Yeast mRNA cap-binding protein Cbc1/Sto1 is necessary for the rapid reprogramming of translation after hyperosmotic shock

Elena Garre,* Lorena Romero-Santacreu,* Nikki De Clercq,* Nati Blasco-Angulo,* Per Sunnerhagen,† and Paula Alepuz*

*Departamento de Bioquímica y Biología Molecular. Facultad de Ciencias Biológicas, Universitat de València. Doctor Moliner, 50. E-46100 Burjassot. Valencia. Spain; and †Department of Cell and Molecular Biology, Lundberg Laboratory, University of Gothenburg, S-405 30 Göteborg, Sweden.

Address correspondence to: Paula Alepuz (paula.alepuz@uv.es), Telephone: +34 963543462, Fax: +34 963544635

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ABSTRACT

In response to osmotic stress, global translation is inhibited, but the mRNAs encoding stress-protective proteins are selectively translated to allow cell survival. To date, the mechanisms and factors involved in the specific translation of osmostress-responsive genes in *Saccharomyces cerevisiae* are unknown. We find that the mRNA cap-binding protein Cbc1 is important for yeast survival under osmotic stress. Our results provide new evidence supporting a role of Cbc1 in translation initiation. Cbc1 associates with polysomes, while the deletion of the *CBC1* gene causes hypersensitivity to the translation inhibitor cycloheximide and yields synthetic “sickness” in cells with limiting amounts of translation initiator factor eIF4E. In *cbc1*Δ mutants, translation drops sharply under osmotic stress, the subsequent reinitiation of translation is retarded and P-bodies containing untranslating mRNAs remain for long periods. Furthermore, osmostress-responsive mRNAs are transcriptionally induced after osmotic stress in *cbc1*Δ cells, but their rapid association with polysomes is delayed. However in cells containing a thermosensitive eIF4E allele, their inability to grow at 37°C is suppressed by hyperosmosis, and Cbc1 relocates from nucleus to cytoplasm. These data support a model in which eIF4E-translation could be stress-sensitive, while Cbc1-mediated translation is necessary for the rapid translation of osmostress-protective proteins under osmotic stress.

Keywords: translation/ Cbc1/ osmotic stress/ Hog1 MAPK/ *Saccharomyces cerevisiae*

INTRODUCTION

One important response of eukaryotic cells to stress is the change in gene expression patterns through a general shutdown and reprogramming of protein synthesis. Stress signals inhibit the translation of ‘housekeeping’ proteins and promote the translation of stress-protective proteins (Spriggs *et al.*, 2010; Yamasaki and Anderson, 2008). Defects in translation reprogramming in response to stress have been associated with disease, including cancer, diabetes and inflammatory processes (Keene, 2007). How specific mRNAs become translated under stress, when general translation is down-regulated, is an important issue to be able to understand the mechanisms regulating gene expression.

Eukaryotic translation is a complex process controlled by a wide range of regulatory factors. Stress signals usually inhibit the process in the initiation step. Normal translation takes place through a cap-dependent initiation mechanism; the 40S ribosome subunit is recruited to a
modified base at the 5’ end of the mRNA (the m^7G cap). This process is coordinated by a complex with eukaryotic initiation factors (eIFs) called eIF4F, in which eIF4G is a scaffold protein that facilitates the interactions between proteins and the small ribosomal subunit, and eIF4E recognizes and binds the cap structure of mRNA. Once Met-tRNA, together with the 40S subunit and the eukaryotic initiation factors, form a complex on capped-mRNA, the start codon is recognized and the large ribosomal subunit joins. One key target for the control of translation in the initiation step is eIF4E, which can be bound by inhibitory proteins which subsequently impede mRNA binding (Wang and Proud, 2007; Lackner and Bähler, 2008; Van Der Kelen et al., 2009).

Whereas global translation is inhibited during stress, the mRNAs encoding stress response proteins must be able to engage with ribosomes and be translated. To achieve this, several mechanisms have evolved in eukaryotic cells. For some of them, the recruitment of ribosomes is regulated by elements in the 5’ and 3’ untranslated regions (UTR) of mRNAs, including the internal ribosome entry sites (IRES) which bypass the cap-dependent recruitment of mRNA, upstream open reading frames, and microRNA target sites (Yamasaki and Anderson, 2008; Van Der Kelen et al., 2009; Spriggs et al., 2010). However, for most stress conditions in which translation reprogramming has been described, the selective translation mechanism of stress-specific response mRNAs remains unknown. The relocalization of ‘housekeeping’ mRNAs to cytoplasmic foci upon stress, where they are stored in a translationally silenced state, would allow the dedication of a basal translation to synthesize protective proteins (Bond, 2006; Buchan and Parker, 2009; Lui et al., 2010).

One of the experimental models for studying the regulation of gene expression by stress is the response of *Saccharomyces cerevisiae* cells to hyperosmotic shock. A sudden increase in external osmolarity produces several rapid effects on yeast cells, such as a rapid decrease in protein synthesis and activation of the HOG MAP kinase pathway. Activation by phosphorylation of MAPK Hog1, which is homologous to mammalian p38 MAPK, is necessary for the control of different cellular processes which will allow cells to adapt and survive the stress condition (reviewed in de Nadal et al., 2002; Hohmann, 2002 and de Nadal and Posas, 2010). One of the main effects of Hog1 activation is a quick, massive change in gene expression. Thus, the levels of around 600 mRNAs (representing 10% of all yeast genes) significantly change after a few minutes of stress as a result of changes in their transcription rates and stability (Posas et al., 2000; Rep et al., 2000; Molin et al., 2009; Romero-Santacreu et al., 2009; Miller et al., 2011). The quick and transient general inhibition of translation observed under osmotic stress occurs in the initiation step by an unidentified mechanism which is apparently independent of Hog1. However, this MAP kinase is involved in the later activation of translation, possibly through Rck2, this being a yeast kinase which resembles mammalian MAPKAP kinases (Bilsland-Marchesan et al., 2000; Teige et al., 2001). Rck2 could affect global translation by directly regulating elongation factor EF-2, but an effect on initiation factors, which occurs in the homologous mammalian p38 MAPK pathway, cannot be ruled out. It is important to note that recent studies have shown that a Hog1-dependent short-term regulation of mRNA translation rates takes place after hyperosmotic stress. Therefore during the global inhibition of translation, important genes for cell survival under this stress condition are translationally up-regulated, hence their protein synthesis increases (Melamed et al., 2008; Halbeisen and Gerber, 2009; Warringer et al., 2010). In this scenario, the connection among translation, mRNA degradation and the formation of RNA-containing granules; stress granules and “processing bodies” (P-bodies); seems to be important because, in parallel to the regulation of translation rates, the stability of functionally-related groups of mRNAs is selectively modified and P-bodies are seen to temporarily increase upon hyperosmotic shock (Greatrix and van Vuuren, 2006; Hilgers et al., 2006; Molin et al., 2009; Romero-Santacreu et al., 2009; Miller et al., 2011). Translationally repressed mRNAs can be degraded in P-bodies by the main mRNA decay pathway, which includes the decapping enzyme Dcp1/Dcp2 and the 5’ to 3’exonuclease Xrn1. Under stress however, mRNAs can be stored together with some translation initiation factors such as eIF4E,
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into P-bodies to return to translation after adaptation (Hoyle et al., 2007; Parker and Sheth, 2007; Buchan and Parker, 2009; Romero-Santacreu et al., 2009). To date, the precise mechanisms and factors modulating the changes in mRNA translation and fate under osmotic stress remain unknown.

The nuclear cap binding complex (CBC) in *Saccharomyces cerevisiae*, comprised of Cbc1 and Cbc2, is recruited cotranscriptionally to the cap structure of mRNAs (Görlich et al., 1996; Wong et al., 2007). The CBC complex is mainly nuclear, but can shuttle between the nucleus and the cytoplasm using the importin α/β system (Görlich et al., 1996). Individual CBC subunits are not essential in yeast, but deletions of the corresponding genes diminish cell growth in glucose media, while mutants show different phenotypes related to different aspects of RNA metabolism (Das et al., 2000); consequently, CBC has been proposed to participate in a wide variety of mRNA-associated processes. Nuclear CBC is involved in splicing (Colot et al., 1996; Fortes et al., 1999; Zhang and Robash, 1999; Görnemann et al., 2005; Hossain et al., 2009), the formation of the preinitiation complex (PIC) at active genes (Lahudkar et al., 2011), transcription termination (Das et al., 2000; Wong et al., 2007), and nuclear mRNA degradation (Das et al., 2003; Kuai et al., 2005). Moreover in mammalian cells, CBC plays a role in exporting capped RNA, but it is not clear in yeast (Izaurralde et al., 1995; Görlich et al., 1996; Wong et al., 2007).

Besides the nuclear roles of Cbc1, its mammalian homolog CBP80 has been proposed to form part of the pioneer translation initiation complex. The bulk of cellular translation initiation is mediated by cytoplasmic cap-binding initiation factor eIF4E, but CBP80-bound mRNA, which is a precursor to eIF4E-bound mRNA, can also be translated during a pioneer round of translation in which aberrant mRNAs are eliminated by the nonsense-mediated mRNA decay pathway (NMD) (Ishigaki et al., 2001; Lejeune et al., 2002; Chiu et al., 2004). Supporting this model, CBP80 can be found to be associated with subpolysomal fractions, and the translation initiation factors eIF4GI, eIF3, eIF4I and eIF2A and poly(A)-binding protein (PAB)1, to coimmunopurify with both CBP80 and eIF4E, which suggests that each factor functions in both translation modes.

In yeast, Cbc1 has also been proposed to mediate a pioneer round of translation; nonetheless, contradictory data provide an unclear view of Cbc1’s role in translation. On the one hand, Gao et al. (2005) showed that NMD occurs in Cbc1-bound mRNAs, and also in eIF4E-mRNAs in yeast. However, Kuperwasser et al. (2004) argued against a Cbc1/Cbc2-mediated pioneer round of translation since they found that NMD occurs minimally in Cbc2-bound mRNA. Moreover, and remarkably, deletion in *CBC1* resulted in synthetic lethality with an eIF4GI version mutated in the sites of interaction with eIF4E and Pab1 (Fortes et al., 2000). These authors’ work showed that eIF4GI can simultaneously interact competitively with Cbc1 and eIF4E, and that Cbc1 stimulates a 2.5-fold translation in extracts of yeast strains which harbor a mutated eIF4GI which interacts only weakly with eIF4E. Nevertheless, later results have revealed that the cells expressing eIF4GI, with a point mutation that abolishes binding to Cbc1, fail to manifest a detectable defect in either the growth or composition of the transcriptome (Baron-Benhamou et al., 2003). Collectively, all these results suggest a connection between yeast Cbc1 and translation, but they do not explain the functional significance of this connection for yeast cells.

The importance of a mammalian CBP80-dependent pioneer round of translation in response to stress conditions has been recently documented. In mammalian cells under prolonged hypoxia or during serum starvation, a steady-state translation dependent of eIF4E is dramatically inhibited; however, nonsense-mediated mRNA decay (NMD) which, in mammalian cells, restricts only the pioneer round of translation via CBP80, occurs efficiently (Oh, et al., 2007a, b). Moreover, CBP80-translation differs from the steady-state translation, and a specific translation initiation factor, CTIF, is preferentially involved in CBP80-translation (Lee et al., 2008; Kim et al., 2009).

For the purpose of identifying those proteins involved in the post-transcriptional regulation of the gene expression in *S. cerevisiae* cells under osmotic stress, we performed an osmotic stress sensitivity screening using around 200 mutants in yeast genes encoding for RNA binding proteins.
One of the most sensitive strains carried a deletion in \textit{CBC1}. Our results show herein that Cbc1 is directly involved in translation under normal conditions, and that Cbc1-mediated translation ensures the rapid synthesis of osmostress-responsive genes upon hyperosmotic shock.

**RESULTS**

\textit{Deletion of the \textit{CBC1} gene results in growth reduction and loss of viability under osmotic stress}

For the purpose of discovering the yeast proteins involved in the regulation of gene expression in response to osmotic stress at the co- and post-transcriptional levels, we checked the tolerance to NaCl of the mutants in those genes encoding for the proteins described to be involved in RNA metabolism. To select mutants, we searched in the SGD (\textit{Saccharomyces Genome Database}) and found 181 genes for which deletions were viable and whose products were described to be involved in mRNA export, translation, degradation, splicing, or were genes of unknown functions whose corresponding proteins contained RNA-binding domains. The NaCl tolerance of these 182 mutants (from the Euroscarf mutant collection \url{http://web.uni-frankfurt.de/fb15/mikro/euroscarf/}) was assayed by determining the NaCl concentration which causes a growth reduction of 50\% (IC50 index; Table S1). One of the mutants which showed reduced tolerance to NaCl contained a deletion of the \textit{CBC1} gene (Figure 1A), which encodes the nuclear cap-binding protein Cbc1, homologous to the mammalian CBP80 protein (Lewis \textit{et al}., 1996). The sensitivity of the \textit{cbc1Δ} mutant to NaCl was due to a reduced tolerance to osmotic stress since the \textit{cbc1Δ} mutant also displayed hypersensitivity to high concentrations of sorbitol (Figure 1B). Moreover, \textit{cbc1Δ} cells showed a strong reduction in viability after treatments with 0.6 M KCl or 1.2 M NaCl when compared to wild-type (wt) cells (Figure 1C; between 40\% in 0.6 M KCl and 30\% in 1.2 M NaCl).

\textit{Osmotic stress-activated mRNAs are induced in \textit{cbc1Δ} mutants upon hyperosmotic shock}

Gene expression patterns are massive, rapid and transiently changed in response to hyperosmotic shock. These changes take place at the transcriptional and post-transcriptional levels and yield, among other things, a variation in the mRNA amounts of around 10\% of all the yeast genes, mostly controlled by Hog1 MAPK (reviewed in de Nadal and Posas, 2010). We reasoned that since the Cbc1 protein is co-transcriptionaly bound to mRNAs through their cap structure, and that cells in which the \textit{CBC1} gene has been deleted are hypersensitive to osmotic stress, regulation of gene expression could be defective in a \textit{cbc1Δ} mutant, which is the case in many osmostress hypersensitive mutants, including mutations in HOG pathway components (O’Rourke and Herskowitz, 2004). To test this possibility, we first investigated the induction of the total mRNA levels of \textit{STL1}, \textit{GPD1} and \textit{HSP12}, which are involved in glycerol transport, glycerol production and in maintaining membrane organization, respectively, and whose corresponding mRNAs are strongly and transiently induced by osmotic stress (Posas \textit{et al}., 2000; Romero-Santacreu \textit{et al}., 2009). As previously reported (Rep \textit{et al}., 1999; Posas \textit{et al}., 2000), the osmostress induction of \textit{STL1} was fully dependent on Hog1, while the inductions of \textit{GPD1} and \textit{HSP12} were partially dependent on this MAPK (Figure 2). In the \textit{cbc1Δ} mutant, the three genes were induced after treatment with 0.6 M KCl. The increase in the levels of \textit{STL1}, \textit{GPD1} and \textit{HSP12} during the first part of the time course (the first 15 minutes for \textit{STL1}, and the first 30 minutes for \textit{GPD1} and \textit{HSP12}) was similar in \textit{cbc1Δ} and wt cells, with a slight delay noted in the mutant (Figure 2B). However, the kinetics of mRNA induction showed differences between \textit{cbc1Δ} and wt at the maximal levels reached and in the length of response. In the \textit{cbc1Δ} mutant, the increase in the \textit{STL1}, \textit{GPD1} and \textit{HSP12} mRNA amounts was around two times higher and peaked 10-20 minutes later than in wt (Figure 2). The amount of mRNAs encoding ribosomal proteins, such as \textit{RPL17A} and \textit{RPS31}, has also been described to lower under osmotic stress (Posas \textit{et al}., 2000; Romero-Santacreu \textit{et al}., 2009). Our results reveal that variation in the mRNA levels of these two genes during osmotic stress treatment with 0.6 M KCl was similar in \textit{cbc1Δ} and wt cells, and only a
delay in the recovery of the basal level of RPS31 mRNA was observed in the cbc1Δ mutant cells (Figure 2). In summary, our results indicate that the growth defect and loss of viability of the cbc1Δ mutant under hyperosmotic stress do not correlate with an inability to induce the mRNA levels of the osmotic stress-responsive genes. On the contrary, our results indicate that this induction in the cbc1Δ mutant is stronger and lasts longer than in the wt strain.

The cbc1Δ mutant shows a defect in the reinitiation of translation after hyperosmotic shock

Gene expression also changed at the translation level upon osmotic shock. In yeast, increased extracellular osmolarity leads to a transient inhibition of translation in the initiation step. After adaptation, protein synthesis is restored through an unknown Hog1-dependent mechanism (Uesono and Toh-E, 2002). This stop in global translation occurs in parallel to a transient increase in the P-bodies due to the incorporation of mRNAs from polysomes, which is delayed and lasts longer in the hog1Δ mutant cells (Teixeira et al., 2005; Romero-Santacreu et al., 2009). Thus, as a second step in the study of gene expression under osmotic stress in the cbc1Δ mutant, we analyzed changes in global translation by obtaining polysome profiles and by following the P-bodies’ status. Under non stress conditions, the cbc1Δ and hog1Δ mutants showed a similar translation profile to that of the wt strain (Figure 3A). After 10 minutes of osmotic stress treatment with 0.6 M KCl, global translation dropped in all three strains, as expected. The cbc1Δ mutant displayed a more marked decrease than the wt or even the hog1Δ mutants (Figure 3A and B). Global translation recovered after 30 minutes of stress in wt cells, while the polysome levels did not recover after 60 minutes in the cbc1Δ mutant. At this time, it was possible to see that global translation in the the hog1Δ mutant had not completely recovered (Figure 3B). The P-bodies’ status under osmotic stress was followed by visualizing a GFP-tagged version of the decapping enzyme Dcp2 (Sheth and Parker, 2003). As previously described (Teixeira et al., 2005; Romero-Santacreu et al., 2009), under treatment with 1 M KCl, P-bodies could be visualized in wt cells after 30 minutes of stress and remained until 60 minutes; meanwhile in hog1Δ cells, an assembly of P-bodies was observed at 60 minutes, while bright foci were still visible after 2 h of stress (Figure 3C and D). In the cbc1Δ mutant, P-body assembly/disassembly kinetics were similar to that of the hog1Δ mutant: P-bodies were clearly visible after 60 minutes of stress, and were still observed with a similar brightness after 2 h of treatment (Figure 3C). Mild osmotic stress conditions, as treatment with 0.6 M KCl or 0.4 M NaCl, cause a quicker and more transitory formation of P-bodies (Romero-Santacreu et al, 2009; Figure 3C). Under both conditions, P-bodies were also visualized for longer periods in cbc1Δ cells if compared to wt cells (Figure 3C and D). These results indicate that Cbc1 is not essential for P-body formation. However, delay in the recovery of global translation and the prolonged permanence of P-bodies in the cbc1Δ mutant suggest a role for the cap-binding protein in P-bodies disassembly, the return of mRNAs to polysomes and the reinitiation of translation. A similar role in the control of mRNAs’ cytoplasmic fate in response to osmotic stress has also been suggested for MAP kinase Hog1 (Romero-Santacreu et al., 2009; Warringer et al., 2010). Altogether, our results indicate that the cbc1Δ mutant is not defective in the induction of osmostress mRNAs, but is defective in the reinitiation of translation during adaptation. Thus, the cbc1Δ mutant resembles only part of the defects in the regulation of gene expression caused by HOG1 deletion.

cbc1Δ cells are hypersensitive to the translation inhibitor cycloheximide and show a genetic interaction with translation initiation factor eIF4E

Previously, it was proposed that Cbc1 could mediate translation (Fortes et al., 2000; Gao et al., 2005), yet other results have argued against this role (Baron-Benhamou et al., 2003; Kuperwasser et al., 2004). Our results which revealed defects of the cbc1Δ mutants in the recovery of translation and P-bodies disassembly after hyperosmotic shock prompted us to further investigate the
involvement of Cbc1 in translation. With this aim in mind, we examined the sensitivity of the \( cbc1 \) mutant to the inhibitor of translation elongation cycloheximide (CHX) (McKeehan and Hardesty, 1969). It has been reported that yeast strains with translational defects are hypersensitive to the growth inhibitory effect of CHX (Gross et al., 2007). This is the case of the mutants in ribonucleoprotein Npl3, which promotes translation termination accuracy, and in Upf1, a component of the NMD RNA surveillance pathway with a role in translation (Leeds et al., 1991; Estrella et al., 2009). As Figure 4A depicts, the \( cbc1\Delta \) strain is hypersensitive to CHX; in fact, it is more sensitive than the \( upf1\Delta \) and \( npl3\Delta \) mutants (Figure 4A).

Translation initiation factor eIF4E replaces Cbc1 in the cap structure of cytoplasmic mRNAs. Nevertheless, it has also been proposed that both eIF4E and Cbc1 conduct pioneer rounds of translation in yeast (Gao et al., 2005). However, while eIF4E is essential for cell survival, deletion of \( CBC1 \) yields only a slight growth defect under optimal conditions (Figure 4A and B), indicating that bulk translation is initiated by eIF4E (Gao et al., 2005). Our results of hypersensitivity to CHX support the hypothesis of a role of Cbc1 in translation under non stress conditions. We hypothesized that Cbc1’s role in translation would become relevant for survival under conditions in which the eIF4E function was compromised. To test this, we introduced a \( CBC1 \) deletion into yeast cells carrying a thermosensitive mutation in eIF4E (\( cdc33 ts \)) (Schwartz and Parker, 1999). Yeast cells carrying the \( cdc33^{ts} \) allele (\( cdc33-42 \)) have previously been shown to perform almost 80% of the wild-type translation at 24ºC, and this ratio lowered to 19% at 38ºC (Schwartz and Parker, 1999). Accordingly, \( cdc33^{ts} \) cells did not show important growth defects on the YPD plates incubated at 25ºC or 33ºC, and were almost unable to grow at 37ºC (Figure 4B). It is interesting to note that the \( cdc33^{ts} cbc1\Delta \) double mutant grew clearly less well than the \( cdc33^{ts} \) mutant at 33ºC (Figure 4B). Additionally, we tested sensitivity to CHX in plates incubated for a longer period to allow the growth of the synthetic sick \( cdc33^{ts} cbc1\Delta \) strain. We observed that the hypersensitive defect of the \( cdc33^{ts} \) and \( cbc1\Delta \) single mutant strains was exacerbated in the double mutant (Figure S1A). Collectively, these results suggest that Cbc1, together with eIF4E, functions in the initiation of translation under optimal conditions, which is consistent with the hypothesis of Cbc1’s involvement in a cytoplasmic pioneer round of translation.

As mentioned earlier, our results indicate that the \( cbc1\Delta \) mutant is hypersensitive to osmotic stress (Figure 1). If both proteins eIF4E and Cbc1 are involved in translation under normal conditions, and Cbc1 is required for the reinitiation of translation after the global translation inhibition caused by osmotic stress (Figure 2), there is every reason to investigate the osmotic stress sensitivity of both the double mutant \( cdc33^{ts} cbc1\Delta \) and the single mutant \( cdc33^{ts} \). As Figure 4B depicts, the \( cdc33^{ts} cbc1\Delta \) strain was unable to grow in media with 0.8 M NaCl at any temperature. On the other hand, growth of \( cdc33^{ts} \) cells at a permissive temperature, where both eIF4E and Cbc1 are functional, considerably reduced by adding 0.8 M NaCl. This suggests that salt retains the ability to affect growth independently of both eIF4E and Cbc1. Unexpectedly, thermosensitive mutant \( cdc33^{ts} \) grew even better than a wt strain under hyperosmotic media, and even at a non permissive temperature (Figure 4B and S1B). The viability of \( cdc33^{ts} \) at 37ºC in hyperosmotic media was not explained by the recovery of the wt levels of protein eIF4E (Figure S1C). In summary, these results suggest that Cbc1 and eIF4E play redundant roles in translation, and that the roles of eIF4E under non stress and of Cbc1 under osmotic stress conditions are prevalent. The fact that, firstly, depletion of most of the eIF4E protein is not deleterious for yeast cells growing under osmotic stress but, the opposite, it makes yeast cells more osmotolerant; and second, that this suppression phenotype is lost when the \( CBC1 \) gene is deleted, suggest a potentiation of Cbc1’s role in translation when the amount of protein eIF4E is limited. This behavior could result from a more stable interaction between Cbc1 and eIF4G for this condition (Fortes et al., 2000).
Cbc1 is located in the nucleus under non stress and osmotic stress, and redistributes to the cytoplasm in the cells lacking either translation initiation factor eIF4E or exonuclease Xrn1

We have shown that the reinitiation of translation and P-bodies disassembly after adaptation to osmotic stress depend on Hog1 and Cbc1 (Romero-Santacreu et al., 2009; Figure 3). One possibility is that the Cbc1 expression or function could be regulated by osmotic stress; this regulation may, or may not, be Hog1-dependent. We observed no change in the amount of Cbc1 protein or in the modification of the protein which could change its electrophoretic mobility during osmotic stress (Figure S2). Previous studies have demonstrated how Cbc1 accumulates in the nucleus under optimal growing conditions (Görlich et al. 1996, Shen et al., 2000). We checked whether the Cbc1 protein changed its cellular localization in hyperosmotic media. To test this possibility, we followed the distribution of a Cbc1-GFP fusion during the treatment with 1 M KCl at the times when global translation stopped and P-bodies appeared (Figure 5A, Romero-Santacreu et al., 2009; Teixeira et al., 2005). We observed that Cbc1-GFP was enriched in the nucleus, regardless of osmotic stress treatment (Figure 5A). Thus, all these results provide no indication of Cbc1 regulation by osmotic stress.

Next, we tested whether the Cbc1 protein changed localization in the cdc33ts strain, since our above results suggest that Cbc1’s role in the initiation of translation becomes relevant in this strain given the limitation in the eIF4E-driven initiation of translation (Schwartz and Parker, 1999). As previously described, P-bodies were observed in cdc33ts cells at any temperature (Figure 5B and S3; Teixeira et al., 2005; Brengues and Parker, 2007). It is noteworthy that Cbc1 was distributed all over the cell in the cdc33ts mutant strain under both non stress and osmotic stress conditions when cells were incubated at 25ºC (Figure 5C). At a restrictive temperature (37ºC), Cbc1 was also distributed all over the cell, but when cdc33ts cells were additionally stressed with 1M KCl for 30 and 60 minutes, part of the Cbc1 protein was found in cytoplasmic foci, which partially colocalized with P-bodies (Figure 5B and C).

The inhibition of mRNA degradation by a mutation of the 5’ to 3’ exonuclease Xrn1 has been described to increase the number of P-bodies in yeast cells under normal conditions (Sheth and Parker, 2003). A role of eIF4E in targeting mRNPs to mammalian processing bodies has been suggested (Andrei et al., 2005; Ferraiuolo et al., 2005), and eIF4E has been detected in P-bodies in yeast cells (Brengues and Parker, 2007). We hypothesize that the amount of eIF4E protein dedicated to active translation would be limited in xrn1Δ cells because of a possible stall in the degradation of the eIF4E-containing mRNPs in P-bodies and, if this were the case, that the localization of Cbc1 in xrn1Δ cells would change. As Figure 5D illustrates, Cbc1 lost the nuclear accumulation pattern in xrn1Δ cells and was localized all over the cell. This distribution of Cbc1 in the cell was also observed in xrn1Δ cells under osmotic stress (Figure 5D).

In summary, our results indicate no change in Cbc1 nuclear localization during osmotic stress treatment, when global translation is transiently inhibited and P-bodies appear due to the incorporation of bulk mRNAs (Uesono and Toh, 2002; Teixeira et al., 2005). Conversely in cells in which eIF4E is limited, as in the cdc33ts and xrn1Δ strains, most Cbc1 relocalizes to the cytoplasm under both non stress and osmotic stress conditions. One simple interpretation of this result is that the recycling of Cbc1 to the nucleus needs the replacement of Cbc1 with eIF4E in the cap structure of cytoplasmic mRNA. However, an alternative and non exclusive interpretation is that more Cbc1 is needed in the cytoplasm to replace the eIF4E functions in translation when the amount of eIF4E is limited. Indeed, resistance of cdc33ts to osmotic stress, even at a restrictive temperature (Figures 4B and S1B), suggests that an increase in Cbc1 in the cytoplasm offers an advantage to cells against osmotic stress, possibly through Cbc1’s competence in translation. Finally, localization of part of the Cbc1 protein in the cytoplasmic foci in cdc33ts cells when simultaneously treated with a high temperature and osmotic stress resembles the visualization of the eIF4E initiation factor in P-bodies when global translation is inhibited (Brengues and Parker,
Garre et al 2007), and could also be indicative of the replacement of the eIF4E function in translation with Cbc1 under this condition.

**Cbc1 associates with polysomes**

Cbc1’s role in translation initiation could be performed through a direct association of Cbc1 with the active translation machinery. In mammalian cells, CBP80 associates with polysomal fractions to carry out the pioneer round of translation (Chiu et al., 2004; Kim et al., 2009). In a study into the Npl3 function in translation in yeast, Cbc1 was not detected in the polysomal fractions (Windgassen et al., 2004). However, we decided to do an in-depth analysis of Cbc1’s ability to cosediment with polysomes under both optimal and osmotic stress conditions. rRNA profiles, measured at A260, indicated the position of ribonucleoproteins, ribosomal subunits, monosomes and polysomes (Figure 6). The Western blot analysis of all fractions revealed that significant amounts of Cbc1 protein (between 15-20% of total Cbc1) were detectable in polysome-containing fractions of the wt cells grown under optimal conditions (Figure 6A). The association of Cbc1 with polysomes was dependent on blocking translation elongation by CHX (Figure 6B and Figure S4), indicating that Cbc1 associates with translationally active ribosomes (Moldave, 1985; Ramirez et al., 1991; Harel-Sharvit et al., 2010). Similarly to CBP80 in mammalian cells, Cbc1 was detected in all the polysomal fractions, but concentrated in the subpolysomal fractions (Figure 6), while eIF4E was mainly detected in higher polysomal fractions (Chiu et al., 2004; Hoyle et al., 2007). These results suggest that Cbc1-bound mRNAs are less efficiently translated than eIF4E-bound mRNAs.

Association with polysomes has also been described for other yeast nucleus-cytoplasm shuttling mRNA-bound proteins, such as Npl3, Gbp2 and Rpb4. For all these proteins, as with Cbc1, their fusions with GFP were only visualized in the nucleus due to the higher concentration of the proteins in this compartment (Windgassen et al., 2004; Harel-Sharvit et al., 2010). In mammalian cells, CBP80 associated with polysomes and was also highly enriched in the nucleus (Kim et al., 2009).

Next, we examined the association of Cbc1 with polysomes under osmotic stress. The proportion of polysome-associated Cbc1 did not significantly change after 15 or 30 minutes of addition of 0.6 M KCl (Figure 6A). Thus, similarly to the nuclear accumulation of Cbc1, and regardless of osmotic stress, the proportion of the Cbc1 associated with polysomes remained unchanged by stress. However, it should be noted that the proportion of the Cbc1 associated with polysomes was constant, independently of the reduction of polysomes due to the translation inhibition provoked by osmotic stress (Figure 6A); thus, it constituted a larger fraction of the polysome-associated proteins.

We also examined the association of Cbc1 with polysomes in the cdc33Δ and xrn1Δ mutants. Our results indicate that the cytoplasmic localization of Cbc1 protein in cdc33Δ and xrn1Δ (Figure 5) does not lead to an increased association of the protein with polysomes (Figure S5A and B).

Together, all these data suggest that: (1) association of Cbc1 with polysomes is independent of osmotic stress; however, the proportion of Cbc1-dependent translation increases under osmotic stress because under this condition, bulk translation is inhibited, although the proportion of the Cbc1 protein associated with polysomes remains constant; (2) association of Cbc1 with polysomes remains constant despite the abrogation of Xrn1 or eIF4E; thus, this result suggests that the proportion of Cbc1-bound mRNA in relation to the total mRNA in active translation increases when the eIF4E translation initiation factor amount is limited in cdc33Δ and putatively in the xrn1Δ mutants.

**Cbc1 is required for a rapid translation of osmotic-induced mRNAs under osmotic stress**

All the results above enabled us to hypothesize that Cbc1 is directly involved in translation initiation under osmotic stress, when bulk translation is inhibited. Given this stress condition,
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Cbc1-dependent translation would still work efficiently and be relevant for cell adaptation. Recently, it has been reported that specific functional groups of mRNAs increase their association with polysomes under osmotic stress conditions in which the global translation is inhibited (Melamed et al., 2008; Halbeisen and Gerber, 2009; Warringer et al., 2010). Some of the genes that are translationally up-regulated are also up-regulated at the transcriptional level, and include genes essential for survival to osmotic stress, such as GPD1 (Melamed et al., 2008; Warringer et al., 2010). One prediction of our model of Cbc1’s role in translation under osmotic stress is that the association of the osmostress-regulated mRNAs with polysomes would be affected in those yeast cells lacking the CBC1 gene. In order to test this possibility, we examined the association of several osmostress-regulated mRNAs with polysomes during an osmotic stress treatment time course (Figure 7). For GPD1, STL1 and HSP12, mRNA amounts increased after 15 minutes of treatment with 0.6 M KCl (Figure 7A and 2). At this time, an important proportion of the three mRNAs was detected in the polysomal fraction in wt cells (Warringer et al., 2010), whereas in cbc1Δ cells, the polysomal association of these osmostress-regulated mRNAs was clearly lower (Figure 7A). It is noteworthy that at 30 minutes of stress, an important proportion of the mRNAs remained associated with the monosomal and the free fraction in cbc1Δ cells, whereas most mRNAs were associated with polysomes in wt cells (Figure 7A). As described earlier (Figure 2), the STL1, GPD1 and HSP12 mRNA levels were still high in the cbc1Δ strain at times of osmotic stress when these levels dropped in the wild-type strain (Figure 7A). The analysis of the polysomal (P) to the free and monosomal (FM) ratios indicates that most of STL1, GPD1 and HSP12 mRNAs were actively translated at 60 minutes of stress in the cbc1Δ strain (Figure 7B). We also analyzed the translational status of two mRNAs, RPL17A and RPS31, encoding ribosomal proteins. It has been reported that a decrease in the levels of the mRNAs encoding for ribosomal proteins (RP) occurs quickly after osmotic stress (Posas et al., 2000; Molin et al., 2009; Romero et al., 2009) and, for many of the RP-encoding mRNAs, this takes place in parallel to a translational down-regulation (Melamed et al., 2008; Halbeisen and Gerber, 2009; Warringer et al., 2010). Accordingly, the levels and the P/FM ratios for RPL17 and RPS31 mRNAs lowered after treatment with 0.6 M KCl in a wt strain (Figure 7A and B). Similar results were obtained with the cbc1Δ mutant strain during the first 30 minutes of stress; however, the recovery in the polysomal association of both the RP-encoding mRNAs was delayed in cbc1Δ compared with wt cells (Figure 7A and B, see the differences in P/FM ratios at 60 minutes).

In order to independently confirm Cbc1’s role in the rapid translation of transcripts after osmotic stress, we monitored translation efficiency in cbc1Δ cells by checking the expression of an osmotic stress inducible reporter gene. We used a fusion gene between the STL1 promoter and the LacZ reporter gene (STL1-LacZ), and measured the beta-galactosidase activity of the corresponding protein in relation to the increase in the LacZ mRNA level during osmotic stress treatment of cells with 0.6 M KCl. As Figure 7C shows, the efficiency of the translation of STL1-LacZ mRNA (proportion of reporter protein activity in relation to the mRNA amount) increased significantly faster in wt than in cbc1Δ cells. According to our previous data of the association of osmostress-induced mRNAs with polysomes (Figure 7B), the translation efficiency of STL1-LacZ needed more time to recover in a cbc1Δ mutant after osmotic shock (Figure 7C). The importance of 3’-UTRs for the proper up-regulation of mRNA translation under osmotic stress has been indicated (Warringer et al., 2010). Our result obtained with STL1-LacZ indicates an impaired reporter-RNA translation in cbc1Δ cells which is independent of the native 3’UTR of STL1 mRNA. One likely interpretation is that STL1-LacZ translation is regulated through its STL1 5’-UTR.

Collectively, all these results indicate that Cbc1 is necessary for the rapid translation of mRNAs induced by osmotic stress, but that the down-regulation of the translation of those mRNAs encoding for ribosomal proteins is independent of Cbc1. In summary, these results suggest that
Cbc1-dependent translation is not inhibited by osmotic stress, but is required for rapid translation reprogramming under this stress condition.

DISCUSSION

In response to osmotic stress, global translation is inhibited in *S. cerevisiae* cells. Nonetheless, many osmostress responsive mRNAs engage with polysomes and their corresponding stress-protective proteins are efficiently synthesized (Uesono and Toh, 2002; Melamed et al., 2008; Halbeisen and Gerber, 2009; Warringer et al., 2010). The results presented herein identify, for the first time, an mRNA binding protein, yeast cap-binding protein Cbc1, involved in this rapid translation reprogramming upon osmotic stress.

The involvement of yeast Cbc1 in translation has been a matter of debate in the past few years (see the Introduction for details). Our results herein favor a direct role of Cbc1 in translation initiation, which is not essential for cell viability under normal conditions in which Cbc1 is replaced with eIF4E in the cap of most transcripts in the cytoplasm (Gao et al., 2005). First, *cbc1* mutants are sensitive to translation inhibitor CHX (Figure 4A). Second, a *cbc1* mutation confers synthetic sickness, at any temperature, with the *cdc33* mutation, which renders translation initiation factor eIF4E thermosensitive (Figure 4B). A functional interaction between eIF4E and Cbc1 is also demonstrated by the synergistic CHX hypersensitivity of the *cdc33* *cbc1*Δ double mutant (Figure S1A). Third, Cbc1 cosediments with polysomes in a CHX-dependent (i.e., translation-dependent) manner. Ribosome-associated Cbc1 is concentrated in subpolysomal fractions and involves only a small fraction of the Cbc1 protein (15-20%) (Figures 6 and S4).

Our data highlight the important role played by Cbc1-dependent translation under osmotic stress conditions. First, the *cbc1*Δ mutant shows low viability in hyperosmotic media (Figure 1). Second, the decrease in the polysome content in response to osmotic stress is more extensive in the *cbc1*Δ mutant than in wt cells, and the recovery of normal translation profiles is delayed in the *cbc1*Δ mutant (Figure 3A and B). Third, the P-bodies formed when global translation is inhibited upon osmotic shock remain for longer periods in *cbc1*Δ mutants than in wt cells (Figure 3C), indicating a defect in recovery. Fourth, osmostress-responsive mRNAs are induced in a *cbc1*Δ mutant in response to osmotic stress (Figure 2); however, their rapid association with polysomes, as observed in wt cells, is significantly delayed in the mutant (Figure 7A). Moreover, the increase in translation efficiency upon osmotic stress of the reporter gene *STL1*-LacZ and the subsequent decline are also delayed in the *cbc1*Δ mutant if compared with wt cells (Figure 7B). Fifth, the growth inability of *cdc33* mutants at 37°C is suppressed by hyperosmosis and, in those cells with limiting amounts of eIF4E, Cbc1 relocates to the cytoplasm. Sixth, the Cbc1 protein fraction associated with polysomes remains constant under osmotic stress, when global translation is inhibited; this indicates that the proportion of Cbc1-bound mRNA in active translation increases upon stress (Figure 6A). All these phenotypes of the *cbc1*Δ mutant in translation under osmotic stress are not merely the result of Cbc1’s role in mRNA metabolism since other mutants in genes involved in splicing, transcription or mRNA export do not show similar effects (Figure S6).

We suggest a model in which the bulk translation driven by eIF4E is stress-sensitive, and is mostly inhibited by osmotic stress, while Cbc1-mediated translation is stress-resistant. Thus, the newly synthesized mRNA encoding osmostress-protective proteins will be bound by Cbc1 in the nucleus and will undergo Cbc1-dependent translation in the cytoplasm. At the same time, the pool of housekeeping mRNAs, which was actively translated before stress mostly by cytoplasmic eIF4E, will be kept in the P-bodies until adaptation. A similar scenario has been proposed for CBP80-mediated translation in mammalian cells under stress conditions provoked by prolonged hypoxia and serum starvation (Oh et al., 2007a, b). Under such stresses, the steady-state translation mediated by eIF4E is drastically abolished, whereas CBP80-mediated translation efficiently
occurs. According to our model, the level of yeast Cbc1 protein remains constant and the protein does not seem to be modified by osmotic stress (Figure S2).

One open question is whether Cbc1-mediated translation mechanistically differs from eIF4E-mediated translation. As stated before, Cbc1 can interact with eIF4GI (Fortes et al., 2000), similarly to the interaction between mammalian CBP80 and eIF4GI (McKendrick et al., 2001; Chiu et al., 2004). However, in mammalian cells, the CBP80-eIF4GI interaction is of little functional relevance, and a new translation factor, CTIF, is specifically involved in CBP80-dependent translation (Chiu et al., 2004; Lee et al., 2008; Kim et al., 2009). In yeast, additional studies should be conducted to determine the Cbc1-mediated translation mechanism.

Another unresolved issue is whether Cbc1 mediates the translation of any mRNA or whether Cbc1-dependent translation is restricted to specific transcripts, e.g., osmostress-responsive mRNAs. Under osmotic stress, we have shown that the rapid translation of newly synthesized endogenous osmo-responsive mRNAs depends on Cbc1, as does the translation of the osmotic stress-induced STL1-LacZ fusion (Figure 7). Finally, the modulation of translation under osmotic stress by Cbc1 could imply a direct role in the movement of mRNAs from P-bodies to polysomes. Consistently, P-bodies remain longer and less polysomes are observed in the cbc1Δ mutant cells under osmotic stress (Figure 2). Moreover, Cbc1 is localized in cytoplasmic foci which partially colocalize with the P-bodies in cdc33Δ cells when translation is abolished by a simultaneous treatment with high temperature and osmotic stress (Figure 5B and C).

The hypersensitivity and loss of viability of cbc1Δ mutant cells under osmotic stress could relate to Cbc1’s role in translation reprogramming. However, we cannot rule out other Cbc1 functions in response of yeast to osmotic stress. Recently, the fact that a cytoplasmic membrane-anchored version of Hog1, which is unable to induce the transcription of osmostress-responsive genes, showed no growth defects in hyperosmotic media has led to the proposal that the regulation of transcription by Hog1 is not essential for yeast survival under osmotic stress (Westfall et al., 2008). However, cytoplasmic Hog1 could still be able to regulate translation; therefore, we do not know how important this regulation is for cell survival.

The translation mediated by mammalian CBP80 seems to be resistant to several stress conditions, e.g., heat shock, prolonged hypoxia or serum starvation (Maquat and Li, 2001; Marín-Vinader et al., 2006; Oh et al., 2007a, b). Whether yeast Cbc1 is involved in translation reprogramming under other stress conditions should be addressed.

The fact that osmo-mRNAs levels exhibit a greater and prolonged increase in cbc1Δ than in wt under osmotic stress (Figure 2) suggests a defect in the transcription shut-down and/or mRNA destabilization which occurs after an initial increase in transcription and stability (Molin et al., 2009; Romero et al., 2009). Future studies will reveal whether Cbc1 plays a role in cytoplasmic mRNA decay and/or there is crosstalk between the translation and transcription mediated by Cbc1. Recently, the Rpb4/7 subunits of Polymerase II have been postulated as mRNA coordinators that couple transcription, mRNA decay and translation (Goler-Baron et al., 2008; Harel-Sharvit et al., 2010). The involvement of nuclear Cbc1 in transcription (Das et al. 2000; Wong et al., 2007; Lahudkar et al., 2011), mRNA splicing (Lewis et al., 1996) and nuclear degradation (Das et al., 2003), and that of cytoplasmic Cbc1 in translation (Gao et al., 2005; this work) and in mRNA decay, suggest that Cbc1 could also be one of the mRNA coordinators connecting the fate of mRNA to cellular circumstances.

MATERIALS AND METHODS

Yeast strains and growth conditions
The genotypes of the strains used in this study are listed in Table 1. The strains containing CBC1 gene deletion and those expressing Cbc1 protein C-terminally tagged with green fluorescent protein (GFP) were constructed following the polymerase chain reaction (PCR)-based gene
Garre et al. (1998). Purified PCR products were used for yeast transformation by the lithium acetate method (Gietz et al., 1995), and transformants were selected by plating on appropriate selective medium plates.

NaCl tolerance screening was done by growing yeast strains in 220 μL of YPD medium (1% yeast extract, 2% peptone, 2% glucose) in 96-well microtiter plates for 16 hours. Then, cultures were diluted 5000 times in 8 cultures of 215 μL of YPD plus NaCl at 0, 0.15, 0.25, 0.4, 0.55, 0.75, 1 and 1.25 M. Plates were incubated at 30°C for 17-24 hours and OD600 (optical density) was measured by an absorbance microplate reader (SpectraFluor, Tecan). IC50 (concentration of NaCl required to inhibit 50% of growth in relation to the maximal growth observed for the same strain in YPD without NaCl) was calculated using the OriginLab software.

Thermosensitive mutants were grown at 25°C and were then transferred to a permissive (25°C or 33°C, as indicated) or a restrictive temperature (37°C). Hyperosmotic stress was provoked in a liquid medium by adding KCl or NaCl to cultures to obtain the indicated final salt concentration. To test osmotic stress sensitivity in a solid medium, YPD was supplemented with NaCl (0.4, 0.8 or 1.2 M) or with sorbitol (1 or 1.5 M). Cycloheximide (CHX) sensitivity tests were performed on solid YPD containing CHX at the indicated concentration. All the tests for growth on solid media were done with exponentially growing cultures (OD600 0.5), then 5- or 10-fold serial dilutions were made and 8 μL of each dilution were spotted on the indicated plates. The incubation times and temperature are indicated in each case.

**Flow cytometry**

Yeast cells were grown until the exponential phase in YPD and were subjected to osmotic stress by adding 0.6 M KCl or 1.2 M NaCl, or they were kept in YPD as the control condition. After stress incubation, 100 μL of the culture were harvested by centrifugation at 13000 rpm for 30 s and cells were resuspended in 400 μL of PBS buffer (140 mM NaCl, 40 mM Na2HPO4, 2.7 mM KCl, 1.8 mM KH2PO4). To identify dead cells, propidium iodide 15 μM (PI) dye was added and fluorescence was measured in a flow cytometer (EPICS XL-MCL, Beckman Coulter) after 5 min of incubation at room temperature. The yeast cells kept in YPD were used as a 100% viability control. The fluorescence of the cells under the control conditions without PI was used as a baseline.

**Polysomal fractionation and analysis of the specific RNAs and proteins from the fractions**

Cells grown exponentially in YPD media to OD600 0.5 were subjected to 0.6 M KCl stress for the indicated times. A portion of the culture (80 mL) was recovered and chilled for 5 min on ice in the presence of 0.1 mg/mL CHX. Cells were harvested by centrifugation at 6000 g for 4 min at 4°C and resuspended in 400 μL of PBS buffer (140 mM NaCl, 40 mM Na2HPO4, 2.7 mM KCl, 1.8 mM KH2PO4). To identify dead cells, propidium iodide 15 μM (PI) dye was added and fluorescence was measured in a flow cytometer (EPICS XL-MCL, Beckman Coulter) after 5 min of incubation at room temperature. The yeast cells kept in YPD were used as a 100% viability control. The fluorescence of the cells under the control conditions without PI was used as a baseline.
ethanol-sodium acetate. Specific RNAs were analyzed by Northern blotting. To analyze the protein content of the fractions, proteins were precipitated by 10% TCA and twice washed with cold anhydrous acetone. Dry pellets were resuspended in SB 1X (360 mM Tris-HCl, 18% SDS, 60% glycerol, 24% β-mercaptoethanol, 0.12% bromophenol blue). The extracted proteins were analyzed by Western blotting as described below.

**Total RNA extraction and Northern blotting**
For the analysis of the cellular mRNA levels, exponentially growing cultures were incubated at the indicated conditions and times, and the cells from 20 ml culture were collected by centrifugation and frozen at -20°C. Total RNA was extracted from the cell pellets using a Fast-Prep device (Fast120, Bio101 Savant) in 0.5 mL LETS buffer (0.1 M LiCl, 0.01 M EDTA pH 8.0, 0.01 M Tris-HCl pH 7.4, and 0.2% SDS [p/v]), 0.5 mL phenol (pH 4.5):chloroform:isoamylic alcohol (25:24:1) (v:v:v) and 0.3 mL glass beads. Supernatants were extracted with phenol:chloroform:isoamylic alcohol (25:24:1) (v:v:v) and chloroform:isoamylic alcohol (24:1) (v:v). RNA was precipitated by adding two volumes of 96% ethanol and 1/10 volumes of 5 M LiCl and by incubating at -20°C for at least 3 h. Isolated RNA from the polysomal fractions and total RNA were separated by electrophoresis in denaturing 1% (w/v) agarose gel (2.2 M formaldehyde, 1X MOPS [20 mM MOPS, 8 mM NaAc, 1 mM EDTA pH 7.0]), and were blotted on Hybond N+ membranes (Amersham) (Sambrook and Russell, 2001). Ethidium bromide-stained gels were read in a phosphorimager scanner (FLA-3000, FujiFilm) to quantify the signal of 18S or 25S ribosomal RNA (rRNA). Then, the membrane was hybridized for 24 h at 65°C in 0.5 M sodium phosphate (pH 7.2), 7% SDS (w/v) and 1 mM EDTA (pH 7.0). DNA probes were obtained by PCR and were labeled by random priming using the Ready-to-Go kit (Amersham) and 33P-dCTP. After hybridization, membranes were washed once for 10 min at 25°C in 1X SSC, 0.1% SDS and once for 15 min at 65°C in 0.5X SSC, 0.1% SDS. Membranes were exposed to an imaging plate (BAS-MP, Fujifilm), which was read in a phosphorimager scanner (FLA-3000, Fujifilm).

**Western blot analysis**
To analyze the total cellular content of the Cbc1 protein under non stress and osmostress conditions, we followed the protein extraction protocol detailed in Alepuz et al. (2001). The total protein extracts and the protein extracts from polysomal fractions were resolved by 10% polyacrylamide-SDS gel electrophoresis. Gels were then transferred to a nitrocellulose membrane (PROTRAN, Whatman) and blocked in 5% nonfat milk overnight at 4°C. The antibodies utilized were α-Cbc1 (1:20,000 dilution; kindly provided by D. Görlich) and α-Rpl5 (1:5,000 dilution; kindly provided by J.L. Woolford). The secondary antibody was anti-rabbit (1:25,000 dilutions, Amersham, GE Healthcare). Membranes were developed using an ECL Advance and Typhoon scanner. Images were quantified using the Image Gauge software.

**β-Galactosidase assays**
The wt and cbc1Δ strains containing STL1-LacZ fusion in a multicopy plasmid (STL1 promoter fused to LacZ, LEU2+ 2μm, a kind gift from E. de Nadal) were grown until the exponential phase in selective media. For the β-galactosidase assays, 3 mL of each culture, either untreated or subjected to the indicated conditions, were recovered by centrifugation. Cells were washed once and resuspended in 500 μL Z buffer (16.1 g/L Na2HPO4·7H2O, 5.5 g/L NaH2PO4·H2O, 0.75 g/L KCl, 0.246 g/L MgSO4·7H2O, pH 7.0). To permeabilize cells, 50 μL of sample were kept in liquid N2 for 1 minute to be then heated at 37°C for 1 min. To start the reaction, 600 μL of Z buffer containing 37.4 mM β-mercaptoethanol and 160 μL of 4 mg/mL ONPG were added to each permeabilized cells sample. Samples were incubated at 30°C until the appearance of a yellow color and the reaction was then stopped by adding 400 μL 1M Na2CO3. The absorbance at 420 nm was measured in the supernatants. The β-galactosidase units were calculated as described (Miller,
The results shown are the mean and standard error of at least three independent experiments.

**Fluorescence microscopy**

Cells expressing Dcp2-GFP or Dcp2-RFP from plasmids (Sheth and Parker, 2003) or Cbc1-GFP from the CBC1 genomic locus were grown overnight in YPD or in SC (synthetic complete) medium supplemented with the appropriate amino acids, respectively. Then, cells were transferred to YPD and grown to an OD600 of 0.5-0.6. Cells were washed and resuspended in SC plus amino acids, without salt or with 1M KCl, as indicated. Observations were made at the indicated times using an Axio Imager Z1 fluorescence microscope (Zeiss), an FV1000 system and a FV10-ASW 2.1 Viewer software for the colocalization experiments.

**ACKNOWLEDGMENTS**

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*Strain derivative of the EUROSCARF wild-type BY4741 strain

b Strain derivative of the wild-type yRP840 strain
FIGURE LEGENDS

**Figure 1.** The *cbc1Δ* mutant shows growth defects and loss of viability under osmotic stress. (A) The NaCl concentration required to inhibit 50% of growth (IC50) as a measure of the osmotic stress sensitivity of the indicated strains. The data are the mean of at least three independent experiments and the standard error is indicated. (B) Sensitivity to different osmotic stress conditions of the *cbc1Δ* and *hog1Δ* mutant cells. Exponentially growing cells were spotted on YPD plates supplemented with NaCl or sorbitol. Plates were incubated at 30°C. (C) Viability analysis of the *cbc1Δ* mutant during osmotic stress. Exponentially growing wt and *cbc1Δ* cells in YPD were subjected to the indicated osmotic stress conditions or kept in YPD without stress. Samples were taken at the indicated times and dead cells were determined by flow cytometry using propidium iodide. The percentage of living cells for each condition is compared to the cells in an equivalent aliquot of the same strain incubated in YPD. The data are the mean and the standard error of two independent experiments.
Figure 2. Increase in the levels of osmostress-responsive mRNAs is delayed, but not inhibited, in the *cbc1Δ* mutants. The wt, *hog1Δ* and *cbc1Δ* strains were grown exponentially in YPD, then cells were subjected to osmotic stress by addition of 0.6 M KCl. Samples were taken at the indicated times and the expression of the indicated genes was analyzed by Northern blotting (A). (B) Quantifications of the mRNA levels normalized to rRNA. The data are the mean and the standard error of three independent experiments.
Figure 3. The *cbc1Δ* mutant shows a defect in translation recovery after hyperosmotic stress. (A) Reinitiation of translation after adaptation to osmotic stress is delayed in the *cbc1Δ* mutant. Polysome profiles show translation inhibition and recovery during osmotic stress in the wt, *hog1Δ* and *cbc1Δ* strains. Cells grown exponentially in YPD were treated with 0.6 M KCl at the indicated times. Polysomal profiles were obtained as
described in Material and Methods. The relative amount of rRNA in each fraction was measured by online UV detection at 260 nm and shows the ribosomal light subunits (40S), heavy subunits (60S), monosomes (80S) and polysomes (2n, 3n, 4n...). Representative absorbance curves (out of 5 repetitions) are displayed. (B) Percentage of the polysomal area during osmotic stress (0.6 M KCl) in wt, hog1Δ and cbc1Δ. The polysome percentage is relative to the polysomal area of each strain at time 0. The data are given as the mean and standard error of three independent experiments. (C) P-bodies assembly and disassembly under osmotic shock are delayed in the cbc1Δ mutant. The wt, hog1Δ and cbc1Δ strains expressing a GFP-tagged version of the decapping enzyme Dcp2 were grown until the exponential phase to be then treated with 1 M KCl, 0.6 M KCl or with 0.4 M NaCl. Dcp2-GFP allowed P-bodies visualization by fluorescence microscopy. (D) The quantification of the percentage of cells with P-bodies is indicated in the graphs. At least 100 cells were quantified for each condition.
Figure 4. Cbc1 is involved in translation under non stress and osmotic stress conditions. (A) Sensitivity to cycloheximide (CHX) of the \textit{cbc1}\textDelta\textit{ mutant}. The wt, \textit{cbc1}\textDelta\textit{ mutant and strains carrying deletions in the \textit{UPF1} and \textit{NPL3} genes were grown exponentially in YPD. An aliquot of each culture was diluted and 8\mu\text{L} of 5-fold serial dilutions were spotted on plates containing YPD or YPD supplemented with 0.05 \mu\text{g/mL}; 0.075 \mu\text{g/mL} and 0.1 \mu g/mL CHX. Plates were grown for 2 days at 30°C. The results in this figure are representative of at least two independent experiments. (B) Synthetic sickness of the double mutant \textit{cdc33}\textsuperscript{ts}\textit{cbc1}\textDelta\textit{ and resistance to osmotic stress of the single mutant \textit{cdc33}\textsuperscript{ts}. Cultures of the wt, \textit{cbc1}\textDelta, \textit{cdc33}\textsuperscript{ts} and \textit{cdc33}\textsuperscript{ts}\textit{cbc1}\textDelta\textit{ strains were treated as in (A), and growth was tested in YPD and YPD supplemented with 0.8 M NaCl. Plates were left to grow for 4 days at the indicated temperatures. The results in this figure are representative of at least two independent experiments.
Figure 5. Cbc1 accumulates in the nucleus in wt cells under non stress and osmotic stress conditions, but is distributed all over the cell in mutants in either translation initiation factor eIF4E or mRNA exonuclease Xrn1. (A and D) The wt and xrn1Δ strains
expressing Cbc1-GFP or Dcp2-GFP (P-body marker) were exponentially grown in YPD. Then, cultures were incubated with 1 M KCl at 30°C. Cbc1-GFP localization and P-bodies (Dcp2-GFP) were visualized at 0, 30 and 60 min of osmotic stress treatment by fluorescence microscopy (left panel). Cells were also photographed by Nomarski optics (right panel). (B) The wt and cdc33ts mutant strains expressing Cbc1-GFP or Dcp2-GFP were exponentially grown in YPD at 25°C. One aliquot of each culture was treated with 1 M KCl and incubated at a permissive temperature (25°C). Another aliquot was incubated at a restrictive temperature (37°C) for 30 min and was then treated with 1M KCl. Cbc1-GFP localization, P-bodies (Dcp2-GFP) and cells were visualized as described in (A and D). (C) Cbc1 partially colocalizes with P-bodies. The cdc33ts cells co-expressing Cbc1-GFP and Dcp2-RFP (P-body marker) were exponentially grown in YPD at 25°C. Afterward, the culture was incubated at a restrictive temperature (37°C) for 30 min and was then treated with 1 M KCl for 60 min. Cbc1-GFP and Dcp2-RFP were visualized by fluorescence microscopy using a FV1000 system and the FV10-ASW 2.1 Viewer software.
Figure 6. Cbc1p associates with polysomes under non stress and osmotic stress conditions. (A) The wt culture growing exponentially in YPD was treated with 0.6M KCl and polysomal profiles were determined at the indicated times. Messenger ribonucleoprotein (RNP), free (F), monosomal (M) and polysomal [P(1-5)] fractions were collected and analyzed by Western blotting with the anti-Cbc1 antibody. (B) Wt cells were treated with 0.6 M KCl for 15 min. Polysomal profiles were obtained from one aliquot of the culture (control), as described in Materials and Methods. Another aliquot of the culture was treated with NaN₃ for 30 min at 30°C, and CHX and MgCl₂ were omitted in the cell extract and sucrose gradient to disrupt polysomes (-CHX/-MgCl₂). An analysis of the Cbc1 protein in the fractions was performed as in (A). Detection of ribosomal protein Rpl5 with a specific antibody was used as a control of polysome disassembly. The polysome profiles and the westerns blot analyses shown in this figure correspond to a representative experiment. The percentage of Cbc1 (A and B) and of Rpl5 (B) in non polysomal (RNP+F+M) and in polysomal fractions (P1-P5) is indicated. The data are provided as the mean and standard error of at least two independent experiments.
Figure 7. Rapid translation reprogramming under hyperosmotic stress is delayed in the cbc1Δ mutant. (A) Association of osmostress response mRNAs and ribosomal proteins mRNAs with polysomes in wt and cbc1Δ under osmotic stress. The wt and cbc1Δ cultures growing exponentially in YPD were treated with 0.6 M KCl, and polysomal
profiles were determined at the indicated times. The fractions corresponding to free and monosomal (FM) and to polysomal (P) were collected and analyzed by Northern blotting using specific probes that recognize the indicated mRNAs. The RNP fraction was excluded because it never gave a signal for the mRNAs tested here (data not shown). (B) The quantified signals from three independent replicates were used to determine the average change in the P/FM ratios for the indicated genes. (C) Translation efficiency of the STLI-LacZ fusion in wt and cbc1Δ under osmotic stress. The wt cells expressing fusion STLI-LacZ from a plasmid (the STLI promoter fused to LacZ, LEU2+ 2mm) were grown in selective media at 30°C until the exponential phase, and were then transferred to YPD and incubated at 30°C for 4 hours. Then, cells were subjected to osmotic stress by adding 0.6 M KCl, while samples were taken at the indicated times. β-galactosidase activity was measured as described in Materials and Methods, and the LacZ mRNA level was determined by Northern blotting. Translation efficiency was calculated as the β-galactosidase activity/LacZ mRNA ratio, and the results were normalized to efficiency at time 0. The data shown are the mean and standard error of three independent experiments.