factor and released into YPD (control, black bars) or 0.4 M NaCl (red bars). Association of Swi4-HA with the *CLN2* promoter over time after release was analyzed using the anti-HA-antibody in ChIP time-course experiments. Immunoprecipitated chromatin was analyzed by real-time PCR using specific primers for the *CLN2* promoter. Data represent the percentage of binding relative to the maximum binding observed and are the average ± SD of three independent experiments.

(D) Swi4 is evicted from the *CLN2* promoter in response to osmostress in a Hog1-independent manner. Association of HA-tagged Swi4 in wild type (white columns), *hog1Δ* (black columns) and *whi5Δ msa1Δ msa2Δ* (grey columns) strains with the *CLN2* promoter over time after release from alpha-factor into 0.4 M NaCl was analyzed as in (C). Binding is depicted as the percent of Swi4 binding under control conditions for each strain. Data are the average ± SD of three independent experiments.

(E) Fkh1 binds to *CLN2* and *CLB5* promoters upon osmostress. Cells expressing Fkh1-HA from its own promoter were synchronized using alpha-factor and released in the presence of 0.4M NaCl. Fkh1 binding to *CLN2* (white columns) and *CLB5* (black columns) promoters was followed by real-time PCR using specific primers. Data are the average ± SD of two independent experiments and were normalized to a telomeric region used as an internal control.

Figure S3. Msa1 is phosphorylated by Hog1 *in vivo*

Wild type yeast cells expressing Msa1-HA or *msa1*^{9A}-HA, as well as *hog1*Δ cells expressing Msa1-HA, were synchronized with alpha-factor and were treated (+) or not (-) with brief osmotic shock (0.4 M NaCl, 10 minutes). Protein extracts were then immunoprecipitated using anti-HA

antibody. SP/TP phosphorylated proteins were detected using an anti-phospho Serine/Threonine antibody (BD Transduction Laboratories).

Figure S4. Coordinated action of Whi5, Msa1 and Msa2 is required for proper transcriptional down-regulation of G1-cyclins

(A) Msa2 has no effect on *CLN2* or *CLB5* transcription. Northern blot assays were performed to follow changes in G1-cyclin mRNA levels in wild type (upper panel), *msa2Δ* (middle panel) or *msa1Δ msa2Δ* (lower panel) cells over time after release from pheromone arrest into 0.4 M NaCl.

(B) Absence of Whi5, Msa1 and Msa2 favors RNA Pol II dependent transcription of *CLN2* after osmostress. Wild type (white columns) or triple null (black columns) cells were synchronized with alpha-factor and released into 0.4 M NaCl. Samples were taken at the indicated times after release and precipitated with the anti-Rpb1 antibody (8WG16, Covance) in ChIP assays of RNA Pol II binding to the *CLN2* promoter. Data are the average ± SD of three independent experiments and were normalized to a telomeric region used as an internal control.

Figure S5. Whi5 and Msa1 binding to *CLN2* promoter upon osmostress and genetic activation of the HOG pathway

(A, B) Whi5 and Msa1 are evicted from *CLN2* promoter upon osmostress. Cells expressing Whi5-HA (A) or Msa1-HA (B) from its own promoter were synchronized using alpha-factor and released in control (black columns) or in the presence of 0.4M NaCl (red columns). Whi5 and Msa1 binding to *CLN2* promoter was followed by real-time PCR using specific primers. Data are the average ± SD of three independent experiments.

(C, D) Whi5 and Msa1 remain attached to *CLN2* promoter upon genetic activation of the HOG pathway. *sln1*^{ts} (blue columns) or *sln1*^{ts} *hog1*Δ (grey columns) *c*ells expressing Whi5-HA (C) or Msa1-HA (D) from its own promoter were synchronized using alpha-factor and released at 37ºC. Whi5 and Msa1 binding to *CLN2* promoter was followed by real-time PCR using specific primers. Data are the average ± SD of three independent experiments and were normalized to a telomeric region used as an internal control.

Figure S6. *CLN2* and *CLB5* promoter inhibition depends on Whi5, Msa1 and Msa2

sln1^{ts} and *sln1*^{ts} *whi5*^{3A} *msa1*^{9A} *msa2*Δ cells were transformed with a fluorescent reporter system for analysis of *CLN2* (upper panel) or *CLB5* (lower panel) promoter activity. Fluorescent-positive cells were synchronized with alpha-factor and released at 37 ºC into YPD, and promoter-associated fluorescence was analyzed by flow cytometry over time up to 120 minutes after release. Distribution of fluorescence at different times after release is shown. Each line in the histogram represents fluorescence distribution from 20000 cells.

Figure S7. Cells depleted for Hog1 are unable to re-enter the cell cycle

Cells from indicated strains were grown to log phase, synchronized using alpha-factor and released in medium containing or not 0.4M NaCl. DNA content was measured every ten minutes. FACS profiles show DNA content of 10000 cells.

Figure S8. Increased Clb5 levels can overcome Sic1 dependent G1 arrest in the absence of Whi5, Msa1 and Msa2

sln1^{ts}, *sln1^{ts} whi5*Δ *msa1*Δ *msa2*Δ and *sln1^{ts} hog1*Δ cells were synchronized using alpha-factor in G1 and released from pheromone arrest into YPD at 37 ºC. Clb5 and Sic1 protein levels were followed over time by Western blotting using specific antibodies (Santa Cruz).

Figure S9. Whi5, Msa1 and Msa2 are required for efficient competition with wild type cells

Wild type cells bearing GFP-tagged Eno1 (green line) and *whi5Δ msa1Δ msa2Δ* (black line) cells were synchronized, mixed together using an equal number of cells and released into 0.4 M NaCl. The percentage of cells of each strain was determined over time after release using flow cytometry. Data are the average ± SD of three independent experiments. At each time point 10000 cells were analyzed.