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RESEARCH PAPER

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Karyopherin Msn5 is involved in a novel mechanism controlling the cellular level of cell cycle regulators Cln2 and Swi5

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ABSTRACT

The yeast β-karyopherin Msn5 controls the SBF cell-cycle transcription factor, responsible for the periodic expression of *CLN2* cyclin gene at G1/S, and the nuclear export of Cln2 protein. Here we show that Msn5 regulates Cln2 by an additional mechanism. Inactivation of Msn5 causes a severe reduction in the cellular content of Cln2. This occurs by a post-transcriptional mechanism, since *CLN2* mRNA level is not importantly affected in asynchronous cultures. Cln2 stability is not significantly altered in *msn5* cells and inactivation of Msn5 causes a reduction in protein level even when Cln2 is stabilized. Therefore, the reduced amount of Cln2 in *msn5* cells is mainly due not to a higher rate of protein degradation but to a defect in Cln2 synthesis. In fact, analysis of polysome profiles indicated that Msn5 inactivation causes a shift of *CLN2* and *SWI5* mRNAs from heavy-polysomal to light-polysomal and non-polysomal fractions, supporting a defect in Cln2 and Swi5 protein synthesis in the *msn5* mutant. The analysis of truncated versions of Cln2 and of chimeric cyclins combining distinct domains from Cln2 and the related Cln1 cyclin identified an internal region in Cln2 from 181 to 225 residues that when fused to GFP is able to confer Msn5-dependent regulation of protein cellular content. Finally, we showed that a high level of Cln2 is toxic in the absence of Msn5. In summary, we described that Msn5 is required for the proper protein synthesis of specific proteins, introducing a new level of control of cell cycle regulators.

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Introduction

Nucleocytoplasmic trafficking is a relevant mechanism for the regulation of different cellular processes. The β-karyopherins are soluble receptors that mediate the transport of proteins into and out of the nucleus through the nuclear pore complex (NPC), which is composed in *S. cerevisiae* of approximately 30 different nucleoporins (Nups)

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Supplementary data can be accessed here.

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subunits [1–3]. β-karyopherins act as importins or exportins by recognizing specific features in their cargoes: nuclear localization signals (NLS) or nuclear export signals (NES), respectively [4,5]. The translocation of cargo-bound β-karyopherins through the NPC is facilitated by specific interaction with a class of nucleoporins rich in phenylalanine-glycine repeat motifs (FG-Nups) [6,7]. The binding and release of cargo proteins by karyopherins is controlled by the Ran GTPase cycle [8].

Msn5 is a member of the β-karyopherin family with many pleotropic functions [9,10]. It controls the nuclear export of the transcription factors Pho4 [11], Crz1 [12], Mig1 [13], Msn2/4 [14], Rtg1/3 [15], Aft1 [16], Maf1 [17], and Haa1 [18], which are involved in phosphate metabolism, calcium signaling, glucose repression, stress response, nitrogen regulation, iron response, RNApol III transcription and lactic acid tolerance, respectively. It also participates in different aspects of cell cycle control by mediating nuclear export of the transcription factors Swi5 [19], Swi6 [20] and Whi5 [21], the CKI inhibitor Far1 [22], the APC activator Cdh1 [23], and the Ste5 scaffold protein [24] and the HO endonuclease [25] involved in mating. In addition to its role in protein export, Msn5 may play a secondary role in the trafficking of tRNA between the nucleus and the cytoplasm [26,27]. In particular, Msn5 is involved in the re-export of mature tRNAs to the cytoplasm in the tRNA retrograde pathway [28]. As a consequence, tRNAs accumulate in the nucleus in *msn5* mutant cells. In spite of this, no general translational defects have been described for the *msn5* mutant; rather, translation of only twelve mRNAs related to methionine and arginine biosynthetic pathways are apparently affected by Msn5 inactivation [29].

Cell cycle progression is governed by the sequential activation of different cyclin-CDK complexes. For the yeast *S. cerevisiae*, nine different cyclins (Cln1-3 and Clb1-6) activate the Cdc28 protein, the only yeast CDK that performs an essential function in cell cycle progression [30]. Kinase activities existing at any time are determined by the levels of cyclins and CDK inhibitors (CKI). Two molecular mechanisms, gene transcription and protein degradation, are fundamental to control these key cell cycle regulators. The coordinated expression of different sets of genes, organized in transcriptional waves along the cell cycle, is a very common strategy in cell cycle control in all eukaryotes [31]. All the cyclins associated with Cdc28 CDK are periodically expressed. In the case of the cyclin studied in this work, *CLN2* gene is expressed during the G1/S transition by the transcription factor SBF, a heterodimer composed by the Swi4 and Swi6 proteins [32]. The second major mechanism involved in the control of the cellular levels of cell cycle regulators is proteolysis by means of ubiquitination and degradation in the proteasome [33,34]. Two ubiquitin ligases play a central role in cell cycle control, APC, which regulate mitosis, and SCF, involved mainly in the control of the G1/S transition. SCF regulates the G1/S transition through the degradation of G1 cyclins and CKI [35]. The SCF complex consists of four subunits: Skp1, Cdc53, Rbx1, and an adapter protein with an F-box, which is responsible for substrate recognition. SCF associated to the F-box protein Grr1 (SCF^{Grr1}) is the main ubiquitin ligase involved in Cln2 degradation [36,37].

In addition to transcription and protein degradation, other cellular processes contribute to the fine-tuning of cell cycle machinery. Spatial regulation is a common strategy to control protein function and this is also the case for cell cycle regulators. Indeed, as commented above distinct cell-cycle transcription factors shuttle between the nucleus and the cytoplasm and functional specificity between cyclins can be determined, at least in part, by the differences in their subcellular localization which targets their associated CDK activity to specific locations. This is well illustrated by the case of Cln cyclins [38–40]. In the case of Cln2, it is present in both the nucleus and the cytoplasm. It contains a NLS sequence in its N-terminal region that is responsible for nuclear import via the Kap95-Kap60 classical nuclear import pathway and a NES region between amino acids 225–299 that mediates its nuclear export by the Msn5 karyopherin [41]. This export mechanism helps to confer specific functionality to Cln2. On the other hand, examples of post-transcriptional regulation of cyclin mRNAs have been described. Thus, the translation of *CLN3* mRNA is repressed under nutrient-deprivation conditions, providing a mechanism coupling cell growth and division [42,43], and the RNA binding

protein Whi3 binds to *CLN3* mRNA to promote turnover and to inhibit translation [44,45]. As regards the other G1 cyclins, it has been reported that Cln1 and Cln2 are down-regulated by iron and that this occurs by translational repression of their mRNA [46]. Also, the degradation of *CLB2* mRNA by the MRP RNA endonuclease is important to promote mitotic exit [47].

The combination of different strategies is, therefore, a common trait in the control of cell cycle regulators in order to ensure the correct order and timing of the successive processes. We have previously described that Msn5 controls SBF, the transcription factor that regulates the periodic expression of Cln2, and the subcellular localization of Cln2 [20,41]. In this work we investigate in more depth the connection between Msn5 and Cln2 cyclin regulation describing a new mechanism by which Msn5 karyopherin regulates Cln2 protein synthesis.

Materials and methods

Strains, plasmids and growth conditions

Yeast strains used in this work are shown in Supplemental material Table S1. The *msn5* mutant strains were obtained by integrating a DNA fragment amplified from the pFA6a series plasmids (from J.R. Pringle) or from strains containing the *msn5Δ3::HIS3* or *msn5Δ2::HIS3* disruption cassette [20]. Strain *GAL1:HA-MSN5* was constructed by integrating at the appropriate position a DNA fragments amplified from the pFA6a series plasmids. The *swi4::LEU2, swi6::TRP1* and *grr1::LEU2* strains derived by using disruption-cassette plasmids Bd194 and Bd197 (from L. Breeden) and pBM1829 (from M. Johnston), respectively. The *cln2::TRP1* mutant strain was obtained by integrating a DNA fragment amplified from the pFA6a series plasmids. Strains expressing the Cln2 protein or truncated versions of it tagged at C-terminal with 3xHA, were constructed by integrating at the appropriate position DNA fragments amplified from the pFA6a series plasmids. Strains expressing the Cln2 protein tagged at C-terminal with 6xHA, was constructed by integrating at the appropriate position a DNA fragment amplified from pGA2256 plasmid (from Dr. G. Ammerer). Strains JCY1775 and JCY1845 were constructed by integrating at the *URA3* locus EcoRV-digested Yip-CLN2p:GFP or Yip-CLN2p:CLN2$^{181-225}$-GFP plasmids, respectively.

Centromeric plasmids pCLN1 and pCLN2, containing HA-tagged versions of Cln1 or Cln2, and plasmids ptetO:CLN2 overexpressing the *CLN2* gene under the control of the *tetO$_2$* promoter, were a gift from Dr. M. Aldea. Centromeric plasmids expressing the chimeric cyclin Cln2$^{1-476}$-Cln1$^{477-546}$, Cln2$^{1-370}$-Cln1$^{371-546}$, Cln2$^{1-299}$-Cln1$^{313-546}$, Cln2$^{1-225}$-Cln1$^{227-546}$ and Cln2$^{1-200}$-Cln1$^{201-546}$, were constructed as previously described [41]. To obtain pCLN2^{4T3S}, a XhoI-KpnI fragment from pCLN2 containing the *CLN2* promoter and the N-terminal part of the protein, was cloned in XhoI-KpnI digested pGAL1:CLN2^{4T3S}-HA plasmid (from C. Wittenberg). Now, Cln2^{4T3S} is expressed under the control of the *CLN2* promoter. Yip-CLN2p:GFP contains, in order, the *CLN2* promoter, the GFP coding region and the *ADH1* terminator in YIplac211. This plasmid was constructed in a three-step process. First, the *ADH1* terminator including the stop codon (+772, +1011), amplified from pFA6a-GFP(S65T) with a forward oligo containing a SalI site and a reverse oligo with a PstI site, was introduced by SalI-PstI digestion in YIplac211. Next, the *GFP(S65T)* coding region without start and stop codons, amplified from pFA6a-GFP(S65T) using a forward oligo containing a BamHI restriction site and a reverse oligo containing a XbaI site, was cloned in frame by BamHI-XbaI digestion. Finally, the *CLN2* promoter (−604 to +3) amplified from pCLN2 plasmid using a forward oligo containing an EcoRI restriction site and a reverse oligo containing a KpnI site was cloned in frame by EcoRI-KpnI digestion. For plasmid Yip-CLN2p:CLN2$^{181-225}$-GFP, a fragment covering Cln2 region from 181 to 225 amino acids was amplified from pCLN2 using a forward oligo containing a KpnI restriction site and a reverse oligo containing a BamHI site and cloned in frame by KpnI-BamHI digestion in YIp-CLN 2p:GFP.

Yeast cells were grown on standard yeast extract-peptone-dextrose (YPD) media or synthetic dextrose (SD) minimal media supplemented as required. To repress the *tetO$_7$* promoter, doxycycline was added to a concentration of 10 µg/ml. To evaluate protein stability, 100 µg/mL cycloheximide was added to exponentially growing cells.

Western blot analysis

Approximately 10^8 cells were collected, resuspended in 100 µL water and, after adding 100 µL 0.2 M NaOH, they were incubated for 5 min at room temperature. Cells were collected by centrifugation, resuspended in 50 µL sample buffer, and incubated for 5 min at 95°C. Extracts were clarified by centrifugation, and equivalent amounts of protein were resolved in an SDS-PAGE gel and transferred onto a nitrocellulose membrane. The primary antibodies used were monoclonal anti-HA 12CA5 antibody (Roche Diagnostics), monoclonal anti-HA peroxidase 3F10 antibody (Roche Diagnostics), monoclonal anti-GFP (Roche Diagnostics), monoclonal anti Cdc2 p34 (PSTAIRE) (Santa Cruz Biotechnology Inc.), and anti α-tubulin antibody (Serotec Ltd.). Blots were developed with anti-mouse IgG and anti-rabbit IgG Horseradish Peroxidase conjugate (Thermo Fisher Scientific,) or anti-rat IgG Horseradish Peroxidase conjugate (GE Healthcare) using the Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Bands were quantified with an ImageQuant™ LAS 4000mini biomolecular imager (GE Healthcare).

Gene expression analysis

RNA was purified from exponentially growing cells using RNeasy®Mini Kit (Qiagen) following the manufacturer instructions. Previously, yeast cells were broken in a FastPrep Precellys24 (Bertin technologies) with glass beads in the recommended kit buffer. The sample was incubated with Turbo DNase (Ambion) and after DNase inactivation and incubation with oligo dT, cDNA was obtained with Improm-II® Reverse Transcriptase and Recombinant RNasin® (Promega) following the manufacturer instructions. The cDNA was analized by quantitative RT-PCR in a DNA Engine Peltier Thermal Cycler (Bio Rad) using the SYBR Premix Ex Taq Tli RNase H Plus Green with ROX (Takara).

Northern analysis were carried out as previously described [19]. *CLN2* and *ACT1* mRNA were detected using ^{32}P-labeled probes obtained with HighPrime (Roche) according to the manufacturer instructions.

Polyribosome profile analysis

Cells were grown to the exponential phase in YPD. Preparation of cells and polysome gradients were performed as previously described [48], with some modifications. Approximately 10^9 cells were chilled for 5 min on ice in the presence of 0.1 mg/mL cycloheximide. Cells were harvested by centrifugation at 6000 × g for 4 min at 4°C and washed twice with 1 mL of lysis buffer (20 mM Tris-HCl, pH 8, 140 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 1% Triton X-100, 0.1 mg/mL CHX, and 0.5 mg/mL heparin). Cells were resuspended in 700 µl of lysis buffer, a 0.5-mL volume of glass beads was added, and cells were disrupted by vortexing 8 times for 30 s with 30 s of incubation on ice in between. Lysates were cleared by centrifugation at 5000 rpm for 5 min at 4ºC, after which the supernatant was recovered and centrifuged at 8000 rpm for 5 min at 4ºC. Finally, glycerol was added to the supernatant at a final concentration of 5% and extracts were frozen in liquid nitrogen and stored at −70° C. Samples of 8.5 A_{260} units were loaded onto 5–50% sucrose gradients and were separated by ultracentrifugation for 2 h 40 min at 35,000 rpm in a Beckman SW41 rotor at 4°C. Gradients were then fractionated by isotonic pumping of 60% sucrose from the bottom and either eleven 1 mL-samples or twenty-two 0.5 mL-samples were recovered. This was followed by a recording of the polysomal profiles by online UV detection at 240 nm (Density Gradient Fractionation System; Teledyne Isco, Lincoln, NE). For the RNA analyzes of the polysomal fractions, 6 µL of mixed *lys* and *spo* mRNAs at 3 ng/µl from *Bacillus subtilis* were added to each fraction before extraction in order to normalize all the values with spiked-in mRNA levels of *B. subtilis lys* and *spo.*

The fractions were mixed with a 2-fold volume of 8 M guanidine-HCl; the RNAs were then precipitated in 3 volumes of 100% ethanol and stored at 20°C overnight. Nucleic acids were collected by centrifugation (13,000 rpm for 20 min at 4°C), followed by a wash step with 70% ethanol. The pellets were then dissolved in 500 µl of water, followed by phenol-chloroform-isoamilic alcohol extraction. After centrifugation (10,000 rpm for

5 min at 4°C), the supernatant was precipitated with LiCl (final concentration, 2.5 M) at −80°C. RNA samples were collected at 12,000 rpm for 15 min at 4°C and washed with 70% ethanol. The RNA pellets were resuspended in nuclease-free H_2O. Equal volumes of RNA from each fraction were retrotranscribed using SuperScript™ IV VILO™ Master Mix with ezDNase (invitrogen, Thermo Fisher Scientific) following the manufacturer instructions. The cDNA was analized by quantitative RT-PCR as described above.

Results

Msn5 regulates Cln2 protein level through a post-transcriptional mechanism

Our group had previously described that Msn5 mediates nuclear export of Cln2 [41] and that Msn5 regulates the SBF transcription factor responsible for periodic expression of the *CLN2* gene [20]. During the course of these works, we envisaged the possibility that Msn5 could affect Cln2 by an additional mechanism. As shown in Figure 1(a), the amount of Cln2 in *msn5* mutant cells is severely reduced compared to the wild-type cells and Cln2 levels are recovered after introduction of a plasmid containing the *MSN5* gene. A similar effect was observed in four additional *msn5* mutant strains (Figure 1(b)), confirming that Msn5 is required for proper levels of Cln2 protein. *CLN2* is a periodically expressed gene; therefore, the change in Cln2 protein level in *msn5* mutant cells could be due to an alteration in the distribution of cells along the different phases of the cell cycle. However, this was not the case, because there are not differences in the cell-cycle distribution of cells between the wild type and *msn5* mutant strains (Supplemental material Figure S1). Alternatively, the reduced cellular level of Cln2 protein could be attributed to a defect in *CLN2* gene expression since Msn5 controls the SBF transcription factor [20]. Strikingly, the inactivation of the SBF components Swi4 or Swi6 in asynchronous cultures has very mild or no effect in Cln2 protein level compared to what is observed in *msn5* mutant cells (Figure 1(b)). This suggests that other mechanisms besides transcriptional regulation must affect the control of cellular content of Cln2 by Msn5. Supporting this, Cln2 protein level also diminished when *CLN2* was ectopically expressed under the control of the *tetO*$_2$ promoter (Figure 1(b)).

In order to confirm that the severe reduction in Cln2 protein level in the absence of Msn5 is caused by a post-transcriptional mechanism, we carried out, in parallel, an analysis of *CLN2* mRNA and Cln2 protein in the wild type and *msn5* mutant strains. Remarkably, *CLN2* mRNA level in the *msn5* mutant strain in asynchronous cultures was only slightly affected in comparison with the severe effect observed in Cln2 protein (Figure 1(c,d)). These results rule out the possibility of a reduced expression or stability of the *CLN2* mRNA as the origin for the reduced Cln2 protein level in *msn5* mutant cells, and clearly demonstrate that Mns5 is involved in a post-transcriptional mechanism important for a proper protein synthesis from the *CLN2* mRNA.

Stabilization of Cln2 does not abolish the reduction of protein level in msn5 mutant cells

Msn5 acts as an exportin of Cln2 and it has been reported in several cases that there are changes in protein stability depending on subcellular localization [49,50]. Taking this into account, it is possible that the reduced amount of Cln2 in *msn5* mutant cells was caused by a higher rate of protein degradation due to Cln2 accumulation in the nucleus. Nevertheless, other results raise doubts on this hypothesis. The analysis of Cln2 protein decay in a transcriptional shut-off assay indicated that the stability of Cln2 is not significantly affected in the *msn5* mutant strain when compared to that observed in the wild-type strain (Figure 2(a)). Degradation of Cln2 is mainly mediated by the SCF^{Grr1} ubiquitin ligase [36,37]. Importantly, the inactivation of Msn5 in mutant strains in Cdc53 or Grr1, components of the SCF ubiquitin ligase, still caused a reduction in Cln2 protein levels (Figure 2(b,c)). Finally, a variant protein mutated in phosphorylation sites, Cln2^{4T3S}, which is stable and accumulates in the nucleus [38,51], also displayed a reduced level upon Msn5 inactivation (Figure 2(d)).

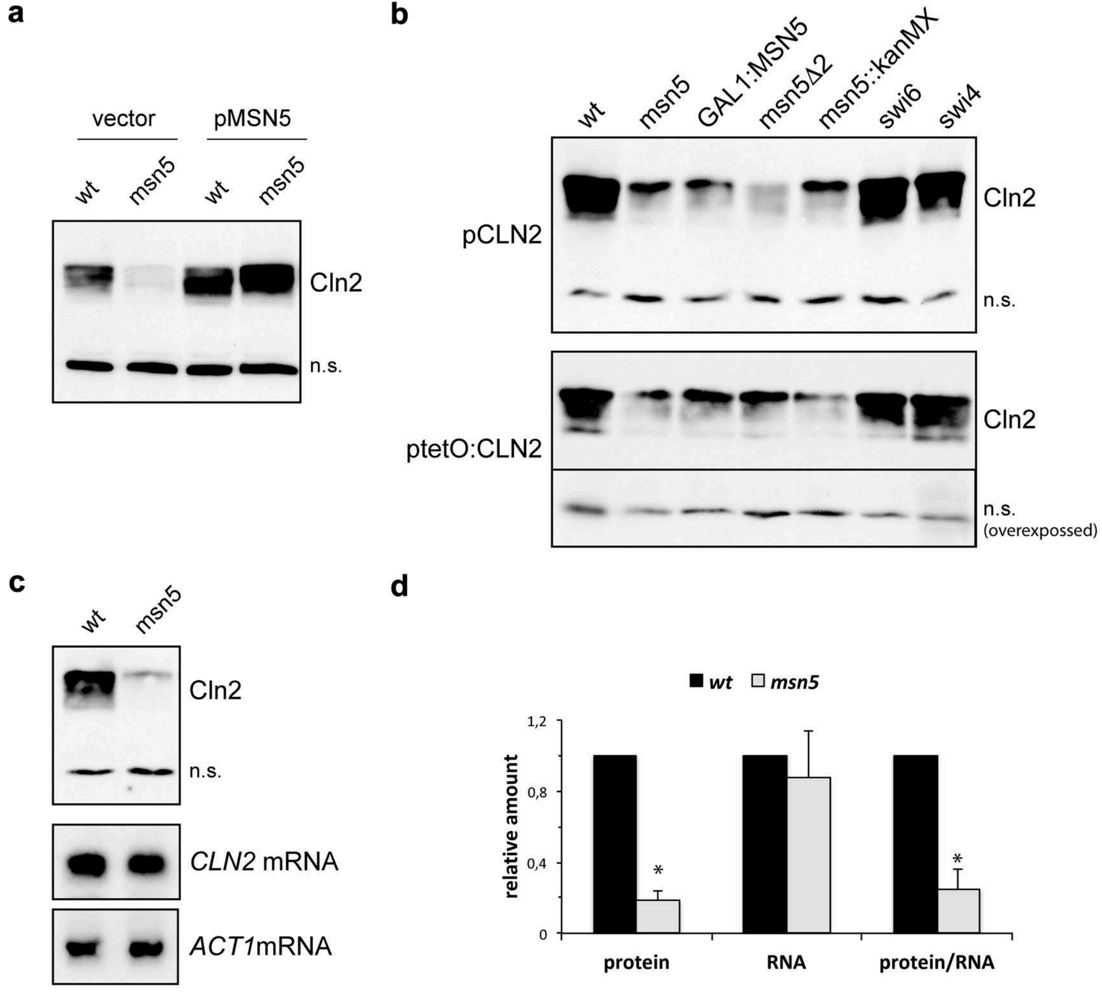

Figure 1. Analysis of Cln2 protein level in the *msn5* mutant cells. (a) The level of Cln2 protein in crude extracts of cells from exponentially growing cultures of the wild type (JCY1357) and the *msn5* mutant (JCY1359) strains expressing a HA-tagged version of Cln2 and transformed with a control vector or a plasmid containing the *MSN5* gene, was determined by western analysis with a specific anti-HA antibody. A non-specific band (n.s.) that cross-reacts with the antibody is shown as loading control. (B) The level of Cln2 protein in crude extracts of cells from exponentially growing cultures of the wild type (W303-1a), *msn5* (JCY1018), *GAL1:MSN5* (JCY313) -note that the *MSN5* gene is not expressed due to glucose repression-, *msn5Δ2* (JCY179) *msn5::kanMX* (JCY705), *swi6* (JCY220) and *swi4* (JCY167) transformed with a centromeric plasmid expressing a HA-epitope tagged Cln2 protein under the control of the *CLN2* (pCLN2) or the $tetO_2$ promoter (ptetO:CLN2), was determined by western analysis. (c) The *cln2* (JCY846) and the *cln2 msn5* (JCY2079) mutant strains transformed with the pCLN2 plasmid, were grown to exponential phase and the level of the Cln2 protein and the *CLN2* mRNA in crude extracts was determined in the same samples by western and northern analysis respectively. A non-specific band (n.s.) and actin (*ACT1*) mRNA are shown as loading controls. (d) Quantitation of the amount of Cln2 protein (relative to non-specific band) determined by western analysis and *CLN2* mRNA (relative to *ACT1* mRNA) determined by qPCR, as well as the protein/mRNA ratio, in the *msn5* mutant strain (JCY1831) relative to the wild type strain (JCY1830). Values are mean and standard deviation derived from 28 (protein), 13 (RNA) or 10 (protein/RNA) replicates. Student's t test was performed from *msn5* to the wild type. *: $P < 0.0001$.

These results strongly support that Msn5 inactivation affects the Cln2 protein level by a mechanism distinct to changes in protein stability. The steady-state level of a protein is the result of the balance between the synthesis and degradation rates. Therefore, our results strongly support that Cln2 protein synthesis takes place at a reduced rate in *msn5* mutant cells.

Msn5 is required for efficient translation of CLN2 and SWI5 mRNAs

The results described above recalls the situation for another cell-cycle regulator protein, the transcription factor Swi5, whose protein synthesis rate is impaired in the absence of Msn5 [19]. It is necessary to clarify that this is a specific response

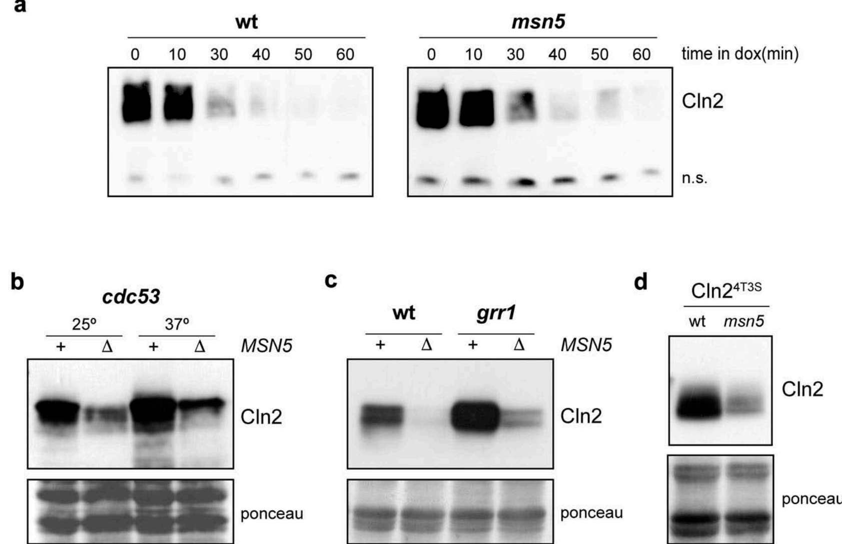

Figure 2. Analysis of Cln2 protein levels in SCF ubiquitin-ligase mutant strains. (a) Doxycycline to a final concentration of 10 µg/mL was added to exponentially growing cultures of the wild type (W303-1a) and the *msn5* mutant (JCY1018) strains transformed with a centromeric plasmid expressing a HA-epitope tagged Cln2 protein under the control of the *$tetO_2$* promoter (ptetO:CLN2). The Cln2 protein level in crude cell extracts was determined at the indicated time by western analysis with a specific anti-HA antibody. A non-specific band (n.s.) that cross-reacts with the antibody is shown as loading control. The shown result is representative of three independent assays. (b) Cells of the thermosensitive *cdc53* and *cdc53 msn5* (JCY684) mutants transformed with a centromeric plasmid expressing a HA-epitope tagged Cln2 protein under the control of the *$tetO_2$* promoter were grown at 25º and transferred for 3 hours at 37°. Cln2 protein level was analyzed by western blot. Ponceau staining of the membrane is shown as loading control. The shown result is representative of five replicates. (c) Cln2 protein level in the wild type (JCY1357), *msn5* (JCY1359), *grr1* (JCY2082) and *grr1 msn5* (JCY2083) strains expressing a HA-tagged version of Cln2. The shown result is representative of three replicates. (d) Cln2 protein level in the wild type (W303-1a) and the *msn5* mutant (JCY1018) strains transformed with a plasmid expressing the stabilized HA-tagged $Cln2^{4T3S}$ variant. The shown result is representative of five replicates.

of Cln2 and Swi5, since Msn5 inactivation did not affect the protein content of other cell cycle transcription factors such as Swi6, Whi5 or Ace2 [19].

In order to investigate how Msn5 could control Cln2 and Swi5 synthesis, we analyzed the association of their mRNA to ribosomes in polysome profiles in sucrose gradient. The polysome profile of the *msn5* mutant was very similar to the wild type profile (Figure 3(a)), in agreement with previously reported results in other experimental conditions [29]. Thus, global translation capacity does not seem to be altered in *msn5* mutant cells, which is consistent with the fact that reduction of protein level is only observed in some proteins like Cln2 and Swi5 and not in other proteins. Interestingly, the cumulative amount of both *CLN2* and *SWI5* mRNAs relative to the control *ACT1* mRNA in the monosomal and polysomal fractions is similar to that observed in total crude extracts (Figure 3(b); crude extracts in Figure 1(d) for *CLN2* and [19] for *SWI5*). This is an important observation that rules out any defect in *CLN2* and *SWI5* mRNAs export or mRNA access to the translational machinery as the cause of the reduced levels of Cln2 and Swi5 proteins; rather, it points to a defect in the translation process itself.

Although global translation was not altered in *msn5* cells, it is possible that translation of a subset of mRNAs was affected. To compare the translational states of particular target mRNAs, the distribution of mRNA between the different fractions was examined. In the wild type cells, *CLN2* and *SWI5* mRNAs manifested a distribution very similar to that of *ACT1* and *HXK2* mRNA, used as positive controls of optimal translation activity. However, in the case of *msn5* mutant cells there is a specific shift of *CLN2* and *SWI5* mRNAs from the heaviest polysome region to the non-polysomal (in particular the 80S monosomes) and light polysomal fractions (Figure 3(c) and Supplemental material Figure S2). This strongly suggests that translational efficiency of *CLN2* and *SWI5* mRNAs is specifically diminished in the absence of Msn5. This could certainly help to

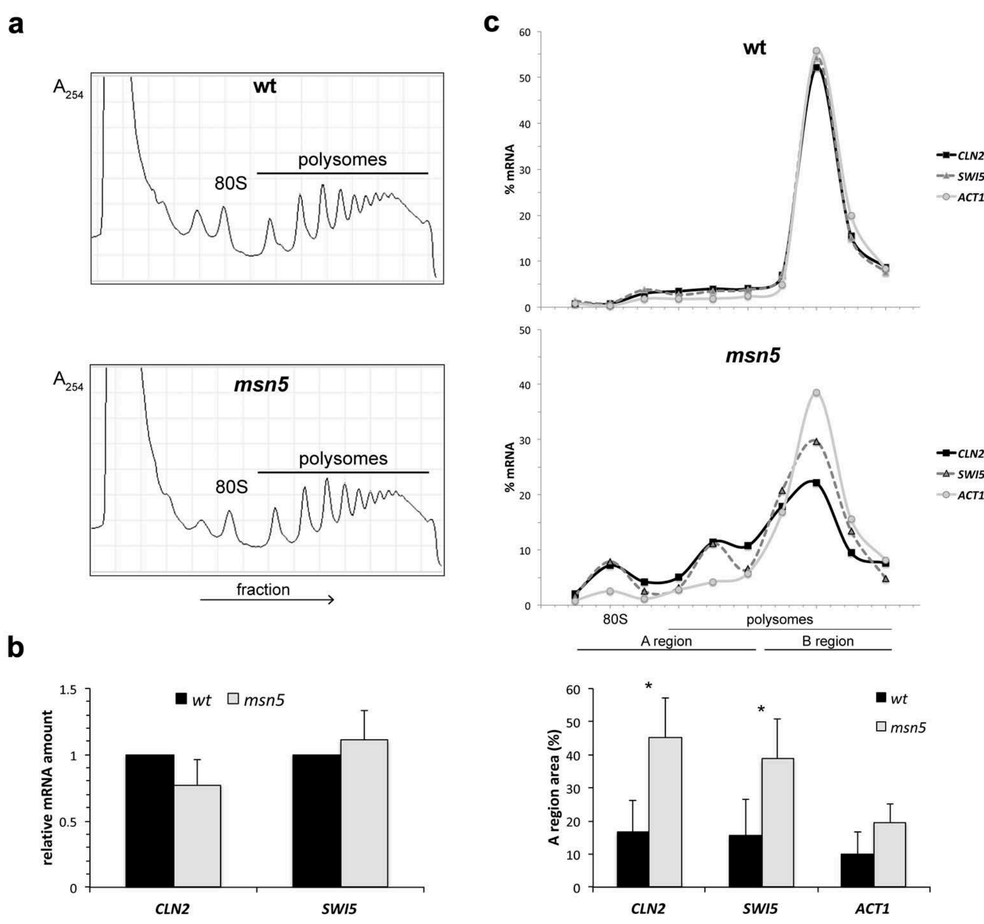

Figure 3. Analysis of mRNA translation in the *msn5* mutant strain. (a) Polysome profiles in sucrose gradient of RNA extracted from exponentially growing wild type (JCY1830) and *msn5* mutant (JCY1831) cells. The profiles shown are representative of three independent experiments. (b) The amount of *CLN2, SWI5* and *ACT1* mRNA in gradient fractions was determined by qPCR. Graph represents the cumulative amount of *CLN2* and *SWI5* relative to *ACT1* mRNA. Values are mean and standard deviation derived from three samples. (c) Distribution of *CLN2, SWI5* and *ACT1* mRNA along the gradient fractions. To quantify polysome association, the gradient profile was divided in two regions: one referred to as A includes the non-polysomal (including 40S and 60S ribosomal subunits and 80S monosomes) and light polysomal fractions prior to the *ACT1* peak, and a second one referred to as B that includes heavier polysomal fractions containing the *ACT1* peak. Graph represents the relative A area for each gene in the wild type and *msn5* mutant strains. Values are mean and standard deviation derived from 3 replicates. Student's t test was performed from *msn5* to the wild type. *: $P < 0.05$.

explain the reduced amount of Cln2 and Swi5 proteins observed in *msn5* mutant cells.

An internal region of Cln2 mediates the Msn5-dependent control of protein level

After establishing that Msn5 controls Cln2 synthesis, we decided to analyze whether a specific region of *CLN2* mRNA/Cln2 protein might be responsive to Msn5. It is important to point out that the decrease in Cln2 levels in the *msn5* mutant strain was observed in constructs lacking the 3′-UTR (C-terminal epitope-tagged proteins) or 5′-UTR (*tetO* constructs), so the effect of Msn5 on Cln2 synthesis must be mediated by sequences in the coding region. In a first approach, the levels of various C-terminal truncated forms of Cln2 were analyzed in both wild type and *msn5* mutant strains (Figure 4(a)). Truncated Cln2 forms encompassing fragments 1–370, 1–299 and 1–225 showed diminished protein level in *msn5* compared to wild type cells. Thus, the C-terminal part of Cln2 was not required for its regulation by Msn5 and, reciprocally, region 1–225 was sufficient to mediate control by Msn5. Interestingly, this control was lost in the $Cln2^{1-181}$ truncated protein, since the protein concentration was the same in both *msn5* and wild-type cells. This result

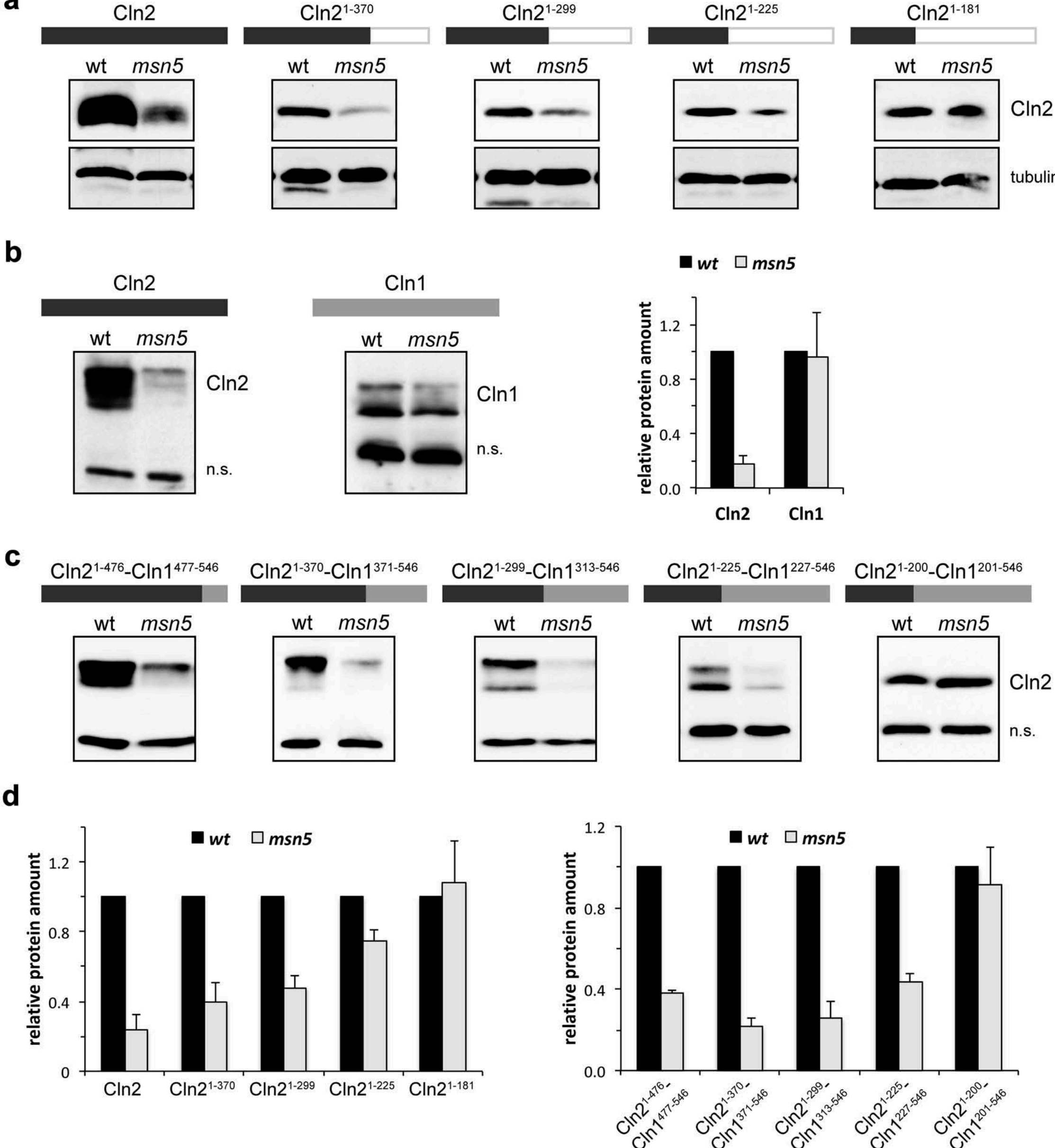

Figure 4. Characterization of a region in Cln2 mediating the regulation of protein level by Msn5. (a) The level of different C-terminal truncated Cln2 proteins in crude extracts of cells from exponentially growing cultures was determined by western analysis. Strains used were *CLN2-HA* (JCY1357), *CLN2*$^{1-370}$*-HA* (JCY1361), *CLN2*$^{1-299}$*-HA* (JCY1415), *CLN2*$^{1-225}$*-HA* (JCY1363), *CLN2*$^{1-181}$*-HA* (JCY1365) and their corresponding *msn5* mutant strains (JCY1359, JCY1367, JCY1417, JCY1369 and JCY1371 respectively). Lower panels for each construct show tubulin protein level as loading control. (b) The level of Cln1 and Cln2 protein in the wild type (W303-1a) and the *msn5* mutant (JCY1018) strains transformed with a centromeric plasmid expressing an HA-tagged *CLN2* or *CLN1* gene was analyzed by western blot. A non-specific band (n.s.) that cross-react with the antibody is shown as loading control. The graph represents the mean and standard deviation of the amount of Cln protein (relative to non-specific band) derived from at least three experiments. (c) The level of Cln2 protein in the wild type (W303-1a) and the *msn5* mutant (JCY1018) strains transformed with a centromeric plasmid expressing distinct HA-tagged chimeric cyclin proteins was determined by western analysis. (d) Quantitation (mean and standard deviation) of the amount of truncated and chimeric cyclin proteins described in A and C derived from three to eight replicates.

supports that the region from 181 to 225 is necessary for this Msn5-dependent regulatory mechanism.

With the aim of addressing this point in the context of a functional cyclin protein instead of in truncated variants, we carried out a second

approach consisting in the construction of chimeric cyclins by interchanging equivalent domains between the Cln2 and Cln1 cyclins. It has to be note that in contrast to what happen in Cln2, Cln1 protein level is not severely affected by Msn5 inactivation (Figure 4(b)). In agreement with the previous results, different chimeric cyclins containing the N-terminal region of Cln2 as far as amino acid 225 manifested a severe reduction in their protein level after inactivation of Msn5 (Figure 4(c)). Importantly, this Msn5-dependent effect was absent in the chimera $Cln2^{1-200}$-$Cln1^{201-546}$. This indicates that region from amino acid 200 to 225 of Cln2 is important for the Msn5-dependent regulation of protein level.

Once we have identified an internal region in Cln2 protein necessary for its regulation by Msn5, we wonder whether this region would be sufficient to confer Msn5-regulated synthesis to a protein. To test this possibility, the $Cln2^{181-225}$ fragment was fused to GFP. The cellular content of control GFP was similar in wild type and *msn5* mutant cells. However, the fusion to GFP of the $Cln2^{181-225}$ fragment caused a reduction in its protein level in *msn5* compared to wild type cells (Figure 5(a)). This is not due to alterations in transcription or mRNA stability since levels of mRNAs are not affected in *msn5* cells (Figure 5(b)). Moreover, it is not related to changes in protein stability since $Cln2^{181-225}$-GFP shows the same degradation rate as GFP (Figure 5(c)). Thus, the synthesis of the $Cln2^{181-225}$-GFP protein is defective in the absence of Msn5.

In short, all our results indicate that a small fragment of Cln2 from residue 181 to 225, or its corresponding mRNA region, imposes a requirement of Msn5 karyopherin for the efficient synthesis of Cln2 protein.

msn5 *mutant cells are sensitive to overexpression of Cln2*

During the described work, we envisaged the possibility that *msn5* cells were sensitive to high levels of Cln2. To explore this possibility in detail, we analyzed growth of wild type and *msn5* mutant cells containing the ptetO:CLN2 plasmid. As it can be seen in Figure 6, while overexpression of *CLN2* from the $tetO_2$ promoter has no effect in wild type cells, the three *msn5* mutant strains tested manifested an impaired cell growth. This result indicates that the *msn5* mutant was sensitive

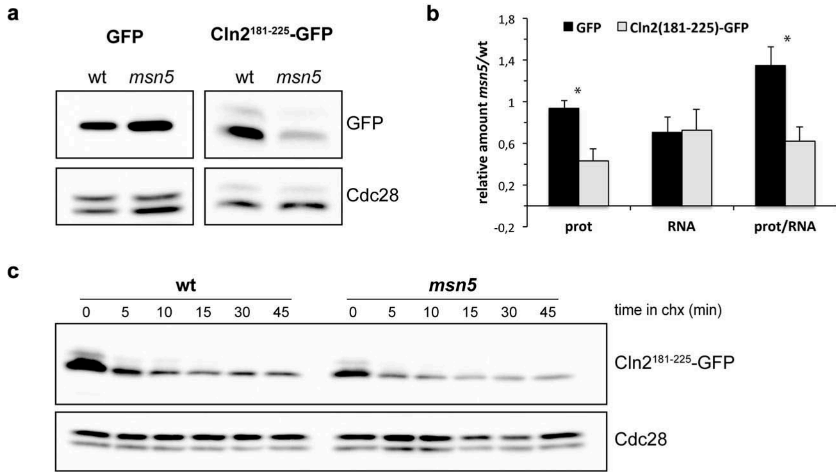

Figure 5. Analysis of $Cln2^{181-225}$-GFP protein level and stability in the *msn5* mutant strain. (a) The level of $Cln2^{181-225}$-GFP and the GFP control protein in the wild type (JCY1775 and JCY1845 respectively) and the *msn5* mutant (JCY1778 and JCY1847 respectively) strains, was determined by western analysis with a specific anti-GFP antibody. Cdc28 protein level is shown as loading control. (b) Graph represents the amount of Cln2 protein (relative to Cdc28) determined by western analysis and *CLN2* mRNA (relative to *CDC28* mRNA) determined by qPCR, as well as the protein/mRNA ratio, in the *msn5* mutant strain relative to the wild type strain. Values are mean and standard deviation derived from 11 replicates. Student's t test was performed from $Cln2^{181-225}$-GFP to the GFP control. *: $P < 0.0005$. (c) Cycloheximide to a final concentration of 100 µg/mL was added to exponentially growing cultures of the wild type (JCY1845) and the *msn5* mutant (JCY1847) strains expressing the $Cln2^{181-225}$-GFP protein. Protein level in crude cell extracts was determined at the indicated time by western analysis with a specific anti-GFP antibody. Cdc28 protein level is shown as loading control.

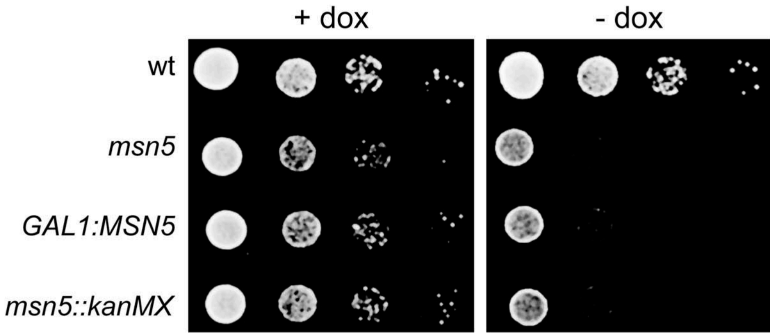

Figure 6. Effect of Cln2 missregulation on cell growth. (a) 10-fold serial dilutions from exponentially growing cultures of the wild type (W303-1a), *msn5* (JCY1018), *GAL1:MSN5* (JCY313) and *msn5::kanMX* (JCY705) strains transformed with a centromeric plasmid expressing *CLN2* under the control of the *tetO*$_2$ promoter (ptetO:CLN2), were spotted onto YPD medium with or without 10 µg/mL doxycycline and incubated at 25° for 3 days.

to Cln2 in a dose-dependent manner. Considering the nuclear accumulation of Cln2 protein in *msn5* mutant cells [41], this observation strongly suggests that abnormal accumulation of high levels of Cln2 inside the nucleus could prove toxic for cells.

Discussion

Previous work in our group connected Msn5 with Cln2 [20,41]. Msn5 controls subcelular localization of Swi6, a component of the cell cycle transcription factor SBF responsible for the periodic expression of many genes in G1/S, among them the *CLN2* gene. On the other hand, Msn5 mediates the nuclear export of Cln2 protein through a NES sequence located between amino acids 225 and 299. In this report, we described that Msn5 also affects Cln2 by an additional mechanism since Msn5 participates in regulating Cln2 protein levels. The *msn5* mutant cells had a severe reduction in Cln2 levels compared to wild-type cells. Initially, the drop in Cln2 protein could be thought to be a consequence of Msn5 controlling *CLN2* gene expression through the regulation of SBF. However, different observations raise this idea into question. Analysis in asynchronous cultures revealed a very small reduction of *CLN2* mRNA compared to the drastic drop of Cln2 protein in *msn5* cells. The slight effect in *CLN2* mRNAs in asynchronous cultures is not a surprise since many other genes regulated by SBF are affected in their cell-cycle regulation but not in their global level of expression [52]. Consistent with this, mutants in the two components of SBF, Swi4 and Swi6, have no significant effect in Cln2 protein level in contrast to what is observed in the *msn5* mutant. Furthermore, protein level of some chimeric cyclins (Cln2$^{1-200}$-Cln1$^{201-546}$) and truncated version of Cln2 (Cln2$^{1-181}$) are not affected by Msn5 inactivation despite being expressed from the *CLN2* promoter. Finally, when *CLN2* is ectopically expressed, although the effect is softened, Msn5 absence still caused a reduction in Cln2 protein level. All these observations strongly indicate that the drastic reduction in Cln2 protein level is not caused by alterations in *CLN2* gene transcription (although it cannot be ruled out that the slight effect in transcription could contribute to some extent); rather, Msn5 control of Cln2 protein level occurs mainly at posttranscriptional level.

Protein stability could be affected by subcellular localization [49,50]. Msn5 mediates nuclear export of Cln2 [41]. Therefore, it is conceivable, in principle, that nuclear accumulation of Cln2 in *msn5* mutant cells could lead to greater protein instability and, consequently, to lower Cln2 cellular levels. However, several results contradict this possibility. The Cln1 and Cln2 cyclins contain a NLS activity in the N-terminal 1–224 region, and Cln2, but not Cln1, contains a Msn5-dependent NES between 225–299 amino acids. However, both cyclins are distributed through the whole cell and the presence of the Cln2-NES only alters the nuclear/cytoplasmic ratio modestly [41]. Because of this,

we would not expect drastic differences in the subcellular localization of chimeric cyclins (all containing Cln2-NLS) based on the presence of the Cln2-NES sequence. Moreover, we observed that a variant of Cln2 (Cln2^{4T3S}) that is stable and accumulates in the nucleus [38,51] also had a reduced protein level in *msn5* mutant cells. Importantly, the protein level of the truncated version Cln2$^{1-225}$, the chimeric cyclin Cln2$^{1-225}$-Cln1$^{227-546}$ or the fusion protein Cln2$^{181-225}$-GFP are affected by the inactivation of Msn5 despite lacking the NES region. All these observations indicate that the control of Cln2 protein level by Msn5 is a new mechanism not related with the regulation of Cln2 subcellular localization. On the other hand, the drop in Cln2 protein levels caused by Msn5 inactivation also occurred in mutant strains in the SCF^{Grr1}, which is the main activity involved in Cln2 degradation. Finally, the shut-off experiments showed that the stability of Cln2 and Cln2$^{181-225}$-GFP was not significantly affected in the *msn5* strain. All of these observations led us to conclude that the reduced amount of Cln2 in *msn5* cells is not caused by an increased rate of degradation as a result of an altered localization. Instead, the results indicate that in the absence of Msn5 there are problems in Cln2 protein synthesis.

The analysis of polysome profiles indicated that inactivation of Msn5 apparently does not have a significant impact on global mRNA translation efficiency. This is consistent with the fact that Msn5 only affects protein level of specific proteins and it is in agreement with previously reported results in other growth conditions [29]. Importantly, a clear shift in the distribution of specific mRNA to lighter polysomal and non-polysomal fractions is observed. This is the case for *CLN2* and *SWI5* mRNAs. It has to be noted that the distribution of *ACT1* and *HXK2* mRNA along the polysomal profile is also affected, although at a minor level, in *msn5* cells compared to wild type cells. This could indicate that Msn5 affects a general aspect of mRNA translation but that specific properties of particular mRNAs (like *CLN2* and *SWI5*) impose a more restrictive need for Msn5 for their efficient translation. Importantly, the altered polysomal association confirms a role for Msn5 in proper translation of *CLN2* and *SWI5* mRNA, and could explain the reduced protein synthesis rate of Cln2 and Swi5 in the *msn5* mutant cells.

We have tried to elucidate the molecular basis of Cln2 protein synthesis control by Msn5. Typically, mRNA is regulated by sequences present in the 5′- or 3′-unstranslated regions. However, the drop in Cln2 levels in the *msn5* mutant strain was observed in constructs lacking the 3′-UTR (C-terminal epitope-tagged proteins) or 5′-UTR (*tetO* constructs) and, some constructs containing the 5′-UTR are not affected by *msn5* mutation. Thus, the effect of Msn5 on the Cln2 synthesis rate must be mediated by sequences in the coding region. In support of this, we show that the region coding for amino acids 181–225 was able to confer Msn5-regulated synthesis when fused to GFP. This is a very similar case to that of Swi5 [19] and suggests that Msn5 could affect synthesis of specific proteins by a common mechanism. Susceptibility to Msn5 regulation could rely on specific properties of mRNAs like sequence, codon usage or secondary structures that could affect mRNA translation. However, the analysis of mRNA sequences coding the Cln2$^{181-225}$ and Swi5$^{325-422}$ fragments that mediate the Msn5-dependent regulation were not conclusive.

Different scenarios can be envisaged on how Msn5 could control the synthesis of Cln2, Swi5 and other putative proteins. Msn5 has a well-defined role in exporting phosphorylated protein cargos. Therefore, it can be argued that the reduced levels of target proteins resulted from defects in Msn5-mediated nuclear export of specific factors required for proper *CLN2* and *SWI5* mRNA translation in the cytoplasm. These factors may act directly, increasing the translation efficiency of these mRNAs, or indirectly, inactivating or removing a specific RNA-binding translational repressor. In any case, the effect on translation would occur in a stage after mRNA export and its association to the translation machinery since the amount of *CLN2* and *SWI5* mRNAs associated to ribosomes is very similar in wild type and *msn5* cells. On the other hand, the tRNAs perform an essential function to decode information in mRNAs into proteins, and Msn5 may affect tRNAs in different ways. Msn5 mediates the nuclear export of Maf1, a repressor of RNApol III transcription [17]. However, *msn5* mutation affected Cln2 protein levels even in the absence of Maf1 (Supplemental material Figure S3). In addition to a role in protein trafficking, Msn5 is

also involved in tRNA export to the cytoplasm, the tRNAs accumulating inside the nucleus in its absence [26,27,53]. It is possible that an altered tRNA nuclear-cytoplasmic trafficking could affect the cytosolic pools of tRNAs in such a way that compromises the translation efficiency of some specific messengers like *CLN2* and *SWI5* mRNAs. It has been suggested that there may be substrate specificity among the different tRNA exporters, so they could influence the cytoplasmic pools of particular tRNAs and hence possibly influence the proteome in different ways [28]. The $tRNA^{Met}$ and $tRNA^{Ser}$ are the most affected tRNAs by *msn5* mutation among the analyzed cases [53]. It is interesting to point out that the $Cln2^{181-225}$ fragment mediating the Msn5-dependent regulation of Cln2 protein level contains a significant higher proportion of Met residues (6.8%) than the average proportion found in proteins (1.7%) and that the $Swi5^{325-422}$ fragment mediating the Msn5-dependent regulation of Swi5 protein level contains a higher proportion of Ser residues (18.8%) than the average proportion found in proteins (7.1%). Moreover, Msn5-mediated tRNA export involves the formation of a quaternary nuclear export complex consisting of Msn5, aminoacylated tRNA, RanGTP and the Tef1/2 protein [54], the yeast ortholog of translation elongation factor eEF1A that functions in delivering aa-tRNAs to the ribosome. In fact, *msn5* mutant cells partially accumulate not only tRNA but also Tef1/2 in the nucleus [26]. Together with the reduced pools of tRNA, the reduced amount of cytosolic Tef1/2 could also contribute to defects in translation.

It is interesting to comment that we have observed a toxic effect of high Cln2 activity in the absence of Msn5, regardless of the reduced amount of the Cln2 protein. This could be because the toxicity of Cln2 was due to its nuclear accumulation. Consistent with this, the stable variant $Cln2^{4T3S}$ that accumulates inside the nucleus affects proper cell growth of a wild type strain [36]. We can speculate that unscheduled Cln2 nuclear function is toxic and that Cln2 export by Msn5 helps to restrict the period during which this activity takes place. For example, Cln2 export by Msn5 could contribute, together with transcriptional shut-off and degradation of the protein, to the inactivation of Cln2 after the G1/S transition. If excess nuclear Cln2 is indeed toxic for cells, the concomitant protein level reduction accompanying its nuclear accumulation when exportin Msn5 is absent may be an evolutionary advantage.

How extended could the case of *CLN2* and *SWI5* be? The protein level of cyclin Cln1 and the transcription factors Swi6, Whi5 and Ace2 [19] are not affected by Msn5 inactivation, indicating that the effect of Msn5 is restricted to specific proteins. In fact, in a genomic approach only 12 genes were identified as having an altered translation profile in a *msn5* mutant strain, all of them being translationally down-regulated compared to wild type cells [29]. It has to be pointed out that in this analysis the translation status was calculated as a ratio between the polysomal and non-polysomal fractions. In the light of our results with *CLN2* and *SWI5*, this is a binary extreme situation in which genes with a shift in mRNA distribution inside the polysomal fractions (i.e. a shift to lighter polysomal fractions) will scape to the analysis. Future work will help to identify more targets for Msn5-dependent regulation of protein synthesis and to clarify the molecular basis of the regulation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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