The TEA Transcription Factor Tec1 Confers Promoter-Specific Gene Regulation by Ste12-Dependent and -Independent Mechanisms

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In Saccharomyces cerevisiae, the TEA transcription factor Tec1 is known to regulate target genes together with a second transcription factor, Ste12. Tec1-Ste12 complexes can activate transcription through Tec1 binding sites (TCSs), which can be further combined with Ste12 binding sites (PRES) for cooperative DNA binding. However, previous studies have hinted that Tec1 might regulate transcription also without Ste12. Here, we show that in vivo, physiological amounts of Tec1 are sufficient to stimulate TCS-mediated gene expression and transcription of the FLO11 gene in the absence of Ste12. In vitro, Tec1 is able to bind TCS elements with high affinity and specificity without Ste12. Furthermore, Tec1 contains a C-terminal transcriptional activation domain that confers Ste12-independent activation of TCS-regulated gene expression. On a genome-wide scale, we identified 302 Tec1 target genes that constitute two distinct classes. A first class of 254 genes is regulated by Tec1 in a Ste12-dependent manner and is enriched for genes that are bound by Tec1 and Ste12 in vivo. In contrast, a second class of 48 genes can be regulated by Tec1 independently of Ste12 and is enriched for genes that are bound by the stress transcription factors Yap6, Nrg1, Cin5, Skn7, Hsf1, and Msn4. Finally, we find that combinatorial control by Tec1-Ste12 complexes stabilizes Tec1 against degradation. Our study suggests that Tec1 is able to regulate TCS-mediated gene expression by Ste12-dependent and Ste12-independent mechanisms that enable promoter-specific transcriptional control.

In Saccharomyces cerevisiae and related yeast species, the transcription factors Tec1 and Ste12 are a paradigm for studying the mechanisms of combinatorial and promoter-specific target gene control (5, 30, 37, 53). These DNA binding proteins can interact with each other and control various developmental programs, including vegetative adhesion, filament formation, and pheromone-induced sexual mating (13, 15–17, 35, 37, 41, 42, 46). Tec1 belongs to the family of the TEA transcription factors, which control several transcriptional programs governing cellular differentiation in many eukaryotes. Common to this family is the TEA DNA binding domain (DBD) composed of a three-helix bundle, which binds to conserved TEA consensus sequence (TCS) elements (2, 37). S. cerevisiae Ste12 is the founding member of the Ste12-like transcription factors that are found preferentially in fungi, where they control cellular development and pathogenesis (15, 33, 34, 50). Ste12 binds to pheromone response elements (PREs), and two or more of these elements are necessary to confer pheromone-responsive transcriptional control by the Fus3/ Kss1 mitogen-activated protein kinase (MAPK) pathway (13, 14, 23, 52).

For combinatorial target gene control, Tec1 and Ste12 form a complex through the C-terminal part of Tec1 (10). For target gene binding, initial in vitro studies have shown that Tec1-Ste12 complex formation enables cooperative binding to combined filamentation and invasion response elements (FREs), which consist of a TCS and a PRE (37). More recent studies show that the promoter regions of many Tec1 target genes do not contain FREs but contain one or several TCSs (10). In addition, Tec1 and Ste12 are often present at the same promoters in vivo (5, 6, 53). It has thus been suggested that Tec1-Ste12 complexes regulate transcription predominantly via TCS elements, with Tec1 providing the DNA binding domain and Ste12 the transcriptional activation domain (AD) (10). Tec1-Ste12 complexes can be further regulated by the protein Dig1, whose inhibitory function on Ste12 is relieved during mating by phosphorylation through the pheromone-stimulated MAPKs Fus3 and Kss1 (4, 10, 12, 45, 49). In addition, Tec1 is directly phosphorylated by the MAPK Fus3 in response to pheromone, which triggers ubiquitin-mediated degradation of the transcription factor (3, 8, 9). Finally, Ste12 directly controls TEC1 gene expression via PREs, and TEC1 transcript levels are pheromone inducible (43). As a consequence, TEC1 transcript and Tec1 protein levels are drastically reduced in the absence of Ste12 (30).

In the study presented here, we have explored the possibility that Tec1 confers Ste12-independent transcriptional regulation, because we have previously shown that overexpression of TEC1 in ste12Δ mutant strains leads to activation of TCS-driven reporter genes (30). We now demonstrate that physiological levels of Tec1 can bind to TCS elements in vitro and regulate TCS-driven genes in vivo in the absence of Ste12. On a genome-wide scale we show that Tec1 is able to control a subset of its target genes without Ste12 through a C-terminal transcriptional activation domain. We also show that complex formation between Tec1 and Ste12 provides Tec1 stability control. Our study suggests that Tec1 is able to regulate TCS-mediated transcription by Ste12-dependent as well as Ste12-independent mechanisms to confer promoter-specific control.
Yeast strains and growth conditions. All yeast strains used in this study are listed in Table 1. Yeast strains carrying the dig1::kanMX4 deletion allele were obtained by transformation with a corresponding deletion cassette and verified by Southern blot analysis. Different TEC1 versions and TEC1-STE12 hybrid constructs were introduced into the yeast genome by targeting of the appropriate plasmid to the leu2::hisG locus. TCS and PRE reporter gene plasmids were targeted to the ura3-52 locus. Yeast culture medium was prepared as described previously (21). Adhesive growth tests were as described earlier (46). For cycloheximide-induced translational shutoff experiments, yeast cultures grown to an

| Strain            | Relevant genotype | Reference |
|-------------------|-------------------|-----------|
| RH2500            | MATa tec1Δ::HIS3  | 30        |
| RH2501            | MATa tec1Δ::HIS3  | 30        |
| RH2765            | RH2500 with TCS-CYC1-lacZ::URA3 | 30 |
| RH2767            | RH2500 with CYC1(ΔUA5)-lacZ::URA3 | 30 |
| RH2768            | RH2500 with TCS-PRE-CYC1-lacZ::URA3 | 30 |
| RH2774            | RH2501 with TCS-CYC1-lacZ::URA3 | 30 |
| RH2806            | RH2501 with TCS-PRE-CYC1-lacZ::URA3 | 30 |
| EGY48-p1840       | MATa ura3 his3 trp1 leu2 lexAop-lacZ::URA3 | 22 |
| YHUM1631          | RH2765 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1634          | RH2765 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1635          | RH2765 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1640          | RH2774 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1641          | RH2774 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1642          | RH2774 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1643          | RH2774 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1644          | RH2774 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1645          | RH2774 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1646          | RH2774 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1647          | RH2774 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1648          | RH2774 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1649          | RH2774 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1656          | RH2501 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1659          | RH2500 with P\_TEC1::TEC1::LEU2 | This study |
| YHUM1696          | RH2500 with tcs\_ACATCTTT-CYC1-lacZ::URA3 | This study |
| YHUM1697          | RH2500 with tcs\_ACATCTTT-CYC1-lacZ::URA3 | This study |
| YHUM1698          | RH2500 with tcs\_ACATCTTT-CYC1-lacZ::URA3 | This study |
| YHUM1699          | RH2500 with tcs\_ACATCTTT-CYC1-lacZ::URA3 | This study |
| YHUM1700          | MATa tec1Δ::HIS3  | 30        |
| YHUM1701          | MATa tec1Δ::HIS3  | 30        |
| YHUM1712          | RH2774 with P\_TEC1::TEC1::LEU2 | This study |
| YHUM1713          | RH2774 with P\_TEC1::TEC1::LEU2 | This study |
| YHUM1718          | YHUM1676 with TCS-CYC1-lacZ::URA3 | This study |
| YHUM1720          | YHUM1649 with dig1::kanMX4 | This study |
| YHUM1721          | MATa tec1Δ::HIS3  | 30        |
| YHUM1722          | MATa tec1Δ::HIS3  | 30        |
| YHUM1723          | MATa tec1Δ::HIS3  | 30        |
| YHUM1724          | MATa tec1Δ::HIS3  | 30        |
| YHUM1727          | MATa tec1Δ::HIS3  | 30        |
| YHUM1731          | RH2500 with tcs\_ACATCTTT-CYC1-lacZ::URA3 | This study |
| YHUM1732          | RH2500 with tcs\_ACATCTTT-CYC1-lacZ::URA3 | This study |
| YHUM1733          | RH2776 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1734          | RH2777 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1735          | RH2777 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1736          | RH2776 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1737          | RH2777 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1738          | RH2777 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1739          | RH2777 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1740          | RH2776 with P\_IRAS\_TEC1::LEU2 | This study |
| YHUM1743          | YHUM1727 with dig1::kanMX4 | This study |
| YHUM1744          | YHUM1718 with dig1::kanMX4 | This study |
| YHUM1745          | YHUM1644 with dig1::kanMX4 | This study |
| YHUM1746          | YHUM1645 with dig1::kanMX4 | This study |
| YHUM1747          | YHUM1648 with dig1::kanMX4 | This study |
| YHUM1749          | RH2768 with P\_TEC1::TEC1::LEU2 | This study |
| YHUM1750          | RH2777 with P\_TEC1::TEC1::LEU2 | This study |
| YHUM1751          | RH2777 with P\_TEC1::TEC1::LEU2 | This study |
| YHUM1752          | RH2777 with P\_TEC1::TEC1::LEU2 | This study |
| YHUM1846          | RH2777 with P\_TEC1::TEC1::LEU2 | This study |
| YHUM1847          | RH2777 with P\_TEC1::TEC1::LEU2 | This study |
| YHUM1848          | RH2777 with P\_TEC1::TEC1::LEU2 | This study |
| YHUM1849          | RH2777 with P\_TEC1::TEC1::LEU2 | This study |
TABLE 2. Plasmids used in this study

| Plasmid          | Genotype* | Reference |
|------------------|-----------|-----------|
| BHUM29           | PGAL1-TEC1 in pRS316 | 30         |
| BHUM30           | TEC1 in pRS202 | 30         |
| BHUM212          | pLG669-Z with TCS-PRE-CYC1-lacZ | 37         |
| BHUM213          | pLG669-Z with TCS-pre-CYC1-lacZ | 37         |
| BHUM214          | pLG669-Z with tcs-PRE-CYC1-lacZ | 37         |
| BHUM388          | MBP-TEC1-FLAG in pMAL-c2 | 37         |
| BHUM389          | MBP-STE12-FLAG in pMAL-c2 | 37         |
| BHUM752          | TEC1Bgl280Xho377 in pEG202 | This work |
| BHUM1258         | P_URA3-TEC1 in Ylplac128 | This work |
| BHUM1259         | P_URA3-TEC1 in Ylplac128 | This work |
| BHUM1347         | P_URA3-TEC1 in Ylplac128 | This work |
| BHUM1350         | P_URA3-TEC1 in Ylplac128 | This work |
| BHUM1357         | P_URA3-TEC1 in Ylplac128 | This work |
| BHUM1358         | P_URA3-TEC1 in Ylplac128 | This work |
| BHUM1359         | P_URA3-TEC1 in Ylplac128 | This work |
| BHUM1360         | P_URA3-TEC1 in Ylplac128 | This work |
| BHUM1362         | P_URA3-TEC1 in Ylplac128 | This work |
| BHUM1363         | P_URA3-TEC1 in Ylplac128 | This work |
| BHUM1364         | P_URA3-TEC1 in Ylplac128 | This work |
| BHUM1365         | P_URA3-TEC1 in Ylplac128 | This work |
| BHUM1384         | MBP-TEC1R164A in pMAL-c2 | This work |
| BHUM1388         | MBP-TEC1R164A in pMAL-c2 | This work |
| BHUM1389         | MBP-TEC1R164A in pMAL-c2 | This work |
| BHUM1401         | MBP-TEC1R164A in pMAL-c2 | This work |
| BHUM1404         | MBP-TEC1R164A in pMAL-c2 | This work |
| BHUM1405         | MBP-TEC1R164A in pMAL-c2 | This work |
| BHUM1406         | MBP-TEC1R164A in pMAL-c2 | This work |
| BHUM1480         | MBP-TEC1K644-K644 in pMAL-c2 | This work |
| BHUM1482         | MBP-TEC1K644-K644 in pMAL-c2 | This work |
| BHUM1483         | MBP-TEC1K644-K644 in pMAL-c2 | This work |
| BHUM1484         | MBP-TEC1K644-K644 in pMAL-c2 | This work |
| BHUM1616         | MBP-TEC1K644-K644 in pMAL-c2 | This work |
| BHUM1622         | MBP-TEC1K644-K644 in pMAL-c2 | This work |
| BHUM1623         | MBP-TEC1K644-K644 in pMAL-c2 | This work |
| BHUM1624         | MBP-TEC1K644-K644 in pMAL-c2 | This work |
| BHUM1625         | MBP-TEC1K644-K644 in pMAL-c2 | This work |
| BHUM1626         | MBP-TEC1K644-K644 in pMAL-c2 | This work |
| BHUM1627         | MBP-TEC1K644-K644 in pMAL-c2 | This work |
| BHUM1687         | MBP-TEC1K644-K644 in pMAL-c2 | This work |
| pEG202           | HIS3-based two-hybrid vector with lex4-DBD | 22         |
| pGI4-5           | TRP1-based two-hybrid vector with B42-AD | 22         |
| pLI4             | CYC1-lacZ in URA3-based integrative vector | 30         |
| pME2051          | CYC1-lacZ in URA3-based integrative vector | 30         |
| pME2055          | CYC1-lacZ in URA3-based integrative vector | 30         |
| pME2068          | CYC1-lacZ in URA3-based integrative vector | 30         |
| pME2102          | CYC1-lacZ in URA3-based integrative vector | 30         |
| pME2289          | CYC1-lacZ in URA3-based integrative vector | 30         |
| Ylplac128        | LEU2-based integrative vector | 19         |
| YCplac111        | LEU2-based CEN vector | 19         |
| YEpplac195       | URA3-based 2μm vector | 19         |

* TCS-PRE corresponds to the sequence of FREG(Tyl) (37).

optical density at 600 nm (OD600) of 1 were treated with 200 μg/ml cycloheximide and samples were taken at the indicated time points.

**Plasmids.** All plasmids used in this study are listed in Table 2. Plasmids BHUM1622 to BHUM1627, carrying point-mutated TCS elements upstream of the CYC1-lacZ reporter gene, were obtained by substituting a 430-bp XhoI fragment containing the CYC1 UAS of plasmid pLI4 for synthetic linkers with the indicated mutated TCS element and Xho-coding ends. Linkers were prepared by annealing of the corresponding primers (see Table S3 in the supplemental material), and a single insertion was confirmed by sequencing. Plasmids BHUM1384, BHUM1388, BHUM1390, BHUM1394, and BHUM1401 were obtained by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and BHUM388 as the template. Similarly, plasmids BHUM1480 to BHUM1484 were obtained by site-directed mutagenesis using pME2289 as the template. Plasmid BHUM1616 was constructed by introduction of a 3.1-kb PstI/BamHI fragment from pME2068, carrying PTEC1, into Ylplac128. For BHUM1258, BHUM1259, BHUM1347, BHUM1350, BHUM1357 to BHUM1365, BHUM1464, and BHUM1465, the URA3 promoter region was amplified from the yeast genome by PCR with primers BHURA3-1 and BHURA3-2, yielding a 430-bp product with a newly created upstream SphII site and a new Sall site behind the AGT start codon. The fragment was inserted into Ylplac128 to obtain plasmid Ylplac128-P_URA3. A 2.1-kb SalI/BamHI fragment carrying TEC1 or TEC1Bgl280Xho377 was released from pME2068 or pME2102 and placed downstream of the TEC1 promoter to create BHUM1258 and BHUM1259. Plasmids with truncated TEC1 versions and TEC1-STE12 fusions were constructed by the following strategy: (1) First, a BgII restriction site was inserted into the TEC1 open reading frame (ORF) behind codon 280 by whole-vector PCR with primers JFTEC1-280-1 and -2 using the 2.1-kb SalI/BamHI TEC1 ORF from pME2068 inserted into pBluescript II KS+ as a template. The PCR product served as a whole-vector PCR template to introduce an additional XhoI site behind codon 377 (primers JFTEC1-377-1 to JFTEC1-377-2) using Phusion HI and XhoI. The ORF was then amplified by PCR with primers JFTEC1-280-2 and -3 using the 2.1-kb SalI/BamHI TEC1 ORF from pME2068 inserted into pBluescript II KS+ as a template. The PCR product served as a whole-vector PCR template to introduce an additional XhoI site behind codon 377 (primers JFTEC1-377-1 to JFTEC1-377-2) using Phusion HI and XhoI.
and -2) or behind codon 486 (primers JFTEC1-486-1 and -2). Mutagenized TEC1 ORFs were released by SalI/BamHI digestion and inserted into Yplac128-PGAL1. To replace a natural XhoI site in the TEC1 terminator region by BamHI, PGAL1-TEC1/10262Xho/974 and PGAL1-TEC1/10262Xho/877 were amplified from these plasmids with primers BHURA3-1 and JFTEC1-1918-BamHI, resulting in 2.4-kb SphI/BamHI cassettes that were subcloned into Yplac128BL to obtain BHUM1347 and BHUM1350. (ii) BHUM1344 and BHUM1465 were obtained by replacing the BglII/Xhol TEC1 fragments in BHUM1347 and BHUM1350 by a synthetic linker which was prepared by annealing of primers BglII/Xhol linker 1 and BglII/Xhol linker 2. (iii) STE12 ORF fragments were amplified from the yeast genome by PCR with primers generating a new BglII site at the 5’ end and a Xhol site at the 3’ end of each product. To obtain the TEC1-STE12 fusion constructs BHUM1357 to BHUM1365, STE12 fragments were inserted into BglII/Xhol sites of plasmids BHUM1347 and BHUM1350.

Protein analysis. (i) Preparation of yeast cell extracts. Preparation of total yeast cell extracts was performed as previously described (32, 51). Briefly, yeast cultures grown to an OD600 of 1 were concentrated to 1 ml, mixed with 150 μl lysis buffer (1.85 M NaOH, 7.5% [vol/vol] dimethylsulfoxide, and incubated on ice for 10 min. Samples were mixed with 150 μl trichloroacetic acid (55%) and centrifuged at 13,000 rpm, 30 min at 4°C. One hundred microliters of the supernatant containing the target proteins was obtained and resuspended in collection buffer (50 mM Tris·HCl [pH 7.5], 500 mM NaCl, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) and disrupted in a fluidizer. GST-Tec11–280 was purified by column chromatography on a glutathione-Sepharose column, and the glutathione-S-transferase (GST) tag was proteolytically removed by TEV protease digestion and Ni-nitrilotriacetic acid (NTA) purification. Polyclonal anti-Tec11–280 antibodies were produced by standard immunization procedures by the Pineda Antibody Service (Berlin, Germany) to obtain polyclonal rabbit anti-Tec11–280 antibodies.

(ii) Immunoblot analysis. Equal amounts of proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. TEC1 and TEC1-Ste12 fusion proteins or Tub1 was detected using an anti-TEC1 polyclonal antibody (Novagen, NJ) containing a C-terminal Epitope-Tagging (PET) epitope and a mouse monoclonal anti-Tub1 antibodies (Calbiochem, Darmstadt, Germany). As secondary antibodies, horseradish peroxidase-coupled goat anti-rabbit (Santa Cruz, CA) or goat anti-mouse (Dianoova, Hamburg, Germany) antibodies were used, respectively. Signals were quantified using a scanner and the ImageQuant TL software (GE Healthcare, Freiburg, Germany).

Electrophoretic mobility shift assays. Maltose binding protein (MBP), MBP-Tec11–280, MBP-Tec11–280, and MBP-Maltose binding protein (MBP) were prepared by expression in E. coli JM109/pMAL-c2, BHUM388, and BHUM389 essentially as described earlier (37). E. coli protein extracts containing different TEC1 variants were obtained using appropriate plasmids. 32P-labeled DNA was prepared by PCR amplification of 97-bp (TCS), 113-bp (TCS-TCS), or 111-bp (TCS-PRE, TCS-pre, and tcs-PRE) DNA fragments using the primer pair BHCYC1-2/BHCYC1-3 and plasmid YHUM1642 and plasmid pMG4-5 (22) was cultivated to exponential growth in liquid SC-Tre. His medium supplemented with 2% galactose before β-galactosidase was measured as described above.

Microarray analysis. Transcriptional profiling of yeast strains YHUM1642, YHUM1676, YHUM1694, YHUM1700, and YHUM1701 was performed using Yeast Genome 2.0 expression arrays (Affymetrix, High Wycombe, United Kingdom) following standard protocols. Yeasts were grown in synthetic complete medium lacking leucine and histidine with 2% glucose. For each strain two independent colonies were used. Overnight cultures were diluted into 30 ml of fresh medium to an OD600 of 0.25 and grown to an OD600 of 1 at 30°C before cells were harvested by centrifugation and rapidly frozen in liquid nitrogen. Total RNA was prepared using a RNeasy mini kit (Qiagen, Hilden, Germany) following Yeast Protocol 1c for mechanical disruption. RNA yield and purity were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). All subsequent steps were conducted according to the Affymetrix Gene Chip Expression Technical Manual (Affymetrix, High Wycombe, United Kingdom). Briefly, one cycle of cDNA synthesis was performed with 8 μg of total RNA. In vitro transcription labeling was carried out for 16 h. The fragmented samples were hybridized for 16 h on Affymetrix Yeast Genome 2.0 expression arrays, washed, and stained using the GeneChip Hybridization, Wash, and Stain Kit (PN 900720) and an Affymetrix Fluidics 450 station. Arrays were scanned on an Affymetrix GeneArrayScanner 3000 7G, and nonscaled RNA signal intensity (CEL) files were generated using the Affymetrix GeneChip Expression Console software. The resulting CEL files were loaded into the Affymetrix Expression Console software, and CHP files were created using Quantile normalization and Probe Logarithmic Intensity Error Estimation (PLIER). Differentially expressed genes were defined as having an average signal intensity of more than 50 and a fold change of at least 1.5 (calculated as the fold change of the average expression in the duplicate measurements).

Quantitative real-time PCR. Quantitative real-time PCR was performed by reverse transcription of 8 μg of total RNA into cDNA using the GeneChip Expression 3’-Amplification One Cycle cDNA synthesis kit (Affymetrix, High Wycombe, United Kingdom). Quantitative real-time PCR experiments were performed in duplicate in 96-well plates using the MyQ single-color real-time PCR detection system (Bio-Rad, Munich, Germany). ΔΔCt method using plasmid pMG4-5 was used. mRNA expression was determined using the same cDNA for both the gene of interest and CDC28 as a reference. Gene-specific primers are shown in Table S3 in the supplemental material. The reaction mix (25-μl final volume) consisted of 12.5 μl of 2× iQ SYBR green supermix (Bio-Rad, Munich, Germany), 1 μl of each primer (0.4 μM final concentration), 0.5 μl fluorescein isothiocyanate (FITC) (0.02 μM final concentration), 9 μl H2O, and 1 μl of a 1/10 dilution of the cDNA preparation. A control without template was incorporated in each assay. The thermocycling program consisted of one hold at 95°C for 3 min, followed by 45 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. After completion of these cycles, melting curve data were collected to determine PCR specificity, contamination, and the absence of primer dimers. The detection of the threshold cycle by was done automatically by the cycler software. Quantification of gene expression was carried out with the Gene Expression Analysis for iQ real-time PCR detection system software (Bio-Rad, Munich, Germany).

Microarray data accession number. Array data are available from the ArrayExpress database (http://www.ebi.ac.uk) under accession number E-MEXP-2207.

RESULTS

In vivo, physiological measurements of Tc1 are sufficient to activate TCS-mediated gene expression and FLO11-mediated adhesion in the absence of Ste12. We have previously shown that Tc1 is able to activate expression of TCS-driven reporters and other reporters
natural target genes in ste12Δ mutant strains (30), suggesting that Tec1 can regulate transcription independently of Ste12. These former experiments were done by expression of TEC1 from high-copy plasmids, because efficient TEC1 transcription depends on Ste12. To more accurately measure the influence of Ste12 on the transcriptional activity of Tec1 in vivo, we created yeast strains that contain physiological amounts of Tec1 even when Ste12 is absent. These strains carry a single genomic copy of TEC1 driven from the Ste12-independent URA3 promoter (PURA3-TEC1) and carrying integrated TCS- or FRE-driven CYC1-lacZ genes. Tec1 protein levels were determined by quantitative immunoblot analysis using specific antibodies against Tec1 and Tub1 as an internal control and are indicated as percentages of Tec1 protein present in a control TEC1 STE12 strain. Relative reporter gene activation corrected to Tec1 protein levels is shown in italic. (B) Adhesive growth of yeast strains of the indicated genotypes was determined by growth on solid SC-Ura medium. Plates were photographed before (total growth) and after (adhesive growth) removal of nonadhesive cells by a wash assay. For high-copy TEC1 expression (hc TEC1) plasmid BHUM30 was used; all other strains carry control plasmid YEplac195. (C) FLO11 expression in strains of the indicated genotypes was determined by quantitative real-time PCR. Expression is presented as the percentage of the level measured in a TEC1 STE12 control strain, which was set to 100. Error bars indicate standard deviations.

FIG. 1. Activation of TCS- and FRE-driven reporter genes and FLO11-dependent adhesion by Tec1 in the presence and absence of Ste12. (A) Reporter gene expression was determined by measuring β-galactosidase activities in STE12 or ste12Δ strains expressing no TEC1 (tec1Δ) or single genomic copies of TEC1 driven from the endogenous promoter (TEC1) or the Ste12-independent URA3 promoter (PURA3-TEC1) and carrying integrated TCS- or FRE-driven CYC1-lacZ genes. Tec1 protein levels were determined by quantitative immunoblot analysis using specific antibodies against Tec1 and Tub1 as an internal control and are indicated as percentages of Tec1 protein present in a control TEC1 STE12 strain. Relative reporter gene activation corrected to Tec1 protein levels is shown in italic. (B) Adhesive growth of yeast strains of the indicated genotypes was determined by growth on solid SC-Ura medium. Plates were photographed before (total growth) and after (adhesive growth) removal of nonadhesive cells by a wash assay. For high-copy TEC1 expression (hc TEC1) plasmid BHUM30 was used; all other strains carry control plasmid YEplac195. (C) FLO11 expression in strains of the indicated genotypes was determined by quantitative real-time PCR. Expression is presented as the percentage of the level measured in a TEC1 STE12 control strain, which was set to 100. Error bars indicate standard deviations.

We next used the PURA3-TEC1-expressing yeast strains to quantify Tec1-dependent activation of TCS- and FRE-driven transcriptional reporters, and we related these activities to the Tec1 protein levels present in the different strains. These quantitative measurements revealed that the presence or absence of Ste12 had no obvious effect on the Tec1-dependent expression of the TCS reporter (Fig. 1A). For the FRE reporter, we found that Tec1 was able to stimulate expression even in the absence of Ste12 (Fig. 1A, compare tec1Δ and PURA3-TEC1) and that Tec1-mediated expression was similar to that found for the TCS reporter. However, Tec1-mediated expression of the FRE reporter, but not that of the TCS reporter, was further stimulated by the presence of Ste12, which reflects the cooperative binding of Tec1 and Ste12 to the combined FRE element. These data suggest that there may be no fundamental difference between the functions of Tec1 at the TCS versus the FRE reporter.

We also measured adhesive growth and FLO11 expression in the different TEC1 and PURA3-TEC1 expressing yeast strains in order to relate these processes to the different Tec1 protein levels. As expected, TEC1 strains grow adhesively in the presence, but not in the absence, of STE12 (Fig. 1B). In contrast, PURA3-TEC1 confers adhesive growth even in a ste12Δ strain, although with lower efficiency than in a STE12 strain or compared to a ste12Δ strain carrying TEC1 on a high-copy plasmid (Fig. 1B). In agreement with these data, we found that Tec1 was able to significantly activate FLO11 transcription in the absence of Ste12 (Fig. 1C) and that in general FLO11 transcript levels correlated with Tec1 protein levels.

Together, these data demonstrate that Tec1 is able to activate TCS-mediated gene expression in the absence of Ste12 and they
suggest that Tec1 can bind to TCS elements independently of Ste12.

**In vitro**, Tec1 binds to TCS elements with high affinity and specificity independently of Ste12. We next investigated the in vitro binding of Tec1 to TCS elements in the absence and presence of Ste12. For this purpose, we performed quantitative gel retardation analysis using Tec1 and Ste12 proteins purified from E. coli and different TCS-containing DNA fragments. Importantly, we used Tec1 protein at nanomolar concentrations, which corresponds to the in vivo amounts of approximately 530 Tec1 molecules per cell (18). Under these conditions, Tec1 efficiently bound to single TCSs without Ste12, and binding was not influenced by a neighboring PRE (Fig. 2A). However, Tec1 DNA binding was much more efficient when a second TCS element was present on the target DNA, and it was drastically reduced when the TCS sequence was altered from the proposed consensus sequence CATTCTT to CAaaCTT (Fig. 2A).

We further quantified the binding affinity of Tec1 to a single TCS by performing titration gel shift analysis (Fig. 2B) and calculating the apparent dissociation constant ($K_d$) of the Tec1-TCS complex. These experiments revealed that Tec1 binds to the consensus TCS with an approximate $K_d$ of 56 nM (Fig. 2C and D).

In higher eukaryotes, systematic analysis of target se-
FIG. 3. Quantitative analysis of sequence requirement for Tec1-TCS binding in vitro and TCS-mediated reporter gene activation by Tec1 in vivo. (A) Effects of single-nucleotide exchanges on Tec1 binding. Systematic competition gel shift analysis was performed with 50 nM Tec1 protein and approximately 1 nM Cy5-labeled TCS DNA premixed with increasing amounts (100-, 500-, or 1,000-fold excess) of nonlabeled competitor DNA carrying the indicated TCS sequences. Nucleotides differing from the consensus TCS sequence ACATTCTT are shown in lowercase letters. The ratio of bound TCS to total TCS was determined by quantitative fluorescence imaging. Values multiplied by 100 are shown to indicate the percentage of bound DNA. (B) Relative Tec1 binding affinities to different mutant TCS sequences. Values are shown as percentage of the control. (C) TCS-CYC1-lacZ

The ratio of bound TCS to total TCS was determined by quantitative fluorescence imaging. Values multiplied by 100 are shown to indicate the percentage of bound DNA. (B) Relative Tec1 binding affinities to different mutant TCS sequences. Values are shown as percentage of the control.
quences for TEAD family proteins revealed strong binding to the consensus sequence ANATDCHN (2). Here, we determined the effects of all possible single-nucleotide exchanges in an ACATTCTT target sequence on the Tec1 binding affinity by performing a quantitative competition titration gel retardation analysis (Fig. 3A and B). This systematic analysis revealed RMATTCYY as the consensus sequence for strong and Ste12-independent Tec1 DNA binding (Fig. 3B). We also found a good correlation between in vitro DNA binding affinity and in vivo target gene recognition when measuring Tec1-dependent activation of CYC1-lacZ reporter genes driven by the consensus TCS ACATTCTT or mutated versions (Fig. 3C).

We next asked whether (i) Ste12 affects binding of Tec1 to single TCSs and (ii) Tec1 influences the binding of Ste12 to repeated PREs. To address these issues, we performed gel retardation experiments using Tec1 together with Ste12. We found that Ste12 did not affect Tec1 binding to individual TCSs in vitro (Fig. 4A), suggesting that Tec1-Ste12 complex formation does not increase the Tec1 DNA binding affinity. As previously described (37), we found that FREs are cooperatively bound by Tec1 and Ste12 but not by Ste12 alone (Fig. 4A and B). Mutation of the PRE sequence in the FRE from TGAAACG to acttACG (pre) resulted in loss of cooperativity and led to a binding behavior of Tec1 as found for the individual TCS (Fig. 4B). However, mutation of the TCS in the FRE from CATTCT to CAaaCT (tcs) did not completely disrupt cooperative binding of Tec1 and Ste12 (Fig. 4B). This indicates that Ste12 can stimulate Tec1 binding at low-affinity TCSs that are flanked by PREs. To further test this hypothesis, we looked for such configurations of Tec1 and Ste12 binding sites in natural yeast promoters. We found that the TEC1 promoter itself contains a TGAAACAGGAGATTCTT sequence element at positions 93 to 77 relative to the translational start site, which consists of a PRE and a low-affinity TCS (Fig. 4C, PRE-tcs*). Gel retardation analysis revealed significant cooperative binding of Tec1 and Ste12 to this element (Fig. 4C), indicating that low-affinity TCSs might be relevant in vivo if they are flanked by Ste12 binding sites. Finally, we measured the effect

TCS affinity and were obtained by building the ratios between the values (1,000-fold excess of competitor) of control and mutant sequences multiplied by 100. The derived consensus sequence conferring strong Tec1 binding is shown at the bottom and was compared to the consensus sequence for the human TEF-1 TEAD (2). (C) Reporter gene activation by different TCS sequences was determined by measuring β-galactosidase activities in yeast strains carrying integrated CYC1-lacZ genes driven by the indicated TCS sequences or no TCS element and expressing TEC1 from a low-copy plasmid. tec1Δ indicates expression of the control TCS reporter gene in the absence of Tec1. Error bars indicate standard deviations.
of Tec1 on Ste12 binding to repeated PREs, which are found in pheromone-stimulated genes. Here, we analyzed a FUS1 promoter element that consists of three tandemly repeated PREs. We found that Ste12 binding was very efficient in both the absence and the presence of Tec1 (Fig. 4D), indicating that Tec1-Ste12 complex formation does not affect Ste12 DNA binding to repeated PREs.

TCS-mediated transcriptional regulation by Tec1 depends on conserved residues in the TEA domain. In mammalian TEF proteins, structural and mutational analyses of the TEA domain have revealed a number of residues that are crucial for DNA binding (2). Because no comparable analysis has been performed for Tec1, we analyzed a number of amino acid residues in the three helices that form the TEA domain for their involvement in TCS binding in vitro and TCS recognition in vivo. We focused on residues either that are conserved between Tec1 and mammalian TEA domains or that in mammalian TEA domains were associated with DNA binding (Fig. 5A). This analysis revealed that two single mutations introduced in helix H1, L144A and L146A, did not affect DNA binding affinity in vitro, indicating that these residues are not crucial for TCS recognition (Fig. 5B). In vivo, the L146A mutation led to a 2-fold reduction in TCS reporter expression (Fig. 5C), which might be caused by the slightly decreased protein levels of this Tec1 variant when expressed in yeast (Fig. 5D). We also introduced two mutations in the highly conserved GRNEL sequence of helix H2 found in most TEA family transcription factors. Both mutations, G163A and R164A, abolished TCS binding in vitro (Fig. 5B) and reporter gene activation in vivo (Fig. 5C), demonstrating the crucial role of these conserved residues in DNA binding. Finally, we found that the conserved residue S187 in helix H3, which has been suggested to confer DNA recognition, is essential for DNA binding and TCS-mediated gene expression.

We also determined the effects of selected mutations in the Tec1 TEA domain on yeast adhesive growth in order to analyze the correlation between in vitro DNA binding, in vivo TCS
reporter gene activation, and regulation of a Tec1-dependent developmental process. We found that G163A, R164A, and S187A, which had severe defects in DNA binding, were also defective in adhesive growth. In contrast, the L146A mutant, which did not exhibit obvious defects in binding to TCS, was competent in adhesive growth (Fig. 5E).

The C-terminal domain of Tec1 confers Ste12-independent transcriptional activation and can be replaced by a Ste12 activation domain. We have previously shown that deletions in the C-terminal part of Tec1 cause a loss of TCS-mediated gene activation (30). Therefore, we further analyzed the function of this part of Tec1 with respect to reporter gene activation. For this purpose, we expressed TEC11–280 from the URA3 promoter, a construct that encodes the TEA DNA binding domain (DBD) and lacks the C-terminal Ste12 interaction domain (Fig. 6A and B). We found that the relative transcriptional activity of Tec11–280 was drastically reduced in the presence and absence of Ste12 (Fig. 6C). We then fused the C-terminal TEC11–280 domain back to TEC11–280 and found a significant, yet not a full, reconstitution of the transcriptional activity in both the presence and the absence of Ste12 (Fig. 6C). Together, these data indicate that the C-terminal part of Tec1 carries a Ste12-independent transcriptional activation domain. To independently corroborate this hypothesis, we performed a yeast one-hybrid analysis using the lexA-based yeast two-hybrid system (22). Here, we found that fusion of the lexA DNA binding domain to the C-terminal Tec11–280 or Tec11–280 portions led to a significant stimulation of a lexAop-driven lacZ reporter gene compared to lexA alone (Fig. 6D). Also, Tec11–280 led to a better activation than Tec11–280, a finding that correlates with the results obtained with the TCS reporter.

We next asked whether fusion of Ste12 or different Ste12 domains that were previously identified as a DNA binding domain and two independent transcriptional activation domains, ADI and ADII (29), would confer transcriptional activity to the Tec1 TEA domain. For this purpose, we constructed a series of TEC1—TEA–STE12 hybrids genes (Fig. 6A and B) and measured the activity of the encoded synthetic proteins with respect to TCS- and FRE-mediated reporter gene activation and adhesion. All genes were expressed as single genomic copies driven by the URA3 promoter in tec1Δ stre12A mutant strains.

With respect to TCS-mediated reporter gene activation, we found that addition of the DBD carrying Ste121–250 portion to Tec11–280 did not render an efficient transcriptional activity (Fig. 6E, construct 4). In contrast, fusion of the Ste12 ADI or ADII to Tec11–280 resulted in hybrid proteins that activated the TCS-driven reporter gene as efficiently as the full-length Tec1 protein in the absence of Ste12 (Fig. 6E, constructs 6 and 8). An even better effect was obtained by fusion of the full-length Ste12 protein to Tec11–280, which stimulated TCS-driven reporter expression more than 6-fold (Fig. 6E, construct 10). We also fused the TEC11–280 domain to the different Tec11–280, Ste12 hybrid proteins, and in all cases we found an increase in the relative TCS-mediated transcriptional activities (Fig. 6E, constructs 5, 7, 9, and 11). This finding further supports the view that the C-terminal part of Tec1 carries an AD. We also determined the relative transcriptional activities of different Tec1-Ste12 hybrid proteins at the FRE reporter. As found for the TCS reporter, we measured a strong stimulation of activities by inserting either Ste12 ADI, Ste12 ADII, or full-length Ste12 into Tec11–280–TEC11–280. Interestingly, insertion of the Ste12 DBD alone into Tec11–280–TEC11–280 conferred a 9.1-fold stimulation of the relative transcriptional activity at the FRE (compare Fig. 6C, construct 3, with Fig. 6E, construct 5), indicating cooperative DNA binding of this construct at FRE sites.

We then determined activation of FLO11-mediated adhesive growth by the different Tec1-Ste12 hybrid proteins. We did not observe a full correlation between stimulation of adhesion and activation of the TCS and FRE reporter genes. Specifically, fusions between the Tec1 TEA domain and Ste12 ADII were unable to activate adhesive growth (Fig. 6F, constructs 6 and 7), although their transcriptional activities at isolated TCS and FRE sites were significantly higher than that of the natural Tec1-Ste12 complex. This unexpected finding indicates that the activity of this hybrid protein not only requires a TCS but might be additionally influenced by neighboring sequence elements for, e.g., further DNA binding proteins that appear to differ between the TCS reporter and the natural FLO11 gene. In the case of fusions between the Tec1 TEA domain and Ste12 ADI or full-length Ste12, reporter gene activation was mirrored by a strong stimulation of adhesion (Fig. 6E, constructs 8, 9, 10, and 11). These data point to a specific function of Ste12 ADI in FLO11-mediated adhesion.

In vivo, Tec1 has been found to form a complex with Dig1 via association with Ste12. Therefore, we measured how Dig1 affects the Ste12-dependent and Ste12-independent activity of Tec1 and that of different Tec1-Ste12 hybrid proteins. In agreement with previous studies, we found that TCS-mediated activity of the natural Tec1-Ste12 complex and adhesive growth are under negative control of Dig1 (see Fig. S1A and B in the supplemental material). However, the Ste12-independent stimulation of the TCS reporter and of adhesion by Tec1 was not influenced by Dig1. We also found a negative effect of Dig1 on the transcriptional activities of hybrids between the Tec1 TEA domain and Ste12 ADII (see Fig. S1A, constructs 6 and 7, in the supplemental material) but not for the Tec1-Ste12 ADI hybrids (Fig. S1A, constructs 8 and 9), indicating that Dig1 inhibits Ste12 activity via ADII.

In summary, the data in this section show that Tec1 is able to activate TCS-mediated transcription in the absence of Ste12 depending on its C-terminal domain, which can be replaced by an activation domain of Ste12.

On a genome-wide scale, Ste12-dependent and Ste12-independent Tec1-regulated genes constitute two distinct classes. Our strains expressing TEC1 from the Ste12-independent URA3 promoter enabled us to analyze and compare genome-wide Tec1-regulated gene expression in the presence and absence of Ste12, an issue which was not addressed in previous studies (6, 38). For this purpose, we compared transcriptional profiles of TEC1 STE12 and tec1Δ STE12 strains as well as of PURA3-TEC1 STE12Δ and tec1Δ ste12A strains grown under nutrient-rich conditions using high-density oligonucleotide arrays (Affymetrix GeneChips). We found 289 genes that are regulated at least 1.5-fold by Tec1 in the presence of Ste12 and 48 genes that are regulated by Tec1 in the absence of Ste12, with 35 genes overlapping between the two groups (Fig. 7A and B; see Table S1 in the supplemental material). Thus, the total of 302 Tec1 target genes can be divided into two distinct classes, with 254 genes (84%) being Tec1 regulated in a strictly Ste12-
Fig. 6. Activities of different Tec1 and Ste12 proteins. (A) Diagrams showing Tec1 with TEA and Ste12-binding domains and Ste12 with the DNA binding domain (DBD) and activation domains (ADI and ADII) (44). (B) Diagrams showing different TEC1-STE12 hybrid constructs driven by the URA3 promoter. Encoded Tec1 and Ste12 amino acid residues and functional domains are indicated. (C) Relative activation of TCS and FRE reporter genes by Tec1 (lanes 1), Tec11–280 (lanes 2), and Tec11–280-Tec1377–486 (lanes 3) was determined in STE12 or ste12/H9004 strains expressing single genomic copies of corresponding TEC1 variants and TCS- or FRE-driven CYC1-lacZ genes as described for Fig. 5. (D) Activation of a lexAop-lacZ reporter gene by lexA (pEG202), lexA-TEC1281–486 (BHUM1687), or lexA-TEC1350–486 (BHUM1687). Yeast strain EGY48-p1840 (22) was transformed with these plasmids and pJG4-5, followed by quantitative measurement of β-galactosidase activities using SC-Trp-His medium supplemented with 2% galactose. Error bars indicate standard deviations. (E) Relative activation of TCS and FRE reporter genes by different Tec1-Ste12 hybrid proteins was determined in ste12Δ tec1Δ strains expressing single genomic copies of corresponding TEC1-STE12 variants and TCS- or FRE-driven CYC1-lacZ reporter genes. (F) Adhesive growth of the yeast strains described for panels C and E was determined after 5 days of incubation on solid yeast extract-peptone-dextrose (YEPD) medium.
dependent manner (Fig. 7C, class I) and 48 genes (16%) that are Ste12-independently regulated by Tec1 (Fig. 7C, class II).

To uncover possible differences in the promoters of class I and class II genes, we performed a concurrent enrichment analysis using the ChIPCodis web server (1), which is based on available chip-on-chip (25) and TFBS data (36) for 203 yeast transcription factors. We found that class I was specifically enriched for a total of 38 genes that are bound by Tec1, Ste12, and Dig1 under nutrient-rich conditions (Fig. 7D) (25). We then analyzed these class I genes for the presence of Tec1 binding sites and found high-affinity TCS elements matching the RMATTCYY consensus sequence in 50% of the promoters (Fig. 7D; see Table S2 in the supplemental material). This indicates that these class I genes are regulated by Tec1-Ste12 complexes via TCS binding. In contrast, class II was specifically enriched for 32 genes that were previously identified to be bound by the transcription factors Yap6, Nrg1, Cin5, Skn7, Hsf1, and Msn4 under hyperoxic stress conditions (Fig. 7D) (25). Notably, most of these class II genes were previously not found to bind Tec1 under nutrient-rich conditions (25). Nevertheless, we detected high-affinity RMATTCYY TCS elements in the promoter regions of 59% of these genes (Fig. 7D). Thus, efficient in vivo binding of Tec1 to these class II genes might depend on nonstandard conditions or additional transcription factors.

Because we found that the Tec1<sup>1–280</sup>-Ste12<sup>1–688</sup> hybrid protein, which represents the Tec1 TEA domain fused to the full-length Ste12 (Tec1<sub>TEA</sub>-Ste12), is a very potent activator of TCS-driven reporters, we wondered how this protein would regulate the different classes of Tec1 target genes. Transcriptional profiling revealed that 199 (78%) of class I genes are normally regulated by Tec1<sub>TEA</sub>-Ste12, whereas only 55 genes (22%) are deregulated (Fig. 7C and D). This indicates that a large proportion of class I genes are regulated by Tec1-Ste12 complexes without the need of the Tec1 C-terminal domain. A significantly higher proportion (72%) of class II genes enriched for Yap6, Nrg1, Cin5, Skn7, Hsf1, or Msn4 binding were deregulated by Tec1<sup>1–280</sup>-Ste12<sup>1–688</sup> (Fig. 7D), indicating that normal regulation of these genes specifically depends on the Tec1 C-terminal activation domain.

In summary, our genome-wide analysis reveals that Tec1 target genes constitute two distinct classes that are defined by
A

Tec1-regulated in presence of Ste12 (289)

254

35

13

Tec1-regulated in absence of Ste12 (48)

B

|    | A   | B   | C   |
|----|-----|-----|-----|
| NDJ1 | 29.5 | 1.0 | -1.4 |
| SRD1 | -22.2 | -1.3 | -1.1 |
| PGU1 | -6.1 | 1.1 | 24.3 |
| PRM5 | -4.9 | 1.0 | 2.1 |
| YAR066W | -4.7 | -1.3 | 1.2 |
| HOS2 | -4.2 | 1.4 | -2.0 |
| CWP1 | -2.6 | 1.1 | -4.2 |
| FUS2 | 2.1 | -1.0 | -1.2 |
| MFA1 | 2.3 | -1.1 | 3.4 |
| PRM1 | 2.4 | -1.2 | -6.9 |
| FIG2 | 3.0 | 1.3 | -1.4 |
| AGA1 | 3.8 | 1.0 | -3.6 |
| PRM3 | 4.6 | -1.1 | 1.1 |
| FIG1 | 5.0 | -1.3 | -1.1 |

YLR042C | -20.4 | -3.7 | -1.3 |
FLO11 | -5.6 | -2.9 | -2.4 |
DDR48 | -4.7 | -2.0 | 2.4 |
TIP1 | -4.7 | -2.3 | 2.1 |
HXX1 | -3.1 | -2.4 | -1.1 |
TMA10 | -3.0 | -2.1 | -1.7 |
RP11 | -2.7 | -1.8 | -1.4 |
BTN2 | -2.4 | -2.3 | -2.9 |
HSP42 | -2.1 | -2.3 | -1.6 |
PHM8 | -2.0 | -2.1 | -1.9 |
HSP30 | -1.3 | -3.1 | -1.2 |
AQY2 | 1.1 | 2.1 | 2.6 |
BSC1 | 3.4 | 2.9 | -4.8 |
PRM7 | 4.7 | 2.9 | -3.1 |

C

| class I | Tec1 regulation strictly Ste12-dependent (254) |
|---------|-----------------------------------------------|
| NDJ1, SRD1, YAR066W, FUS2, FIG2, PRM3, FIG1 | YLR042C, HXX1, RP11, HSP30 |
| (199)   | (21)  |
| PGU1, PRM5, HOS2, CWP1, MFA1, PRM1, AGA1 | FLO11, DDR48, TIP1, TMA10, BTN2, HSP42, PHM8, PRM7 |
| (55)    | (27)  |

| class II | Tec1 regulation Ste12-independent (48) |
|----------|----------------------------------------|
| normal regulation by Tec1<sup>Δ</sup>-Ste12 (220) |
| altered regulation by Tec1<sup>Δ</sup>-Ste12 (82) |
their Ste12-dependent and Ste12-independent regulatory patterns and their promoter characteristics.

Ste12 controls Tec1 protein stability independently of Dig1 and the Cdc4 phosphodegron (CPD) at Thr273 but involves the Tec1 C-terminal part. We noticed that Tec1 protein levels are lower in the absence of Ste12, even when TEC1 is expressed from the Ste12-independent URA3 promoter (Fig. 1A). Because Ste12 does not affect TEC1 transcript levels in URA3-TEC1-carrying strains, we tested whether Ste12 might influence Tec1 protein stability. Therefore, we determined in vivo decay rates of Tec1 in the presence and absence of Ste12 by performing cycloheximide-induced translational shutoff experiments. We followed the decrease of endogenous Tec1 protein levels in the anaplatinically expressing STE12 and ste12Δ strains after addition of cycloheximide by quantitative immunoblot analysis (Fig. 8A). These experiments revealed that the rate of decay of Tec1 increases more than 5-fold when STE12 is deleted (Fig. 8B), indicating that Ste12 positively controls Tec1 protein stability. To corroborate this finding, we performed a promoter shutoff experiment using the TEC1 gene under the control of the glucose-repressible GAL1 promoter. Again, we found that the Tec1 half-life decreases roughly 5-fold in strains lacking STE12 (see Fig. S2 in the supplemental material), supporting the view that TEC1-Ste12 complex formation significantly enhances Tec1 protein stability. We also explored the possibility that the Ste12-dependent Tec1 stability control is conferred by Dig1, which associates with TEC1-Ste12 complexes (10). However, Dig1 had no effect on the Tec1 half-life in either the presence or absence of Ste12 (Fig. 8C).
Previous studies have shown that Tec1 stability is decreased in response to pheromone by Fus3-mediated phosphorylation of a Cdc4 phosphodegron (CPD) motif centered at position Thr273 of Tec1 and that mutation of Thr273 or absence of Fus3 increases Tec1 stability (3, 8, 9). Therefore, we tested whether Ste12-dependent Tec1 stability control might also be mediated by Thr273. For this purpose, we constructed \( \text{STE12} \) and \( \text{ste12} \)/H9004 strains carrying a single genomic copy of the \( \text{TEC1T273M} \) allele under the control of the \( \text{URA3} \) promoter as the single source for functional Tec1 protein. We found that Ste12 affected the decay rate of Tec1T273M comparably to that of regular Tec1 (Fig. 8D), indicating that the CPD motif at T273 is not involved in regulation of Tec1 stability by Ste12.

Finally, we tested whether the C-terminal part of Tec1 might affect protein stability, because we noticed that steady-state levels of the Tec1\(^{1-280}\) variant are significantly increased (Fig. 6C). Indeed, we found that the half-life of Tec1\(^{1-280}\) lacking the C-terminal part is 3-fold higher than that of full-length Tec1 and, more importantly, completely uncoupled from Ste12 control (Fig. 8E). Moreover, fusing the Tec1\(^{177-486}\) part back to Tec1\(^{1-280}\) not only lowered the half-life 3-fold to match the value observed for regular Tec1 but also reconstituted stability control by Ste12 (Fig. 8E).

In summary these data indicate that Ste12-mediated Tec1 stability control involves the C-terminal part of Tec1 but not Dig1 or the CPD at Thr273.

**DISCUSSION**

The mechanisms by which the \( S. \) _cerevisiae_ transcription factors Tec1 and Ste12 regulate their target genes have become a valuable paradigm for combinatorial and program-specific transcriptional control. Previous studies have shown that these mechanisms include cooperative binding of Tec1 and Ste12 to combined TCS-PRE target sequences (37), Tec1-Ste12 complex formation and interaction with the inhibitor protein Dig1 (4, 10, 12, 45, 49), control of Tec1 protein stability by the MAPK Fus3 (3, 8, 9), and regulation of \( \text{TEC1} \) transcription by Ste12 (30, 43). In addition, both regulators have been found to colocalize at many target gene promoters \( \text{in vivo} \), suggesting that Tec1-Ste12 complex formation confers program-specific target gene control (6, 53). Our study provides (i) new insights into the mechanisms by which Tec1-Ste12 complex formation contributes to TCS-mediated target gene control and (ii) evidence that Tec1 is capable of controlling target gene expression by previously unknown Ste12-independent mechanisms.
With respect to regulation by Tec1-Ste12 complexes, we have identified a large class of Tec1 target genes that are regulated by Ste12-dependent mechanisms. Our bioinformatic analysis reveals that many of these genes contain high-affinity TCS elements, often without a flanking PRE, and that many class I genes are bound by Tec1, Ste12, and Dig1 in vivo (25, 53). We further find that Tec1 efficiently binds to TCSs in vitro and that this activity is not affected by Ste12. Also, our Tec1 deletion and Tec1-Ste12 hybrid analyses have revealed that TCS reporters and many TCS-carrying class I genes are normally regulated by chimeras between the Tec1 TEA domain and Ste12. Together, these findings suggest that TCS-containing class I genes can be regulated by Tec1-Ste12 complexes by at least two different mechanisms (Fig. 9). We conclude that at TCS promoters without PREs, Tec1-Ste12 complexes can bind via the Tec1 TEA domain and activate transcription through the different ADs of Ste12. This conclusion is in agreement with the study performed by Chou et al. (10). Our data do not rule out, however, that additional factors present at TCS promoters might affect recruitment or transcriptional activity of Tec1-Ste12 complexes. At promoters carrying TCSs flanked by a PRE, we find that Tec1-Ste12 complexes bind cooperatively, which is in line with initial studies (37). However, our study has revealed that there might be no fundamental difference in Tec1 functions at TCS- versus TCS-PRE-containing promoters. Finally, we show that class I genes can be regulated by Dig1. Our data indicate that Dig1 regulation is mediated by Ste12 ADII (Fig. 9), a finding that is supported by the fact that Ste12 associates with Dig1 through the ADII domain (44).

A completely novel outcome of our study is that Tec1-Ste12 complex formation confers Tec1 stability. So far, only a few examples of stability control by interacting transcription factors have been found in S. cerevisiae, e.g., in the case of the mating type transcription factors MATα1 and MATα2 (26). Here, we find that Ste12-mediated Tec1 stability control is independent of T273 phosphorylation and Fus3 (3, 8, 9) but instead involves the C-terminal part of Tec1. Given the fact that Tec1-Ste12 complex formation depends on the C-terminal portion of Tec1 (10), our results indicate that Ste12 might mask a destabilizing signal in this part of Tec1 (Fig. 9). What is the role of this mechanism? An interesting possibility is that this might be a regulatory device that ensures appropriate discrimination between class I and class II Tec1 target genes. For instance, such a mechanism might prevent erroneous activation of class I genes in the absence of Ste12 and/or ensure efficient expression in its presence. It might also stabilize class I gene expression over a broader range of Tec1 concentrations, e.g., when Tec1 protein levels fluctuate due to varying Fus3 activity within a population (11). Thus, future studies of Ste12-mediated Tec1 stability control could reveal novel insights into the significance of such mechanisms in population development and dynamics.

A central and novel finding of our investigation is the discovery of a second class of Tec1 target genes that can be regulated by Ste12-independent mechanisms. How does Tec1 regulate these genes? Our biochemical and genetic analyses have shown that Tec1 efficiently binds TCSs without Ste12 and that class II gene activation depends on the C-terminal domain of Tec1. Our bioinformatic analysis has revealed that a large number of class II gene promoters contain high-affinity TCSs. Yet, we find that only a few class II promoters have previously been identified to be bound by Tec1 under nutrient-rich conditions (25). Therefore, efficient Tec1 recruitment to class II promoters might depend on nonstandard conditions and on the interaction with further regulators. So far, no genome-wide Tec1 binding analysis has been performed under a regimen of different stress conditions. However, we found that class II is enriched for genes that are bound by the transcriptional regulators Yap6, Nrg1, Cin5, Skn7, Hsf1, and Msn4 under hyperosmotic stress (25). This opens the possibility that Tec1 might be recruited to class II promoters under certain stress conditions and interact with these stress-related DNA binding proteins. Based on previously identified functions of these regulators, such conditions might include oxidative and salt stress (e.g., Yap6, Cin5, and Skn7) (40, 48), heat shock (e.g., Hsf1 and Msn4) (24, 39) or glucose starvation (e.g., Nrg1) (31). Whether and how Tec1 might interact with any of these regulators remains to be investigated. Alternatively, Tec1 might regulate class II genes also indirectly via transcription factors that are under direct Tec1 control. With respect to this possibility, we noticed that class II includes RPI1, a gene which encodes a putative transcriptional regulator involved in the heat shock response and entry into stationary phase (28, 47). Interestingly, the promoter of RPI1 contains high-affinity TCS elements and is bound by Tec1, but not Ste12, under nutrient-rich conditions (25). This opens the possibility that class II genes might be regulated by Tec1 via Rpi1.

In summary, our study reveals that the mechanisms by which Tec1 is able to control target gene expression in S. cerevisiae are much more complex than previously anticipated. Because we find that TCS-mediated transcriptional control by Tec1...
depends on conserved residues in the TEA domain, our findings also contribute to a better understanding of the functions of TEA transcription factors in eukaryotes in general.

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