Genome-Wide Mapping of Binding Sites Reveals Multiple Biological Functions of the Transcription Factor Cst6p in *Saccharomyces cerevisiae*

Guodong Liu, David Bergenholm, Jens Nielsen

ABSTRACT  In the model eukaryote *Saccharomyces cerevisiae*, the transcription factor Cst6p has been reported to play important roles in several biological processes. However, the genome-wide targets of Cst6p and its physiological functions remain unknown. Here, we mapped the genome-wide binding sites of Cst6p at high resolution. Cst6p binds to the promoter regions of 59 genes with various biological functions when cells are grown on ethanol but hardly binds to the promoter at any gene when cells are grown on glucose. The retarded growth of the *CST6* deletion mutant on ethanol is attributed to the markedly decreased expression of *NCE103*, encoding a carbonic anhydrase, which is a direct target of Cst6p. The target genes of Cst6p have a large overlap with those of stress-responsive transcription factors, such as Sko1p and Skn7p. In addition, a *CST6* deletion mutant growing on ethanol shows hypersensitivity to oxidative stress and ethanol stress, assigning Cst6p as a new member of the stress-responsive transcriptional regulatory network. These results show that mapping of genome-wide binding sites can provide new insights into the function of transcription factors and highlight the highly connected and condition-dependent nature of the transcriptional regulatory network in *S. cerevisiae*.

IMPORTANCE  Transcription factors regulate the activity of various biological processes through binding to specific DNA sequences. Therefore, the determination of binding positions is important for the understanding of the regulatory effects of transcription factors. In the model eukaryote *Saccharomyces cerevisiae*, the transcription factor Cst6p has been reported to regulate several biological processes, while its genome-wide targets remain unknown. Here, we mapped the genome-wide binding sites of Cst6p at high resolution. We show that the binding of Cst6p to its target promoters is condition dependent and explain the mechanism for the retarded growth of the *CST6* deletion mutant on ethanol. Furthermore, we demonstrate that Cst6p is a new member of a stress-responsive transcriptional regulatory network. These results provide deeper understanding of the function of the dynamic transcriptional regulatory network in *S. cerevisiae*.
mids (10), while its overexpression results in chromosome instability (11). Currently, the only well-studied direct target of Cst6p is NCE103 (12), which encodes a carbonic anhydrase converting CO₂ to HCO₃⁻, the latter serving as a substrate of several carbonylation reactions. Under low-CO₂ conditions, Cst6p binds to the promoter of NCE103 and activates its expression. In some other fungal species, the orthologs of Cst6p have pleiotropic functions. Thus, in Candida albicans, Rca1p also directly activates carbonic anhydrase gene expression (12), with other phenotypes being altered hyphal formation, membrane ergosterol content, antifungal responses, and chemical resistance (12, 13). In Candida glabrata, Cst6p negatively regulates the expression of the main adhesin gene EPA6 and, thereby, biofilm formation (14).

Despite extensive phenotypic studies on CST6 mutants, the genome-wide in vivo targets of Cst6p still remain unknown. The ChIP-chip study of 203 TFs in S. cerevisiae (4) included Cst6p, but the target list identified seems questionable, because the known target NCE103 was not found and no consensus binding motif could be enriched from the target sequences. We therefore mapped the binding sites of Cst6p during growth on both glucose and ethanol, using ChiP with lambda exonuclease digestion followed by sequencing (ChiP-exo), which allows for identifying the location of DNA-binding proteins at high resolution (15). Following this, we measured the regulatory effect of Cst6p on its target genes, and the biological functions of these regulatory effects were investigated. Our results provide deeper understanding of the function of Cst6p and how it integrates with the transcriptional regulatory network in S. cerevisiae.

RESULTS

Identification of Cst6p binding sites by ChiP-exo. A cst6Δ strain was reported to have a more severe growth defect on respiratory carbon sources (including ethanol and galactose) than on glucose (5, 16), indicating a stronger role of this TF during growth on ethanol than during growth on glucose. We therefore aimed to identify the binding targets of Cst6p during growth on ethanol. To enable immunoprecipitation of Cst6p, we tagged it with CBP-ProtA in situ. The tag does not seem to affect the function of Cst6p during growth on ethanol, as growth was unaffected (see Fig. S1 in the supplemental material). Using ChiP-exo (Fig. 1A), we identified 40 binding sites distributed on 14 chromosomes when the protein products are major HCO₃⁻-consuming enzymes in S. cerevisiae.

Cst6p functionally regulates the expression of its targets. To investigate the regulatory effects of the Cst6p binding events identified, the expression levels of 17 target genes in the cst6Δ strain were compared with their expression levels in the wild type. The CST6 deletion mutant in the CEN.PK genetic background is viable on ethanol (see below), allowing the measurement of gene expression under this growth condition where we found Cst6p to be most active. As expected, the expression of CST6 itself was not detected in the cst6Δ strain (data not shown). The expression of NCE103 had a 92.6% decrease in the cst6Δ strain relative to its expression in the wild type, while a smaller decrease of 50.2% was observed during growth on glucose (Fig. 3). For the other target genes investigated, seven (AHPI, PHDI, YAP6, ACC1, ROX1, HAP4, and PYC1) had significantly decreased expression (approximately 15% to 45%) relative to their expression in the wild type for growth on ethanol. RPS3 had a 30.9% increase in expression,
while the other eight genes tested did not show significant changes in expression between the \textit{cst6}\textsubscript{H9004} strain and the wild type. No relation could be found between the extent of the expression changes of the target genes and the binding strength (signal-to-noise ratio of ChIP-exo peaks) of Cst6p on their promoters.

The function of Cst6p during growth on ethanol is dependent on \textit{NCE103} expression. In a medium with 1% (vol/vol) ethanol as the sole carbon source, the \textit{cst6}\textsubscript{H9004} strain had a longer lag phase, whereas the specific growth rate was only slightly lower later in the cultivation (Fig. 4A). This growth defect is less severe than that reported earlier. Thus, Garcia-Gimeno and Struhl observed no growth on ethanol (5), probably due to differences in strain backgrounds and in ethanol concentrations (3% ethanol was used in their study). In a glucose medium, the growth of the \textit{cst6}\textsubscript{H9004} strain was similar to that of the wild type and respiratory growth after the diauxic shift was not affected (Fig. 4B).

Since the expression of \textit{NCE103} was decreased to a greater extent than the expression of the other target genes with growth on ethanol (Fig. 3), we evaluated whether the low expression of this gene was contributing to the slower growth of the \textit{cst6}\textsubscript{H9004} strain. Constitutive expression of \textit{NCE103} using the \textit{TEF1} promoter in the \textit{cst6}\textsubscript{H9004} strain partially restored the growth on ethanol at the early stage (Fig. 4A), and this implies that the carbonic anhydrase activity or the subsequent HCO\textsubscript{3}\textsuperscript{-}/H\textsubscript{11002} concentration in the \textit{cst6}\textsubscript{H9004} strain is the limiting factor for the initial growth of the \textit{cst6}\textsubscript{H9004} strain on ethanol. The physiological role of Nce103p has been well documented: the \textit{NCE103} deletion mutant is inviable in atmospheric air but can be complemented by supplementing the medium with

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FIG 1 The identification of Cst6p binding sites by ChIP-exo. (A) Schematic representation of the ChIP-exo experiment. Cst6p with C-terminal tag is shown in the form of a homodimer. (B) Binding site motif enriched from the bound region sequences (\(E\) value \(= 8.9 \times 10^{-10}\)). (C) Distribution of the distances of binding events from the start codons of putative target genes. (D) Binding of Cst6p on the \textit{NCE103} promoter. (E) Binding of Cst6p on the \textit{ROX1} promoter. \textit{YPR064W} is a dubious gene upstream from \textit{ROX1}. Data shown in panels B to E are based on the binding events that occurred when cells were grown on ethanol. In panels D and E, the coverage graph showing the 5' ends of reads was created from the aligned BAM file using “Depth Graph (Start)” in IGB (46). The putative binding sites of Cst6p are marked by triangles. The core binding sequences of the ATF/CREB family TFs (consensus sequence, 5' TGACGT 3') are underlined.
some nutrients whose biosynthesis needs \( \text{HCO}_3^- \), such as fatty acids, \( \text{l}-\text{aspartic acid} \) (providing oxaloacetate), uracil, and \( \text{l}-\text{arginine} \) (Fig. 4C) (21). Since the supplementation of \( \text{l}-\text{aspartic acid} \) is not essential for the complementation of the \( \text{nce103}^- \) strain grown on ethanol (21), we chose fatty acids, uracil, and \( \text{l}-\text{arginine} \) to test whether they could also improve the growth of the \( \text{cst6}^- \) strain on ethanol. While the growth of the wild type was slightly affected by the supplementation of nutrients, the \( \text{cst6}^- \) strain did show enhanced growth (Fig. 4D).

\textbf{Cst6p is a member of the stress-responsive transcriptional regulatory network.} Since Cst6p and Aca1p bind to a sequence similar to the sequence bound by the stress-responsive Sko1p \textit{in vitro}, they were at first supposed to be involved in response to stress. However, phenotypic and gene expression analyses showed that Cst6p and Aca1p are not involved in the responses to various stresses (5). Another study reached a similar conclusion based on studies of osmotic stress but showed that the \( \text{cst6}^- \) strain is more sensitive to oxidative stress than the wild type (22). Both these studies used YPD medium, and an effect of the brand of yeast extract and peptone on phenotype was noticed in the latter study.

As our ChIP-exo results show that Cst6p binds to some stress-related genes during growth on ethanol, we wondered if Cst6p is involved in stress response under this condition. Growth test on ethanol showed that the \( \text{cst6}^- \) strain is more sensitive than the wild type to \( \text{H}_2\text{O}_2 \) (Fig. 5A) and high concentrations of ethanol (Fig. 5B). On glucose, the \( \text{cst6}^- \) strain showed sensitivity to \( \text{H}_2\text{O}_2 \) similar to that of the wild type (Fig. 5C). Hypersensitivity to ethanol stress was also observed for the \( \text{cst6}^- \) strain grown on glucose but was less apparent than when it was grown on ethanol (Fig. 5D).

This indicates that Cst6p is indeed a member of the stress-response regulatory network in \textit{S. cerevisiae}. We therefore explored the relationship between Cst6p and other members of this extensively studied network (23, 24). As mentioned above, \( \text{YAP1} \) and \( \text{YAP6} \), encoding stress-responsive TFs, are direct targets of Cst6p. Furthermore, overlap analysis of the Cst6p targets with the known bound targets of all the TFs in \textit{S. cerevisiae} suggests that Cst6p coregulates gene expression with 106 other TFs (see Table S4 in the supplemental material). Notably, among the top 10 TFs with the highest number of shared targets with Cst6p (Fig. 6A), five (\( \text{Sko1p, Msn2p, Skn7p, Cin5p, and Yap6p} \)) are known as stress-responsive TFs and two (\( \text{Ste12 and Sok2p} \)) are involved in the regulation of pseudohyphal growth. The targets of Cst6p with extensive coregulation include the peroxiredoxin-encoding \( \text{AHP1} \), heat shock protein-encoding \( \text{HSP12} \), molecular chaperone-encoding \( \text{MDJ1} \), and stress-responsive TF genes like \( \text{YAP6} \) (Fig. 6B).

Considering that Cst6p and Sko1p bind to the same sequence \textit{in vitro}, we compared the binding sites for Cst6p that we identified in the work presented here with those identified by chromatin immunoprecipitation sequencing (ChIP-seq) for Sko1p (8). For most of the shared targets, the ChIP-exo peak of Cst6p was found inside the broader ChIP-seq peak region of Sko1p. Combined with the result of binding motif search, the two TFs appear to bind to the same site (Fig. 6C shows an example). On the other hand, some well-studied direct targets of Sko1p, such as \( \text{GRE2} \) (22), were not identified as direct targets of Cst6p.

\textbf{DISCUSSION}

TFs in \textit{S. cerevisiae} have been studied extensively for their functions and the underlying molecular mechanisms. However, knowledge of the specific functions of some TFs is still limited, and therefore, we performed a genome-wide binding-site mapping of Cst6p. Using this information, we revealed its role in regulating
diverse cellular processes that can explain different phenotypes reported earlier and further studied here. For example, the binding of Cst6p to chromosome segregation-related genes CBF2, GIP3, and RMI1 could be linked to the chromosome instability of a CST6 overexpression mutant. Furthermore, we find that the regulatory function of Cst6p is highly condition dependent, as almost no binding event was identified in cells grown on glucose but several were identified in cells grown on ethanol.

From our analysis, the carbonic anhydrase gene NCE103 was for the first time identified as a direct target of Cst6p in S. cerevisiae, although a putative Cst6p binding site on its promoter has been shown to be responsible for the regulation by CO2 (12). From quantitative analysis of gene expression, we showed that Cst6p contributes to most of the activation of NCE103 expression for growth on ethanol (Fig. 3). This is consistent with the result in C. albicans, where Rca1p (ortholog of Cst6p) was identified as the only TF activating NCE103 expression by screening a TF knockout mutant library (12). Furthermore, the Cst6p regulation of NCE103 expression is the major determinant of the phenotype of a CST6 deletion strain grown on ethanol (Fig. 4A). While NCE103 is essential for HCO3− supply and growth under atmospheric air, the phenotype of the cst6Δ strain is only obvious at the early stage of the growth on ethanol. When ethanol is used as the sole carbon source, the slow CO2 production by respiration (Fig. 4C) and the markedly decreased Nce103p level may not be able to provide enough HCO3− for key biosynthetic reactions required for cell growth. The growth on glucose is almost not affected, probably due to the fact that NCE103 expression is not significantly affected during growth on this carbon source (Fig. 3) or because the rapid CO2 production from fermentation ensures sufficient provision of HCO3−. In C. albicans, growth delay in the early stage was also observed for the rca1Δ mutant grown on glucose, especially when a synthetic medium was used (13). Taken together, the results suggest that Cst6p/Rca1p is important for the growth of yeast under conditions with low carbonate levels and that this is due to its activation of the conserved target NCE103. From a biotechnological point of view, the expression level of the carbonic anhydrase gene NCE103 is a potential target for metabolic engineering to increase the production of related metabolites (Fig. 4C), and Cst6p could be used to design regulatory circuits that are responsive to CO2 levels.

The binding to stress-related genes and the results of stress resistance tests establish Cst6p as a stress-responsive TF. As seen by the results in Fig. 6B, the binding of several TFs to target genes associated with stress response makes the stress-responsive regulatory network in S. cerevisiae complex. Genetic analyses have
given greater functional understanding of this network: the con-
tributions of different TFs to the transcription of a specific gene
are quite different, and the regulatory effect is critically dependent
on the growth condition and type of stress (8, 22). From our re-
sults, Cst6p is positively involved in the expression of its stress-
related targets during growth on ethanol, where oxidative stress
might arise from respiration in the mitochondria (25). Except for
NCE103, the target genes identified have moderate decreases in
expression in the \textit{cst6}/H9004 strain (Fig. 3), indicating the presence of
other transcriptional activators or repressors controlling the ex-
pression of these genes. The number of binding sites, binding
strengths, and regulatory effects of Cst6p may increase if the cells
are exposed to a higher level stress (e.g., the addition of H$_2$O$_2$).

Previous studies showed that Cst6p, Aca1p, and their paralog
Sko1p have the same binding sequence \textit{in vitro}, and the activating
effects of Cst6p and Aca1p on basal expression of stress-
response genes are only observable in the \textit{sko1}/H9004 background (5,
22). Thus, a competition model between the three TFs was pro-
posed. Here, we provide \textit{in vivo} evidence for this model by show-
ing that Cst6p and Sko1p can bind at the same site on target pro-
moters (Fig. 6C). Considering that other TFs, such as Yap1p and
Skn7p, also bind directly to many of these genes (Fig. 6B), inte-
grated ChIP-exo and transcriptome analyses of all the TFs in-
volved under various stress conditions (26) would provide new
insight into this complex combinatorial regulation. On the other
hand, collected microarray data showed that the expression of
\textit{CST6} itself is hardly affected by environmental changes (27),
which suggests that Cst6p is connected to the transcriptional reg-
ulatory network in \textit{S. cerevisiae} mainly through its targets.

Cst6p/Rca1p was identified as a negative regulator of genes
involved in hyphal growth and cell adhesion in the human patho-
gens \textit{C. albicans} (13) and \textit{C. glabrata} (14). In \textit{C. albicans}, the tar-
gets of Rca1p identified from transcriptome analysis have a con-
siderable overlap with the targets of Efg1p, which is a major
activator of hyphal growth. Here, the functionally conserved ho-
mod of \textit{EFG1} in \textit{S. cerevisiae}, \textit{PHD1} (28), was identified as a
direct target of Cst6p. This provides a hint that Cst6p/Rca1p may
mediate the suppression of hyphal growth by low levels of CO$_2$
(13, 29) through Phd1p/Efg1p, which could be tested in \textit{Candida}
species. While Cst6p appears to activate the expression of \textit{PHD1}
(Fig. 3), both Cst6p and Phd1p may act as repressors in other
yeasts or under specific conditions (6).

Here, we demonstrated that ChIP-exo can be used as an effi-
cient method for mapping the precise locations where TFs bind to
the genome. The present work offers three lessons about the un-
derstanding of transcriptional regulatory network: (i) it is impor-
tant to use functionally active conditions to map the binding sites
of TFs, (ii) genome-wide mapping of targets can reveal novel
functions of TFs and suggest additional conditions for target map-
ping, and (iii) the transcriptional regulatory network may be far
more complex than previously imagined due to the extensive
combinatorial regulation and existence of TF cascades. Integrative
analysis of data from different types of study is therefore critically

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5}
\caption{The function of Cst6p in stress resistance. (A) Growth on 1\% (vol/vol) ethanol (EtOH) with or without supplementation of 0.5 mM H$_2$O$_2$. (B) Growth
on different concentrations of ethanol. (C) Growth on 2\% (wt/vol) glucose with or without supplementation of H$_2$O$_2$. (D) Growth on 2\% (wt/vol) glucose with
or without supplementation of ethanol. The data represent the average results and standard deviations from biological duplications.}
\end{figure}
transforming CEN.PK 113-5D with a *Kluyveromyces lactis* URA3 (KlURA3) marker gene (from vector pWW01042 [31]) flanked by 45-bp upstream and downstream sequences of the CST6 coding region and screening on synthetic complete medium lacking uracil (SC−Ura; Formedium). Similarly, the prototrophic wild-type strain was constructed by integrating KIURA3 into the *ura3*-52 locus of CEN.PK 113-5D by homologous recombination. The tagged strain CST6-TAP was constructed by integrating a tagging cassette containing the tandem affinity purification (TAP) tag CBP-ProtA coding sequence and KIURA3 (as described in reference 32) into the CST6 locus, allowing the tag to be fused in-frame to the C-terminal end of Cst6p, connected by a six glycine linker (G6). For constitutive expression of NCE103 in the CST6 deletion background, CST6 in CEN.PK 113-5D was first replaced by a 159-bp fragment downstream from CST6, followed by KIURA3 to obtain strain *cst6ΔU*, and then the KIURA3 marker gene in *cst6ΔU* was looped out by the homologous recombination of the two direct-repeat 159-bp fragments on 3-fluoroorotic acid medium to obtain strain *cst6ΔL*. Plasmid pRS416-NCE103, constructed by inserting the NCE103 gene flanked by the TEF1 promoter and its own terminator between the XbaI and KpnI sites of a centromere plasmid, pRS416 (33), was transformed into *cst6ΔL* to get the *cst6Δ(NCE103)* strain. All the printers for cassette constructions and PCR identifications are listed in Table S5 in the supplemental material.

**Media and cultivations.** Single colonies from fresh agar plates were inoculated into 3 ml YPD medium or minimal medium (as described below) with 2% (wt/vol) glucose in tubes and grown for 12 to 24 h. Cells were harvested by centrifugation and resuspended in sterile water to obtain precultures. For growth in liquid medium (total volume of 20 ml in 100-ml shake flasks), strains were cultivated on a rotary shaker at 200 rpm and 30°C in minimal medium containing (liter−1) 7.5 g (NH₄)₂SO₄, 14.4 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, vitamins and trace metals as used in reference 34, and carbon sources as indicated above. For nutrient supplementation, a 100× stock containing 50 mM palmitic acid and 50 mM stearic acid dissolved in 1:1 (vol/vol) ethanol-Tween 20 was added to get a final concentration of 0.5 mM of each fatty acid, and uracil and l-arginine were added to a final concentration of 20 mg liter−1 of each compound.

For growth assays on agar plates, cell suspensions were adjusted to an optical density at 600 nm (OD₆₀₀) of 1.0 and diluted four times in 10-fold series. Three microliters of each dilution was spotted onto SC−Ura; Formedium) supplemented with carbon sources as indicated above, and photographs were taken after 2 to 3 days of growth at 30°C.

**ChIP-exo.** Cells were cultivated in shake flasks in minimal medium with 2% (wt/vol) glucose or 1% (vol/vol) ethanol as the sole carbon source to an OD₆₀₀ of 1.5 to 1.8 (mid-log phase). Formaldehyde with a final concentration of 1% (wt/vol) and distilled water were added to the cultures to cross-link protein-DNA complexes with an OD₆₀₀ of 1.0 and a total volume of 100 ml. Cross-linking was performed for 12 min at room temperature with shaking and quenched by adding 2.5 M glycine to a final concentration of 125 mM. After 5 min, cells were washed twice with 20 ml cold TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) and frozen with liquid nitrogen.

ChIP-exo was performed according to the methods in references 35 and 36, with some modifications. Briefly, cells were disrupted with glass beads on a FastPrep 24 (MP Biomedicals) and the crude cell lysate was sonicated to shear chromatin, using a Branson digital Sonifier 250 (Branson Ultrasonics). After centrifugation, the supernatant, containing chromatin fragments, was applied to IgG Sepharose 6 fast flow

TABLE 1 Strains used in this study

<table>
<thead>
<tr>
<th>Strain or genotype</th>
<th>Genotype or description</th>
</tr>
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<tbody>
<tr>
<td>CEN.PK 113-5D</td>
<td>MATa SUC2 MAL2-8′ ura3-52</td>
</tr>
<tr>
<td><em>cst6Δ</em></td>
<td>CEN.PK 113-5D *cst6Δ:*KIURA3</td>
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<tr>
<td>Wild type</td>
<td>CEN.PK 113-5D *ura3-52:*KIURA3</td>
</tr>
<tr>
<td>CST6Δ-TAP</td>
<td>CEN.PK 113-5D CST6-G6-TAP:KIURA3</td>
</tr>
<tr>
<td><em>cst6Δ(NCE103)</em></td>
<td>CEN.PK 113-5D <em>cst6Δ</em> pRS416-P_TEF1−NCE103</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**Strains.** All the *S. cerevisiae* strains used in this study (Table 1) are derived from the uridine auxotrophic strain CEN.PK 113-5D (30), provided by P. Kötter (Frankfurt, Germany). The *cst6Δ* strain was constructed by

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**FIG 6** Cst6p as a member in the stress-response transcriptional regulatory network. (A) Top 10 TFs with the highest number of targets shared with Cst6p. All the coregulatory TFs and their shared targets with Cst6p are listed in Table S4 in the supplemental material. (B) Heat map showing coregulation of Cst6p targets. For regulators, 7 of the TFs in panel A and Phd1p are included. For target genes, the nine regulators themselves and Cst6p targets encoding TFs or stress-related proteins are included. Targets with known functions in stress response are marked by dots. Filled squares indicate TF-target binding relationships. (C) Binding of Cst6p (on ethanol) and Sko1 (under 0.4 M KCl stress) at the SKSI-RMI1 divergent promoter region. The putative binding site of ATF/CREB family TFs (S′ TGACGT 3′) in this region is marked by the triangle.

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needed in order to reconstruct complete transcriptional regulatory networks.
beads (GE Healthcare) for immunoprecipitation at 4°C with gentle rocking overnight. NEBNext end repair module, NEBNext dA-tailing module, NEBNext quick ligation module, PreCR repair mix, lambda exonuclease, and RecI (all from New England Biolabs) were used for on-bead end repair, incorporation of 3′-d(A) DNA tails, first adaptor ligation, nick filling, and chloramid trimming, respectively. The first adaptors contain unique 6-bp index sequences (see Table S6 in the supplemental material). To elute and reverse cross-link the bound complexes, TE buffer containing 1% SDS was added to the beads and samples were incubated overnight at 65°C. After protease K (Thermo Scientific) digestion and DNA extraction with phenol-chloroform-isomyl alcohol (Amresco, USA), the single-strand DNAs were subjected to primer extension using phi29 DNA polymerase (New England Biolabs). The products were given d(A) tails and ligated with the second adaptor using the same reagents as in on-bead reactions and then amplified by PCR for 20 to 22 cycles using Phusion high-fidelity DNA polymerase (New England Biolabs). The GeneRead size selection kit (Qiagen) was used to purify DNA before and after the second incorporation of d(A) tails, second adaptor ligation, and PCR. The final DNA samples were measured by using a Qubit double-stranded DNA (dsDNA) high-sensitivity (HS) assay kit (Thermo Scientific) and 2200 TapeStation (Agilent Technologies), pooled in equimolar amounts, and sequenced on the NextSeq 500 system (2 × 75 bp, mid-output mode; Illumina). All adapters and primers used in ChIP-exo are listed in Table S6.

ChIP-exo sequencing reads were mapped to reference genome assemblies R64-2-1 of S. cerevisiae S288C with Bowtie2 (37) using the default settings to generate Sequence Alignment/Map (SAM) files. SAM files were treated with SAMtools (38) option -q 20 to remove low-quality reads and then converted to sorted BAM files. BAM files were trimmed 70 bp from the 3′ end using trimBam (http://genome.sph.umich.edu/wiki/BamUtil:_trimBam) to increase the resolution. To identify peaks and compare biological duplicates, the program GEM (39) was used. The noise level was calculated from averaged noise throughout each replicate. Binding events were manually curated in the Integrative Genomics Viewer (40) to remove redundant events representing the same binding and false-positive events with poor peak shape or poor duplication (41). Identification of target genes was done with BEDTools (42) using the closest function with the parameters -io -iU -s or -io -id -S to find any downstream or upstream targets, respectively. Gene targets with a distance of more than 1,200 bp from the binding event were sorted out.

Bioinformatic analyses. The binding site motif was enriched by analyzing the 100-bp sequences upstream and downstream from binding sites using MEME-ChIP 4.10.2 (43). The putative binding sites of Sko1p on promoters were predicted on the YeTFaSCo website (18). The overlaps between Cst6p targets and targets of other TFs were obtained using the “Rank Genes by TF” tool in Mapper from SGD (27). The overlaps between Cst6p targets and targets of other TFs were obtained using the “Rank Genes by TF” tool in YeASTRACT (44).

Quantitative real-time PCR. Cells were cultivated to an OD600 of 1.5 to 1.8 (mid-log phase) in minimal medium with 2% (wt/vol) glucose or 1% (vol/vol) ethanol as the sole carbon source, harvested by centrifugation after mixing with crushed ice, frozen in liquid nitrogen, and then stored at −80°C. A FastPrep-24 homogenizer (MP Biomedicals) was used to disrupt cells, and total RNA was isolated using the RNeasy minikit (Qiagen). cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen). For quantitative PCR (qPCR), the DyNaMo flash SYBR green qPCR kit (Thermo Scientific) was used with a reaction mixture volume of 20 μL. Previously published primers for CST6 and NCE103 (12) were used, while primers for other genes were designed using IDT’s PrimerQuest tool. All the primers in qPCR are listed in Table S5 in the supplemental material. The PCR was performed on the Mx3005P qPCR system (Agilent Technologies). The thermal program consisted of an initial denaturation of 15 min at 95°C, 40 cycles of 10 s at 95°C and 30 s at 60°C, and a final segment of 1 min at 95°C and 30 s at 55°C followed by a ramp up to 30 s at 95°C (for the dissociation curve). ACT1 was used as the reference gene for gene expression level comparison using the cycle threshold (ΔΔCt) method (45). Statistical significance tests were done with a one-tailed equal variance (homoscedastic) t-test in Microsoft Excel 2013.

Sequence data accession number. The ChIP-exo data have been deposited in the Gene Expression Omnibus database under the accession number GSE76154.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl doi:10.1128/mBio.00559-16/-/DCSupplemental.

Figure S1, DOCX file, 0.3 MB.
Table S1, XLSX file, 0.01 MB.
Table S2, XLSX file, 0.02 MB.
Table S3, XLSX file, 0.01 MB.
Table S4, XLSX file, 0.01 MB.
Table S5, DOCX file, 0.02 MB.
Table S6, DOCX file, 0.01 MB.

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REFERENCES

 Binding Sites and Functions of Cst6p in Yeast


