The Ccr4-Not Complex Regulates Skn7 through Srb10 Kinase

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The Ccr4-Not complex is a multifunctional regulatory platform composed of nine subunits that controls diverse cellular events including mRNA degradation, protein ubiquitination, and transcription. In this study, we identified the yeast Saccharomyces cerevisiae osmotic and oxidative stress transcription factor Skn7 as a new target for regulation by the Ccr4-Not complex. Skn7 interacts with Not1 in a two-hybrid assay and communoprecipitates with Not5 in a Not4-dependent manner. Skn7-dependent expression of OCH1 and Skn7 binding to the OCH1 promoter are increased in not4Δ or not5Δ mutants. Skn7 purified from wild-type cells but not from not4Δ cells is associated with the Srb10 kinase. This kinase plays a central role in the regulation of Skn7 by Not4, since increased OCH1 expression in not4Δ cells requires Srb10. These results reveal a critical role for the Ccr4-Not complex in the mechanism of activation of Skn7 that is dependent upon the Srb10 kinase.

Eukaryotic cells must adapt to continually changing environments. Therefore, they have adapted molecular mechanisms for very rapid responses that involve in most cases transcriptional activation of stress genes. Responses to stress are particularly critical for unicellular organisms such as the yeast Saccharomyces cerevisiae that must adapt to rapid and extreme changes, for instance, changes in nutrient availability, osmolality, and temperature. Stresses are sensed at the cell membrane and through specific signaling pathways induce transcriptional activation of stress-responsive genes. Expression of these genes then protects the cells against damage. In yeast, it has been well documented that exposure of cells to one type of stress protects them from exposure to other stresses. This stems from the induction of a similar subset of genes (about 10% of the genome) upon exposure of yeast to any type of stress (12). For the most part, these genes are controlled by the STRE (stress response element) promoter element, which is recognized by two partially redundant transcription factors, Msn2 and Msn4 (8, 22, 38, 46). This transcriptional response is called the environmental stress response.

We recently determined that the S. cerevisiae environmental stress response is activated in cells growing in high glucose that lack an intact Ccr4-Not complex (31). This complex consists of at least nine subunits (Not1 to Not5, Ccr4, Caf1, Caf40, and Caf130) that exist in complexes of 1.2 and 2 MDa from yeast to human. Two of its subunits are enzymes, namely, Not4, which is an E3 ubiquitin ligase, and Ccr4, which is the major yeast deadenylase. Genetic and molecular analyses have suggested that the complex may be composed of different functional modules. Indeed, it has been proposed that the Caf1 and Ccr4 subunits are physically and functionally separated from the Not subunits (1). Nevertheless, the role of the different subunits of the Ccr4-Not complex still remains to be clarified, since it is presently known that multiple cellular functions are under the control of the Ccr4-Not complex. For instance, in addition to its role in protein ubiquitination (44), this complex regulates mRNA metabolism, both at the level of mRNA degradation, via the Ccr4 deadenylase subunit, and at the level of transcription initiation, via a control of the distribution of TFIIID across the genome (for reviews, see references 14, 17, and 19). In the case of the environmental stress response, the Ccr4-Not complex controls transcription initiation indirectly through the Msn2 transcription factor. Indeed, Msn2-dependent transcriptional activation is increased and the Msn2 protein is post-translationally modified in mutants of the Ccr4-Not complex (31). Both of these effects require the Gic7 type I protein phosphatase and its regulatory subunit Bud14 (30).

In this work, we determine that in addition to controlling the environmental stress response in unstressed cells, the Ccr4-Not complex regulates the response to specific stress signals. Indeed, we show that the Ccr4-Not complex controls the Skn7 transcription factor, which is one of the transcription factors that yeast cells have evolved to respond to specific stress responses in addition to the general response mediated by Srb10. Skn7 contains a receiver domain typical of two-component signal transduction systems (26, 32), which, though commonly used for environmental sensing in eubacteria, are restricted in S. cerevisiae to the response to osmotic and oxidative stresses. Indeed, in response to hypo-osmolality, Sln1, a membrane-associated histidine kinase, is autophosphorylated, and through its intermediary phosphorylase protein Ypd1, it transfers its phosphate residue to Skn7 on a specific aspartate residue, D427, within the Skn7 receiver domain (33). It has been shown that Skn7 in this pathway is involved in cell wall integrity and the cell cycle (7, 9, 10, 40). However, Skn7 also plays a role in response to oxidative stress (28). Its activation in response to oxidative stress is independent of
MATERIALS AND METHODS

Media and strains. All media were standard. The strains used in this work derive from MY1 (16) (Table 1). Single-step deletions and/or tagging of genes was performed by PCR according to the method in reference 35, and many strains were created by mating of haploid strains and sporulation of the diploids. MY2048 and MY2052 result from crosses with null mutant strains described in reference 43. To create strains expressing Skn7 fused to a tandem affinity purification (TAP) tag at its C terminus, we amplified the cassette with the TAP tag and the marker genes from pF6a-taptop.8.8.6 (a kind gift from D. Bartford) using oligonucleotides with Skn7 sequences just prior to and beyond the stop codon, leading to strains MY5037 and MY5041. Strain MY4858, derived from BY4741 (see http://web.uni-frankfurt.de/fb15/mikro/euroscarf/data/by.html) and expressing a TAP-tagged Caf40, was a kind gift from Klaus Mulder and Marc Timmers, and MY4910 was created from MY4858 by PCR according to the method in reference 35.

Plasmids. The plasmid expressing B42-Skn7cl was obtained by subcloning the Smal-Xhol fragment of a partial SKN7 clone in pACT2 obtained from a large two-hybrid selection into pG4-5 digested with EcoRI, treated with Klenow fragment, and digested with XhoI. We used gap repair to create a prey plasmid expressing the entire Skn7. Using an oligonucleotide with sequences homologous to the pG4-5 vector (48) at the end of the B42 activation domain and hemagglutinin (HA) epitope, followed by Skn7 sequences starting at the start codon on one hand, and an oligonucleotide with sequences homologous to the pG4-5 vector in the ADH1 3’ untranslated region followed by Skn7 sequences covering the stop codon, we amplified Skn7 using genomic DNA. We cotransformed the linearized pG4-5 vector together with the PCR fragment into the reporter two-hybrid strain EGY48 (48). Transformants that could grow in the absence of tryptophan were selected for, and the expression of the Skn7 fusion protein was verified from transformants growing in glucose or galactose by Western blot analysis with an anti-HA antibody. The plasmid was recovered from transformants expressing a fusion protein, and the EcoRI-SalI fragment covering the entire Skn7 open reading frame (ORF) could be subcloned into pRS316, pRS306, or pBSKS for further use. Site-directed mutagenesis was performed with the SKN7 sequences cloned in pRS306, leading to pMAC561, pMAC562 (D427N) and pMAC566 (D427E), and the mutations were verified by sequencing. The mutant SKN7 sequences from pJG4-5 including the fused N-terminal HA tag by using a 5’ oligonucleotide containing the end of NCB2 promoter sequences and the sequence for the HA tag and the beginning of SKN7 sequences and a 3’ oligonucleotide containing ADH1 3’ sequences that then allowed us to do gap repair in yeast, by cotransforming a plasmid with the NCB2

| Strain | Description | Reference |
|-------|-------------|-----------|
| EGY48 | MATa his3 trp1 ura3 LEU2::pLexOp6-LEU2 | 48 |
| MY1   | MATa ura3-52 trp1 leu2::PET56 gcn4 gal2 | 30 |
| MY2   | MY1 MATa | This work |
| MY3   | MY1 his3::TRP1 | This work |
| MY4   | MY2 his3::TRP1 | This work |
| MY2048| MY4 not4::LEU2 | This work |
| MY2052| MY4 not5::LEU2 | This work |
| MY3246| MY1 srb10::kanMX4 ura3::STRE-lacZ-URA3 | This work |
| MY3247| MY2 srb10::kanMX4 ura3::STRE-lacZ-URA3 not4+3::LEU2 | This work |
| MY3496| MY2 msn2::TRP1 | 30 |
| MY3498| MY3 not4+3::LEU2 msn2::TRP1 | 30 |
| MY3595| MY2 not4::kanMX6 | This work |
| MY3688| MY3 skn7::SKN7-Myc13-HIS3 | This work |
| MY3725| MY1 skn7::TRP1 | This work |
| MY3726| MY1 not4::kanMX6 skn7::TRP1 | This work |
| MY3812| MY3 not5::LEU2 skn7::TRP1 | This work |
| MY3819| MY3 not3::URA3 skn7::SKN7-Myc13-HIS3 | This work |
| MY3820| MY3 not5::LEU2 skn7::SKN7-Myc13-HIS3 | This work |
| MY3824| MY3 bud14::kanMX6 skn7::SKN7-Myc13-HIS3 | This work |
| MY3825| MY4 not4::kanMX6 bud14::kanMX6 skn7::SKN7-Myc13-HIS3 | This work |
| MY3826| MY3 not5::LEU2 bud14::kanMX6 skn7::SKN7-Myc13-HIS3 | This work |
| MY4829| MY3725 pHA-SKN7-LEU2 | This work |
| MY4844| MY3726 pHA-SKN7-LEU2 | This work |
| MY4858| MATa leu2Δ20 ura3Δ met15Δ his3Δ1 caf40::CAF40-TAP tag-URA3 | 41 |
| MY4910| MY4858 not4::HisMX4 | This work |
| MY5037| MY3 skn7::SKN7-TAP tag-kanMX4 | This work |
| MY5041| MY3 skn7::SKN7-TAP tag-kanMX4 not4+3::LEU2 | This work |
| MY5187| MY3726 pHA-skn7-D427N-LEU2 | This work |
| MY5188| MY3726 pHA-skn7-D427E-LEU2 | This work |
| MY5189| MY3725 pHA-skn7-D427N-LEU2 | This work |
| MY5190| MY3725 pHA-skn7-D427E-LEU2 | This work |

Aspartate 427 (32, 39). Furthermore, different sites in the promoters are recognized by Skn7 in this response, such as the OSRE (oxidative stress response element), which shares a GCC repeat with the SSRE (47).

In this study, we show that Skn7-dependent transcriptional activation is increased in exponentially growing cells lacking subunits of the Ccr4-Not complex and that this increase correlates with increased binding of Skn7 to specific target promoters. We find that Skn7 purified from wild-type cells is associated with the Srb10 kinase, which mediates the regulation of Skn7-dependent OCH1 transcription by Not4. The Srb10 kinase is part of a module of the mediator that includes three other proteins, Srb8, Srb9, and the Srb11 cyclin (5), and which has both positive and negative effects on transcription regulation (2; for a review see also reference 11). Our present results demonstrate that the Ccr4-Not complex plays a widespread role in the regulation of the cellular response to stress, not only through the general Msn2-dependent stress response as previously published but also via the more specific stress response mediated by Skn7.
promoter and ADH1 3' sequences (pMAC399) digested with PstI and SphI and the PCR fragments. This allowed the creation of plasmids expressing wild-type (pMAC582) or D427-mutated (pMAC636 for D427N and pMAC661 for D427E) Skn7 with an N-terminal HA tag from the NCR2 promoter. Oligonucleotide sequences are available upon request.

Two-hybrid selection and assay. For the large-scale two-hybrid selection, full-length and N-terminally truncated Not1 was expressed as a fusion to the Gal4 DNA binding domain and tested with the library described in reference 21. Transfectants (6.2 × 10⁶) were obtained and selected for 50 mM or 100 mM 3-aminitriazole resistance. Of the positive clones initially obtained, 78 were still positive after plasmid rescue and retransformation and corresponded to 33 different genes. Most genes were represented only once, and this was the case for Skn7 as well as NOT4, suggesting that the selection was by far not saturated. At most four clones covering the same gene were obtained in two cases. For subsequent two-hybrid analyses, strain EGY48 was transformed with derivatives of plasmid pLex202 expressing full-length or partial Skn7 fused to the B42 activation domain. Transformants were spotted on glucose or galactose plates lacking or containing leucine and grown for 3 to 10 days.

Coimmunoprecipitations. To perform coimmunoprecipitation experiments with Skn7, Not1, or Not5, wild-type or not4a strains expressing genomically Myc-tagged Skn7 were grown exponentially to an optical density at 600 nm of 0.8 to 1.0. The cells were resuspended in a 10 mM HEPES buffer (50 mM NaCl, 0.1% Tween), pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8, 1% Triton, 0.1% sodium deoxycholate, and phenylmethylsulfonyl fluoride at 1 mM. Following addition of an equal volume of glass beads, cells were broken with a cell disruptor for 15 min at 4°C. The extracts were clarified for 15 min in a microcentrifuge at 14,000 rpm, and the protein concentration of the supernatant was measured by the Bradford assay. One milligram of total lysate was incubated with antibodies against Not1, Not5, or Skn7 (OCH1-Scottish) overnight at 4°C. Fifteen microliters of protein G-Sepharose beads were resuspended in 40 µl of 2× sample buffer (62.5 mM Tris [pH 6.8], 1% glycerol, 2% sodium dodecyl sulfate [SDS], 0.01% bromophenol blue, 5% β-mercaptoethanol), boiled for 5 min, and loaded on a 9% polyacrylamide-SDS gel. Twenty-five micrograms of the total lysate was also loaded on the gel. After electrophoresis, proteins were transferred to nitrocellulose for Western blot analysis.

S1 analysis. Total cellular RNA was extracted by the hot acid phenol method, and 50 µg was hybridized to DED1 or OCH1 probes (sequences available upon request) and then digested with S1 nuclease and analyzed on a sequencing gel as described previously (15).

ChIP experiments. Chromatin immunoprecipitation (ChIP) experiments and quantitative real-time PCR were performed as described previously (18). Monoclonal anti-Myc and polyclonal anti-TATA binding protein (anti-TBP) antibodies were used for the immunoprecipitations of cross-linked extracts. Oligonucleotide primers were used for the immunoprecipitations. Oligonucleotide sequences used for quantitative real-time PCR are available upon request; see Materials and Methods. We prepared total protein extracts from this strain growing in high glucose and immunoprecipitated Not1 or Skn7 from the total extract. We found that tagged Skn7 was in the immunoprecipitate (Fig. 1B), demonstrating that in vivo Not1 can interact with Skn7. We then investigated whether another subunit of the Ccr4-Not complex, Not5, was able to coimmunoprecipitate with Skn7. We chose to look at Not5 because, like Skn7, it interacts in the two-hybrid assay with Not1 in the two-hybrid assay (see Materials and Methods). We chose Not1 as a bait because it is the scaffold of the complex and might lead to the isolation of proteins interacting with any of the subunits of the complex. In turn, this might give clues to the mechanism of function of the Ccr4-Not complex. In this screen, we identified a clone expressing a portion of Skn7 that could interact with a clone expressing full-length Not1 and with a clone expressing an N-terminal truncation of Not1, namely, amino acids 1319 to 2108. Sequencing of the isolated Skn7 clone revealed that it expressed amino acids 191 to 300 of Skn7, containing a predicted coiled-coil domain of the protein, fused to the Gal4 activation domain. This result was confirmed using a different two-hybrid system in which we fused Not1 to the LexA DNA binding domain (vector pLex202) and the interacting Skn7 amino acids to the B42 activation domain (vector pJG4-5) (48) (Fig. 1A). We also created a fusion of the B42 activation domain to full-length Skn7 (622 amino acids) and observed that full-length Skn7 also gave a two-hybrid interaction with the C-terminal region of Not1. In contrast, we could not detect a two-hybrid interaction between the two full-length fusions (Fig. 1A), suggesting that in the two long fusion proteins, the interaction may not be favorable.

We then created a strain expressing Myc-tagged Skn7 from its own promoter and its own locus (according to reference 35; see Materials and Methods). We prepared total protein extracts from this strain growing in high glucose and immunoprecipitated Not1 or Skn7 from the total extract. We found that tagged Skn7 was in the immunoprecipitate (Fig. 1B), demonstrating that in vivo Not1 can interact with Skn7. We then investigated whether another subunit of the Ccr4-Not complex, Not5, was able to coimmunoprecipitate with Skn7. We chose to look at Not5 because, like Skn7, it interacts in the two-hybrid assay with the C-terminal domain of Not1 (37). We found that indeed Skn7-Myc coimmunoprecipitated with Not5 (Fig. 1C).

To determine whether this interaction required an integral Ccr4-Not complex, we created a not4a strain expressing tagged Skn7. We chose this strain because Not4, like Not5, is part of the Not module of the Ccr4-Not complex. The deletion of NOT4 led to the loss of coimmunoprecipitation between Not5 and Skn7 (Fig. 1C) and to the loss of coimmunoprecipitation between Not1 and Skn7 but did not abolish the interaction between Not5 and Not1 (data not shown). We also immunoprecipitated Myc-tagged Skn7 from wild-type and not4Δ cell extracts and detected low levels of Not5 in the immunoprecipitate from wild-type cell extracts but none in the immunoprecipitate from not4Δ cell extracts, despite efficient immunoprecipitation of Skn7 itself from both cell extracts (data not shown). Taken together these results show that Skn7 can interact, directly or indirectly, with at least two subunits of the Ccr4-Not complex in vivo and that the association of Skn7 with Not5 requires Not4.
Skn7-dependent expression of *OCH1* is increased in mutants of the Ccr4-Not complex. Skn7 is important for the response to osmotic stress, and it activates genes such as *OCH1* in response to hypotonic stress. We tested the expression of *OCH1* in wild-type and *not4*Δ cells, by S1 analysis of total cellular RNA, and for the same amount of total cellular RNA we compared *OCH1* mRNA levels in the different strains. The levels of *DED1* mRNA were measured as an internal control. We observed that *OCH1* mRNA levels were increased in cells lacking *NOT4*. This increased expression of *OCH1* required Skn7, as could be determined by measuring expression of *OCH1* in *not4*Δ cells lacking *SKN7* (Fig. 2A). This result was confirmed in multiple experiments (data not shown).

We then investigated whether Msn2 contributed to increased *OCH1* expression in *not4*Δ cells by measuring *OCH1* mRNA levels in wild-type or *not4*Δ cells, expressing or lacking *MSN2*. The deletion of *MSN2* had no effect on *OCH1* mRNA levels in wild-type or *not4*Δ cells (Fig. 2B, compare lanes 1 and 5 and lanes 4 and 6), whereas, as before, we saw that increased *OCH1* mRNA levels in *not4*Δ cells required Skn7 (Fig. 2B, compare lanes 1 and 2 to lanes 7 and 8). As in *not4*Δ cells, *OCH1* mRNA levels were increased in *not5*Δ cells through Skn7 (Fig. 2B, compare lanes 1 and 3 to lanes 7 and 9). Deletion of the Glc7 type I protein phosphatase regulatory subunit Bud14, which mediates increased activity of Msn2 in mutants of the Ccr4-Not complex, did not reduce *OCH1* expression in either *not4*Δ or *not5*Δ cells (Fig. 2B, compare lanes 1 to 3 to lanes 10 to 12). On the contrary, in the absence of Bud14, the activation of *OCH1* in *not4*Δ and *not5*Δ cells was remarkably higher.

Taken together, these results show that Skn7-dependent expression of *OCH1* is increased under normal growth conditions upon mutation of the Ccr4-Not complex. Msn2 plays no role in this increase. Furthermore, Bud14, which mediates Msn2 activation upon mutation of the Ccr4-Not complex (30), is in contrast inhibitory to Skn7-dependent expression of *OCH1* in *ccr4-not* mutants.

Residue 427 of Skn7 and Not4 independently set thresholds of Skn7-dependent *OCH1* expression. Previous studies have demonstrated that a reporter construct driven by the *OCH1* promoter responds to activated alleles of *SLN1* (called *sln1*Δ) in an *Skn7*-D427-residue-dependent manner (33). Indeed, D427N Skn7 led to reduced activity and D427E Skn7 resulted in increased activity in this reporter system. To test if the Ccr4-Not complex might impact on D427-dependent transcriptional activation by Skn7, we created plasmids expressing HA-tagged wild-type or D427N or D427E mutant forms of Skn7 (see Materials and Methods). These plasmids were transformed into wild-type or *not4*Δ cells lacking the chromosomal copy of *SKN7*. We then grew the transformants in high glucose to exponential phase and extracted total cellular RNA for S1 analysis of *OCH1* mRNA levels. We also measured the levels of RNA in these reactions as a loading control. We observed that in wild-type cells the expression of *OCH1* was higher in cells expressing D427E Skn7 and lower in cells expressing D427N Skn7 than in cells expressing wild-type Skn7 (Fig. 3, lanes 1 to 3). Similarly, in *not4*Δ cells, expression of *OCH1* was higher in cells expressing D427E Skn7 and lower in cells expressing D427N Skn7 than in *not4*Δ cells expressing wild-type Skn7 (Fig. 3, lanes 4 to 6). Thus, *not4*Δ cells retain the response to residue D427 modification observed in wild-type cells.
These results suggest that residue D427 and Not4 independently set thresholds of Skn7-dependent expression of OCH1.

**The binding of Skn7 to the OCH1 promoter specifically is increased in cells lacking NOT4.** The results presented above suggest that increased Skn7-dependent expression of OCH1 in cells lacking NOT4 is not mediated by activation of residue D427. This would suggest that the Ccr4-Not complex controls Skn7 by a mechanism that is distinct from the control of Skn7 by hypotonic stress. We thus decided to look at the binding of Skn7 to DNA. Indeed, Skn7 has been reported to be exclusively nuclear (36), making it unlikely that the Ccr4-Not complex regulates the localization of Skn7. Moreover, the binding of Skn7 to DNA has been shown to not depend upon residue D427, and thus, an effect of the Ccr4-Not complex on Skn7 regulation of the localization of Skn7. Moreover, the binding of Skn7 to DNA has been shown to not depend upon residue D427. This would suggest that the Ccr4-Not complex controls Skn7 by a mechanism that is distinct from the control of Skn7 by hypotonic stress. We thus decided to look at the binding of Skn7 to DNA. Indeed, Skn7 has been reported to be exclusively nuclear (36), making it unlikely that the Ccr4-Not complex regulates the localization of Skn7. Moreover, the binding of Skn7 to DNA has been shown to not depend upon residue D427, and thus, an effect of the Ccr4-Not complex on Skn7 binding seemed a reasonable hypothesis. To assay for this, we performed ChIP experiments with wild-type, not3Δ, not4Δ, or not5Δ cells expressing Myc-tagged Skn7. We grew the cells exponentially in high glucose, cross-linked the cells with formaldehyde, prepared total extracts, shared chromatin, and immunoprecipitated Skn7. The presence of several DNA sequences in the immunoprecipitates was investigated by real-time PCR. On one hand we looked at the OCH1 promoter with primers that amplify a region containing one SSRE and two HSEs (heat shock promoter elements), which can all be recognized by Skn7, which has a DNA binding domain with similarity to that of the heat shock factor (10, 40, 45) (Fig. 4A), and on the other hand we amplified an intergenic region that contains no distinguishable HSEs or SSRES. We then expressed the amount of the OCH1 promoter, likely to be bound by Skn7, relative to the intergenic region (unlikely to be associated with Skn7) and observed that there was a significant increase in the association of Skn7 with the OCH1 promoter in cells lacking Not4 compared to wild-type cells (Fig. 4B). A similar increase was observed in cells lacking Not5 but not in cells lacking Not3 (Fig. 4B). No significant change in the association of TBP with OCH1 was found in cells lacking either NOT4 or NOT5 compared to wild-type cells (Fig. 4B). This finding suggests that activation of OCH1 by Skn7 in cells lacking Not4 is likely to act at a step subsequent to TBP binding. These results demonstrate that increased binding of Skn7 to the OCH1 promoter correlates with increased transcription of the OCH1 gene in cells lacking NOT4. We next wanted to determine whether binding of Skn7 to other promoters, in particular to those of oxidative stress-inducible genes that are mediated by different Skn7 binding sites (23, 47), is also increased in cells lacking Not4. Thus, we repeated the ChIP experiments with wild-type and not4Δ cells and evaluated the presence of oxidative stress-inducible promoters, namely, TSA1, AHP1, and CCP1, in addition to that of the OCH1 promoter and intergenic DNA, in the immunoprecipitates by real-time PCR. As before, we found that there was an increase in the association of Skn7 with OCH1 (Fig. 4C). A milder but measurable increase of Skn7 association with CCP1, but not with TSA1, and not reproducibly with AHP1, relative to intergenic DNA, in cells lacking Not4 was also observed (Fig. 4C). In good correlation with the finding of no general effect of Not4 on the association of Skn7 with oxidative stress-responsive promoters, we observed that not4Δ cells had resistance to 2.5 mM hydrogen peroxide similar to that of wild-type cells, resistance that is Skn7 dependent (Fig. 4D). Taken together these findings suggest that Not4 controls preferentially the association of Skn7 with the OCH1 osmotic stress-responsive promoters.
Srb10 mediates \textit{OCH1} activation in cells lacking Not4. To investigate further the regulation of Skn7 by Not4, we purified by tandem affinity chromatography Skn7 TAP tagged at its C terminus from its endogenous promoter and locus. Interestingly, when we visualized the proteins by Coomassie blue staining of the gel, we observed that the profiles of proteins purified with Skn7 from wild-type and \textit{not4} cells were different (Fig. 5A), and analysis by mass spectrometry of the purified proteins prior to separation by SDS-polyacrylamide gel electrophoresis revealed the presence of a kinase, namely, Srb10, in the preparation of Skn7 from wild-type cells only. We also purified the Ccr4-Not complex from wild-type or \textit{not4} cells expressing TAP-tagged Caf40 from its endogenous promoter and locus (Fig. 5B). This revealed the absence of Caf130, Not3, and Not2 in Caf40-purified Ccr4-Not complexes when cells lacked \textit{NOT4} as determined by both Western blotting and mass spectrometry analysis of the purified material, but most excitingly it revealed the presence of Srb9 in the purification of Ccr4-Not complexes from wild-type but not mutant cells. Indeed, the interest of this finding lies in the fact that Srb9 and Srb10 are part of the same module of the mediator that has been shown to play both negative and positive roles in the regulation of transcription (5). Thus, we considered the possibility that the Srb10 kinase might play a role in the regulation of Skn7 by the Ccr4-Not complex. We created wild-type and \textit{not4} strains lacking the \textit{SRB10} gene and analyzed the expression of \textit{OCH1} in these strains. There was no increase of \textit{OCH1} expression in \textit{not4} cells relative to wild-type cells in the absence of Srb10, yet the absence of \textit{SRB10} did not seem to have an effect per se on the expression of \textit{OCH1} in wild-type cells (Fig. 5C).

Taken together, these results show that Srb10 is required for Skn7-dependent increased expression of \textit{OCH1} in cells lacking Not4. This correlates with the importance of Not4 for the integrity of the Ccr4-Not complexes that can be purified through Caf40 and for the association of components of the Srb10 kinase module with either the Ccr4-Not complex or Skn7.

**DISCUSSION**

Skn7, a new target for regulation by the Ccr4-Not complex. In this work, we have determined that Skn7, a transcription factor involved in the oxidative and osmotic stress responses, is a new component of the transcription machinery that is controlled by the Ccr4-Not complex. We investigated its relationship to the Ccr4-Not complex after it was isolated in a two-hybrid selection with Not1 as bait. We were able to confirm an
interaction between Skn7 and two subunits of the Ccr4-Not complex, namely, Not1 and Not5, by coimmunoprecipitation, and we could show that the interaction between Not5 and Skn7 requires Not4. We further found that strains with deletions of NOT4 or NOT5 present an increase in expression of OCH1, a gene that is activated by Skn7 in response to a low osmotic signal, in mutant cells under normal growth conditions. Finally, we confirmed that the induction of OCH1 is mediated by Skn7, because the deletion of SKN7 mostly abolished the induction of OCH1 in cells lacking Not4. This gene is activated by Skn7 in response to hypotonic shock through Skn7 binding to the SSRE. Residue D427 of Skn7 is required for activation of Skn7 through SSREs, but it does not affect Skn7 binding to DNA (33). Consistently residue D427 determines the level of OCH1 expression, even in cells lacking Not4. These results show that residue D427 of Skn7 and Not4 independently set thresholds for the expression of OCH1 by Skn7.

Skn7 binds to a number of different sites besides the SSRE, including HSEs (10, 40, 45) or the OSRE, which mediates Skn7-dependent expression of TRX2 and GPX2 in response to oxidative stress (47). Recently yet another Skn7 binding site has been identified in the CCP1 promoter that is responsible for its activation in response to oxidative stress (23). Our work shows that it is mostly the binding of Skn7 to OCH1, and also to some extent to some oxidative stress-responsive promoters such as CCP1, but not to others such as TSA1, that is increased in cells lacking Not4. It could be that Not4 regulates the capacity of Skn7 to associate specifically with SSRE- rather than with OSRE-containing promoters. Alternatively, Not4 might regulate the capacity of Skn7 to associate with other factors present at the OCH1 promoter and other regulated promoters specifically. In any case, it is clear that these findings demonstrate the capacity of Not4 to contribute to the regulation of Skn7 in specific responses, rather than to overall Skn7 activity. Thus, this work provides evidence not only for a new target of the Ccr4-Not complex but also for an additional level of control of the Skn7 transcription factor acting upon its DNA binding to specific target genes.

FIG. 5. Purification of TAP-tagged Skn7 and Caf40 in wild-type and not4Δ cells reveals a connection to the Srb10 kinase that mediates activation of OCH1 in not4Δ cells. (A) Wild-type (MY5037) and not4Δ (MY5041) cells expressing TAP-tagged Skn7 were grown to exponential phase, for total extract preparation and TAP. The eluates were loaded on SDS-polyacrylamide gels and stained with Coomassie blue. The indicated proteins (**) were confirmed by mass spectrometry. The most prominent bands in both eluates correspond to heat shock proteins of the Hsp90 and Hsp70 families found in most TAPS. Srb10 cannot be attributed to a particular band as it was identified by mass spectrometry in the purification prior to separation of the proteins by SDS-polyacrylamide gel electrophoresis. (B) Wild-type (MY4858) and not4Δ (MY4910) cells expressing TAP-tagged Caf40 were grown to exponential phase, for total extract preparation and TAP. The eluates were loaded on SDS-polyacrylamide gels and stained with Coomassie blue. The indicated proteins (**) were confirmed by mass spectrometry. The Hsp70 proteins comigrate with Not4; therefore, one does not see Not4 disappear in the not4Δ background. (C) Wild-type (MY1), not4Δ (MY3595), srb10Δ (MY3246), or srb10Δ not4Δ (MY3247) cells were grown exponentially for total RNA preparation. Equal amounts of total cellular RNA were analyzed by S1 digestion for the levels of OCH1 mRNA and DED1 as an internal control.
plex is lost. In this mutant, association of Skn7 with the Ccr4-Not complex, interaction between Skn7 and the Ccr4-Not complex is evolutionarily conserved and integrates signals from multiple components of the preinitiation complex (PIC).

The Srb10 kinase mediates increased activation of OCH1 by Skn7 in cells lacking Not4. An exciting finding in this study is the positive role of the Srb10 kinase in the Skn7-dependent activation of OCH1 in cells lacking Not4. Srb10 and its cyclin Srb11 form a stable complex with Srb8 and Srb9 that loosely associates with the 21-subunit core mediator complex (5). This complex is evolutionarily conserved and integrates signals from sequence-specific activators and repressors to the general transcription machinery (3, 4, 27). A recent study has suggested that the Srb10 module may sterically block mediator interactions with RNA polymerase II to inhibit transcription (20). However, Srb10 can phosphorylate the C-terminal domain of the polymerase, but it has many other substrates that may be more relevant for its function in vivo. For instance, negative regulation of transcriptional activators such as Ste12, Gcn4, and Msn2 by Srb10 has been reported (13, 42). Srb10 phosphorylation promotes the rapid degradation of Gcn4. It also promotes Msn2 degradation, but in addition it promotes Msn2 nuclear export, and these different effects are mediated through separable domains (6, 13, 29). In contrast, positive regulation of Gal4 activation by Srb10 has been documented (24). Our current study identifies another positive role for Srb10, namely, promotion of OCH1 activation by Skn7 that correlates with increased DNA binding of Skn7 to this specific promoter.

The copurification of the Srb10 kinase with Skn7 and that of Srb9 with the Ccr4-Not complex, in wild-type but not mutant cells, lead us to propose a model (Fig. 6) in which the interaction of the Srb10 kinase module with the Ccr4-Not complex inhibits the activation of OCH1 by Skn7. Activation might rely on phosphorylation of Skn7 itself or on phosphorylation of other components of the transcription machinery by Srb10 at the OCH1 promoter. In this context it should be mentioned that Skn7 extracted from wild-type cells or not4Δ cells migrates with different apparent sizes on SDS-polyacrylamide gels, suggesting that it exists in different posttranslational states (data not shown).

**Model for regulation by the Ccr4-Not complex.** Our present finding that a component of the Srb10 module is associated with wild-type but not mutant Ccr4-Not complexes raises the question of whether the Ccr4-Not complex regulates specifically the role of Srb10 in Skn7 activation of OCH1 or whether it regulates globally the activity of Srb10. One previous study has already connected the Ccr4-Not complex to the Srb10 module of the holoenzyme by two-hybrid experiments (34), and we have several observations compatible with the idea that the Ccr4-Not complex may have a global rather than specific regulatory impact on the function of the Srb10 kinase. First, GAL1 transcripts accumulate more rapidly in not4Δ cells than in wild-type cells upon transfer from glucose to galactose (data not shown), and Srb10 is known to play a role in GAL1 activation. Second, though we have published that activation of Msn2 in mutants of the Ccr4-Not complex is mediated by the Glc7 type 1 protein phosphatase and its regulatory subunit Bud14 (30), we have found that Srb10 also contributes to STRE activation in these mutants (data not shown). Thus, we can imagine that the Ccr4-Not complex, which has been reported to be both nuclear and cytoplasmic (for reviews see references 14, 17, and 19), serves as a scaffold for Srb10 and its substrates in response to the appropriate physiological signals, a situation possibly mimicked by mutation of the complex. Alternatively, the complex might function to sequester or inhibit components of the kinase module, releasing or activating them only upon the appropriate signals (Fig. 6). Investigating these different exciting models will most certainly help us understand the mechanism and function of the Ccr4-Not complex.

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