Biochimica et Biophysica Acta 1823 (2012) 959–970

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Regulation of cell cycle transcription factor Swi5 by karyopherin Msn5

Francisco José Taberner [1], Inma Quilis [1], Josep Sendra, María Carmen Bañó, Juan Carlos Igual *

Departament de Bioquímica i Biologia Molecular, Universitat de València, 46100 Burjassot, Valencia, Spain

ARTICLE INFO

Article history:
Received 30 September 2011
Received in revised form 20 January 2012
Accepted 13 February 2012
Available online 21 February 2012

Keywords:
Swi5
Msn5
Cell cycle
Saccharomyces cerevisiae
β-karyopherin

ABSTRACT

Inactivation of *S. cerevisiae* β-karyopherin Msn5 causes hypersensitivity to the overexpression of mitotic cyclin Clb2 and aggravates growth defects of many mutant strains in mitotic exit, suggesting a connection between Msn5 and mitotic exit. We determined that Msn5 controlled subcellular localization of the mitotic exit transcription factor Swi5, since it was required for Swi5 nuclear export. Msn5 physically interacted with the N-terminal end of Swi5. Inactivation of Msn5 caused a severe reduction in cellular levels of Swi5 protein. This effect occurred by a post-transcriptional mechanism, since *SWI5* mRNA levels were not affected. The reduced amount of Swi5 in *msn5* mutant cells was not due to an increased protein degradation rate, but to a defect in Swi5 synthesis. Despite the change in localization and protein level, Swi5-regulated transcription was not defective in the *msn5* mutant strain. However, a high level of Swi5 was toxic in the absence of Msn5. This deleterious effect was eliminated when Swi5 nuclear import was abrogated, suggesting that nuclear export by Msn5 is important for cell physiology, because it prevents toxic Swi5 nuclear accumulation.

1. Introduction

The periodic oscillation of several cyclin-dependent kinase (CDK) activities governs progression through the different cell cycle transitions. A common hallmark of mitotic exit in all eukaryotic cells is a dramatic reduction in overall CDK activity [1]. In *Saccharomyces cerevisiae*, Clb2-Cdc28 kinase is the most important mitotic CDK activity. Its inactivation at the end of mitosis is accomplished by two mechanisms: degradation of Clb2 and the other Clb cyclins by the ubiquitin-ligase APC, and kinase inhibition by the CDK inhibitor Sic1. This process is controlled by the Cdc14 phosphatase, which is activated at the end of mitosis by the mitotic exit network (MEN). Cdc14 acts on the APC activator Cdh1, the Sic1 protein, and the transcription factor Swi5 (responsible for expression of the *SIC1* gene) to trigger cyclin degradation and Sic1 accumulation [2-5].

Sic1 abundance is cell cycle-regulated and peaks at the end of mitosis. This periodicity results from stage-specific degradation of the protein and regulated transcription of the gene [6]. *SIC1* gene expression is controlled by the Swi5 transcription factor [7,8]. Swi5 was originally identified as one of the transcription factors involved in HO endonuclease expression [9]. However, it plays a more general role, since it is responsible, together with transcription factor Ace2, for a transcriptional wave at the end of the cell cycle, which includes many genes that encode important regulators of the M/G1 transition, such as the above-mentioned inhibitor Sic1 [10]. Swi5 and Ace2 are related proteins. They contain a similar zinc finger domain and show functional redundancy [11,12]. In fact, both factors jointly activate the transcription of many genes. However, the expression of other genes is specifically regulated by either Swi5 or Ace2 [11,13,14]. Specific functions may arise from differences in their spatial regulation [15-17] or interacting partners [12,18].

The participation of Swi5 in both sexual differentiation and cell cycle regulation has resulted in extensive studies of this transcription factor. Swi5 is a 709-amino acid protein with a modular architecture. The C-terminal third of the protein contains both a zinc finger domain [19] and a classical nuclear localization signal (NLS). The central segment of the protein meditates interactions with Pho2 and plays an important role in the full transcriptional activation of the *HO* gene [18,20]. Swi5 also contains a region rich in serines, which are phosphorylated by Pho85 CDK and mediate Swi5 activity and stability [16,21]. However, little is known about the function of the N-terminal third of Swi5.

Swi5 is regulated at several levels. The *SWI5* gene is periodically transcribed at the G2-M transition [22] by Fkh2–Mcm1–Ndd1 transcription factors [10]. The newly synthesized Swi5 is located in the cytoplasm until the end of mitosis, when Swi5 enters the nucleus to act as a transcriptional activator [23]. Some of the mechanisms controlling the subcellular localization of Swi5 have been determined. The Swi5 NLS is inactivated by phosphorylation of the serines 552, 646, and 664 by Clb-Cdc28 CDK, which prevents Swi5 nuclear accumulation in the G2/M phase [24]. At the end of mitosis, Cdc14 phosphatase dephosphorylates these serine residues, which triggers the nuclear import of Swi5 by the classical import pathway [25,26]. The Swi5 nuclear signal decreases during G1, which led to the idea that Swi5 would be degraded once it enters the nucleus [23]. Recently, Swi5

* Corresponding author at: Dept. Bioquímica i Biologia Molecular, Facultat de Ciències Biològiques, Universitat de València, C/ Dr. Moliner 50, E-46100 Burjassot, Spain. Tel.: +34 963543012; fax: +34 963544635.
E-mail address: jcigual@uv.es (J.C. Igual).
[1] These authors contributed equally to this work.

0167-4889/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2012.02.009

was described as being ubiquitinated by the SCF^{Cdc4} ubiquitin-ligase; this process is controlled by the Pho85-dependent phosphorylation of the serine-rich central region [16,27]. Swi5 degradation seems to be important for entry into S phase [27].

Transport of proteins into and out of the nucleus is an active process that occurs through the nuclear pore complex (NPC). It is facilitated by soluble transport receptors of the β-karyopherin family. β-karyopherins act as importins or exportins by recognizing specific features in their cargoes: NLS or nuclear export signals (NES), respectively [28-30]. The binding and release of cargo proteins by karyopherins is controlled by the Ran GTPase cycle. Importins bind cargo in the cytosol and release it in the nucleoplasm by interactions with Ran-GTP. In the export process, the binding of cargo by karyopherins in the nucleus is stabilized by Ran-GTP and, once in the cytoplasm, hydrolysis of GTP to GDP triggers dissociation of the cargo [31].

Msn5 is a member of the β-karyopherin family and has been associated with different cellular processes. It participates in different aspects of cell cycle control by mediating nuclear export of the Start transcription factors Swi6 and Whi5 [32,33], the CKI inhibitor Far1 [34], the APC activator Cdh1 [35] and the HO endonuclease [36]. Msn5 also controls nuclear export of the transcription factors Pho4 [37], Crz1 [38], Mig1 [39], Msn2/4 [40], Rtg1/3 [41], Aft1 [42], and Maf1 [43], which are involved in phosphate metabolism, calcium signalling, glucose repression, stress response, nitrogen regulation, iron response and RNApol III transcription, respectively. In addition to its role in protein export, Msn5 may play a secondary role in the export of tRNA [44]. Consistent with this, Msn5 and its mammalian orthologue Exportin-5 can bind tRNA and other RNAs [45].

In the present work, we described a new connection of Msn5 with cell cycle control. Msn5 is involved in the regulation of the subcellular localization and protein levels of the late mitosis transcription factor Swi5.

2. Materials and methods

2.1. Yeast strains and plasmids

The yeast strains used in this study are shown in Supplemental Material Table 1. The *msn5* mutant strains were obtained by integrating a DNA fragment amplified from a strain containing the *msn5Δ3::HIS3* disruption cassette, originally obtained by using plasmids p335-Δ3::HIS3 (from Dr. F. Estruch). The *swi5*, *sic1* and *cdh1* mutant strains were obtained by integrating a DNA fragment amplified from plasmid pFA6a-kanMX6 (from Dr. J.R. Pringle). Strains expressing the Msn5, the Swi5 protein or truncated versions of the protein from the *GAL1* promoter, N-terminal or C-terminal tagged with HA or myc epitopes, were constructed by integrating at the appropriate position a DNA fragment amplified from the pFA6a series plasmids (a gift from Dr. J.R. Pringle).

Plasmids used in the two-hybrid assay expressing different fragments of Swi5 fused to the Gal4 activation domain were obtained by cloning DNA fragments coding for the indicated regions of Swi5, which were amplified from genomic DNA using a forward oligo containing an EcoRI restriction site and a reverse oligo containing a BamHI restriction site, into EcoRI–BamHI digested pGAD-C1. pBD-MSN5 expressing Msn5 fused to the Gal4 DNA-binding domain was a gift from Dr. M. Johnston.

Plasmid $pSWI5^{AAA}$ expressing a mutated Swi5 protein in which Ser 522, 646 and 664 have been substituted by Ala was a gift from Dr. L.H. Johnston. Plasmid HO(31)-CYC1-lacZ (M1853) contains the Swi5 binding site from the HO promoter in front of the lacZ gene [18].

2.2. β-galactosidase assays

Approximately 10^8 cells were harvested, washed with water and resuspended in 200 μL of Z buffer (60 mM Na_2HPO_4, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4$, 0.1% β-mercaptoethanol, pH 7) containing 1 mg/mL of zymolyasa-20 T. After 20 min of incubation at 30 °C, extracts were centrifuged and the supernatants were conserved. Different quantities of extracts (20–180 μL) were taken and Z buffer was added up to 900 μL. Reaction was started by the addition of 180 μL of 4 mg/mL ONPG (o-nitrophenyl-β-D-galactopiranoside). Samples are incubated at 30 °C until they become yellow colored. The reaction was stopped with 450 μL of Na_2CO_3 1 M and the A_{420} was determined. Activity is expressed in U/mg of protein where 1U is defined as $A_{420} \times 10^3$/min of incubation. Protein concentration was determined by Bradford assay.

2.3. Northern blot analysis

Approximately 10^8 exponentially growing cells were collected, resuspended in 500 μL of LETS (0.1 M LiCl, 10 mM EDTA, 0.2% SDS, 10 mM Tris–HCl pH 7.4), mixed with 500 μL of phenol:chloroform: isoamylic alcohol (125:24:1) and broken by vigorous shaking with glass beads. After two extraction with one volume of phenol:chloroform:isoamylic alcohol and one volume of choloform:isoamyl alcohol (24:1), RNA was precipitated with 0.1 volume of 5 M LiCl and 2.5 volume of cold 100% ethanol at least for 3 hours at −20 °C. RNA was collected by centrifugation, washed with 70% ethanol, resuspended in 30 μl of water and quantified. 5 μg of total RNA was fractionated on agarose gel containing formaldehyde and transferred to Hybond-N membrane (Amersham). *SWI5*, *SIC1* and *ACT1* mRNA were detected using ^{32}P-labeled probes obtained with HighPrime (Roche) according to the manufacturer instructions and quantified in a FLA3000 PhosphorImager (Fujifilm).

2.4. Coimmunoprecipitation

Approximately 5×10^8 cells expressing Swi5-myc and either untagged or HA epitope-tagged versions of Msn5 were resuspended in 100μL of 50 mM Tris–HCl pH 8, 250 mM NaCl, 5 mM EDTA, 0,1% Triton X-100, 1 mM PMSF and 5 μg/mL Complete mixture (Roche Applied Science) and broken with vigorous shaking in the presence of glass beads. Cellular debris was removed and the supernatant was clarified by centrifugation at 13,000 × *g* for 5 min. Dynabeads Protein G magnetic beads (50 μL) were sequentially washed twice with phosphate-buffered saline containing Tween 0.02%, incubated with rat monoclonal anti-HA 3F10 antibody for 20 min at room temperature and after extensive washing incubated with the cell extract for 20 min at room temperature. After washing beads, the immunoprecipitated proteins were eluted by boiling the beads for 5 min in loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis.

2.5. Miscellaneous

Indirect immunofluorescence and western blot analysis were carried out as described previously [46]. Antibodies used in westerns assays were anti-HA 12C5A antibody (Roche), an anti-Swi5 antibody (form Dr. D. Stillman), anti-PSTAIRE antibody (Santa Cruz) or anti α-tubulin antibody (Serotec Ltd.). Bands were quantified in non-saturated films using Quantity One software (Biorad) or in an Image-Quant LAS4000 biomolecular imager (GE Healthcare). Antibodies used in immunofluorescence assays were anti-HA 3F10 antibody (Roche) or mouse anti-myc 9E10 (Roche).

3. Results

3.1. Genetic interactions connect Msn5 karyopherin with mitotic exit

Impaired growth in the presence of high levels of the cyclin Clb2 is a typical characteristic of strains containing mutations in genes

involved in mitotic exit [47]. To characterize phenotypic traits of the *msn5* mutant strain in relation to cell cycle progression, we investigated the effect of Clb2 overexpression. When high levels of Clb2 were induced by galactose in cells containing a *GAL1:CLB2* gene, growth was severely impaired in the absence of Msn5 (Fig. 1A). This defect was not due to a change in the carbon source, because it was not observed in cells containing a control plasmid; rather, it indicates that *msn5* cell growth was hypersensitive to high levels of Clb2. In support of this, defective growth was rescued by reintroduction of a functional copy of the *MSN5* gene. The growth defect observed in the *msn5* mutant was very similar to that observed in strains with mutations in genes involved in mitotic exit, such as the *sic1* and *swi5* mutants; however, the growth defect was less severe than that seen in a mutant in APC activator Cdh1.

The previous result suggested that the Msn5 karyopherin might be associated with mitotic exit. To further investigate this connection, we carried out a synthetic interaction assay between the *msn5* mutation and the mutations in several genes involved in mitotic exit. The *MSN5* gene was inactivated in strains with mutations in the phosphatase Cdc14, the kinase Cdc15 (a component of the MEN pathway) or the APC ubiquitin ligase subunits Apc2, Cdc20, or Cdh1. Growth at high temperature revealed that none of the double mutants, *msn5 cdc14*, *msn5 cdc15*, *msn5 apc2*, *msn5 cdc20* or *msn5 cdh1*, grew properly compared to the single mutant strains (Fig. 1B). Defective growth was rescued by the reintroduction of a functional copy of the *MSN5* gene. Msn5 was also inactivated in the CDK inhibitor Sic1 and mitotic exit transcription factor Swi5 mutant strains. However, no synthetic growth defect was observed in the *msn5 sic1* and *msn5 swi5* double mutant strains. These results indicate a synthetic interaction between the *msn5* mutation and the *cdc14, cdc15, cdc20, apc2,* and *cdh1* mutations, which reinforces the idea that Msn5 participates in an essential function with mitotic exit genes.

3.2. Msn5 controls the subcellular localization of Swi5

Msn5 has been reported to be involved in the nuclear export of Cdh1. However, two observations suggest that this mechanism alone cannot explain the link between Msn5 and mitotic exit: first, accumulation of Cdh1 in the nucleus in a *msn5* mutant strain is associated with higher APC^{Cdh1} activity [35], which is inconsistent with hypersensitivity of *msn5* to high levels of Clb2; second, the synthetic interactions between *msn5* and *cdh1* mutations suggest that Msn5

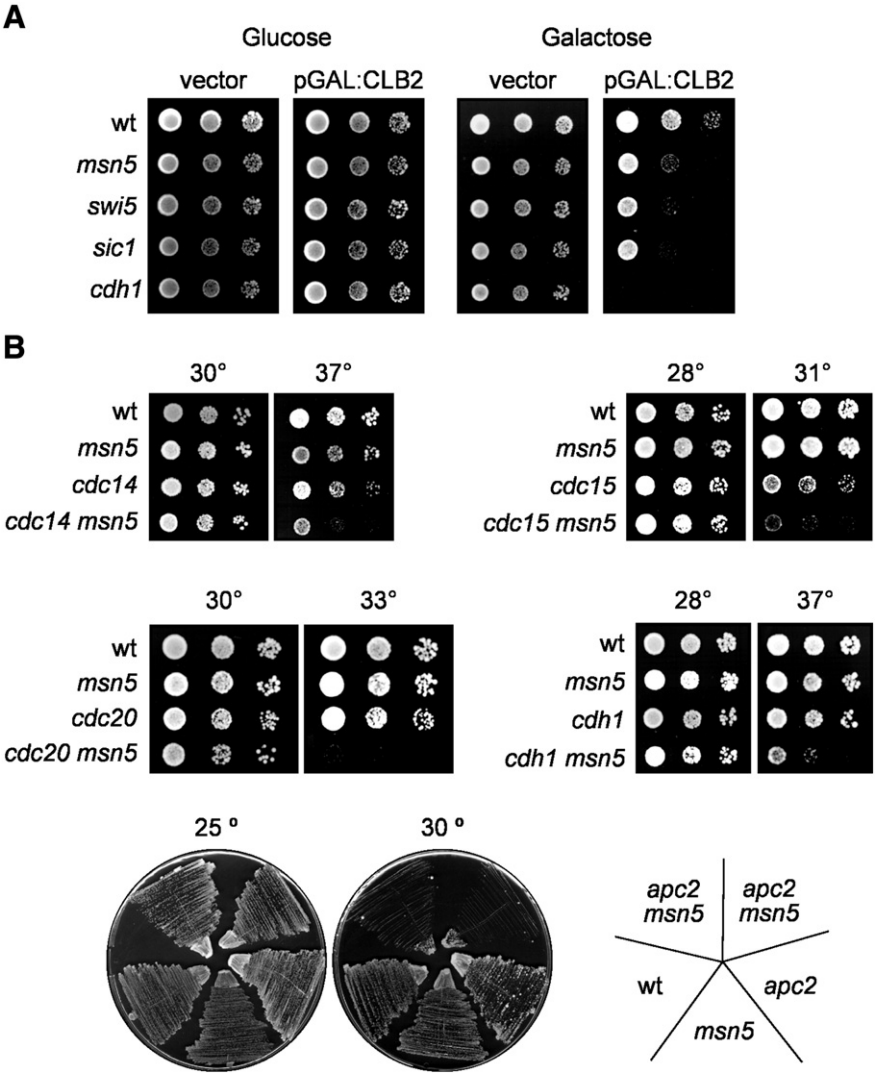

Fig. 1. Genetic interactions between *MSN5* and mitotic exit genes. A) 10-fold serial dilutions from exponentially growing cultures of wild type (W303-1a), *msn5* (JCY1018), *swi5* (JCY920), *sic1* (JCY316) and *cdh1* (JCY1012) strains transformed with an empty vector or a plasmid expressing the *CLB2* gene under the control of the *GAL1* promoter were spotted onto YPD or YPGal medium and incubated at 28° for 3 days. B) 10-fold serial dilutions from exponentially growing cultures of wild type (W303-1a), *msn5* (JCY1018), *cdc14*, *cdc14msn5* (JCY676), *cdc15*, *cdc15msn5* (JCY674), *cdc20*, *cdc20msn5* (JCY672), *cdh1* (JCY1012) and *cdh1msn5* (JCY1014) strains were spotted onto YPD medium and incubated at the indicated temperature for 3 days. The *apc2* (KTM200U) and *apc2msn5* (JCY744) strains were streaked in YPD plates and incubated at the indicated temperatures.

should affect other mitotic exit proteins. One good candidate is the transcription factor Swi5, which is responsible for a transcriptional wave at the end of mitosis that includes the *SIC1* gene. A putative interaction between Msn5 and Swi5 was reported in two-hybrid global analysis [48]. Given the high number of false positives in the genome-wide analysis, our first aim was to confirm the existence of a physical interaction between Msn5 and Swi5 by a two-hybrid assay. The strategy we designed involved the expression of Swi5 fused to the Gal4 activation domain (GAD) in the presence of Msn5 fused to the Gal4 DNA-binding domain (BD), and analysis of the expression of a *GAL7:lacZ* gene. It should be noted that the presence of Msn5-BD led to a significant level of β-galactosidase activity, which was probably due to Msn5 interacting with several transcription factors. However, β-galactosidase activity significantly increased when both the Swi5-GAD and Msn5-BD proteins were present (Fig. 2A), demonstrating that Msn5 physically interacts with Swi5 *in vivo*.

To further investigate the interaction between Msn5 and Swi5, we carried out a coimmunoprecipitation experiment. Crude extracts were prepared from cells expressing an HA-tagged Msn5 protein and from control cells with untagged Msn5. The HA-Msn5 protein was immunoprecipitated, and the presence of Swi5 in the purified fraction was determined by western blot analysis. As shown in Fig. 2B, Swi5 selectively coimmunoprecipitated with HA-Msn5. These results provide further support for Msn5 physically interacting with Swi5 *in vivo*. We also carried out an *in vitro* binding experiment between GST-Msn5, His_6-Swi5 and His_6-Gsp1/Ran expressed in bacteria. No specific interaction between Msn5 and Swi5 could be detected, which raises the possibility that the *in vivo* interaction between Msn5 and Swi5 could involve either additional bridging protein(s) or, most probably, a post-translational modification of Swi5 as occurs with other Msn5 cargo proteins [36-39,42,43].

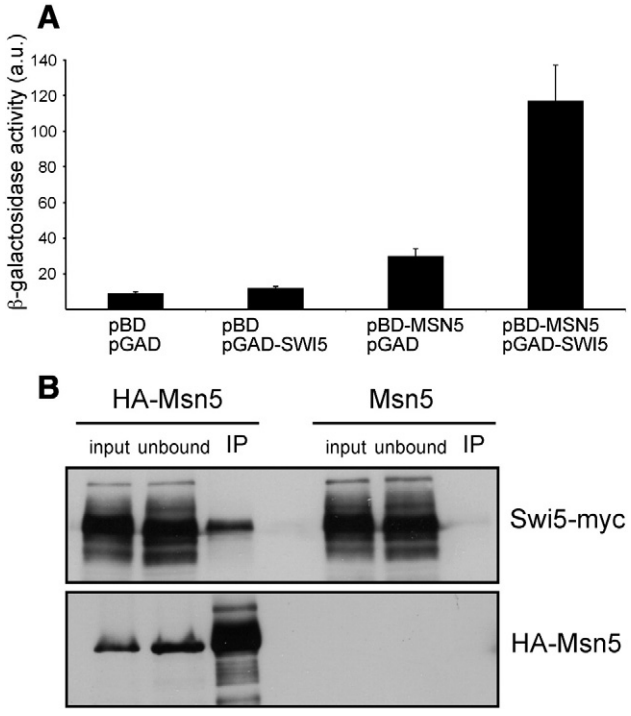

Fig. 2. Physical interaction between Swi5 and karyopherin Msn5. A) The two-hybrid strain PJ69-4A was transformed with plasmids expressing Swi5 fused to the Gal4 activation domain (pGAD–SWI5), Msn5 fused to the Gal4 DNA-binding domain (pBD-MSN5) and the corresponding empty vector (pGAD and pBD) as indicated. The ability of the proteins to induce expression of a *GAL7:lacZ* gene was tested by measuring β-galactosidase activity in extracts from exponentially growing cells. B) Msn5 was immunoprecipitated with anti-HA antibody from crude extracts of cells expressing a HA-tagged Msn5 (JCY520) and the control strain (JCY927). The presence of myc-tagged Swi5 and HA-tagged Msn5 in the input, unbound and immunoprecipitated fractions was determined by western analysis with a specific anti-myc or anti-HA antibody. Note that both Msn5 and Swi5 are over-expressed.

Given that Msn5 is a karyopherin, its *in vivo* physical interaction with Swi5 raises the possibility that Msn5 may control the subcellular localization of the transcription factor. Therefore, we decided to investigate the localization of Swi5 in the absence of Msn5. Swi5 is a periodic protein whose levels oscillate throughout the cell cycle. As with other cell cycle regulators, the localization of Swi5 also varies throughout the cell cycle. The protein begins to accumulate in the cell in the G2/M phase. During G2/M, Swi5 remains cytosolic until it enters the nucleus at the end of mitosis. It remains nuclear during G1, which is coincident with its degradation [19,23]. To analyze the possible effects of Msn5 on Swi5 localization, we expressed Swi5 under the control of the *GAL1* promoter to ensure constitutive expression during the cell cycle. This altered expression of the gene did not change the cell cycle-regulated localization pattern of Swi5 [23]: the wild-type strain showed nuclear localization of Swi5 only during telophase and G1, but the protein was cytosolic in the other cell cycle stages. In contrast, in the *msn5* mutant cells, Swi5 was detected inside the nucleus during all cell cycle stages, although the nuclear signal was weaker or absent in a number of large-budded cells (Fig. 3A). This result was confirmed by analysis of Swi5 localization in α-factor synchronized cells (Fig. 3B). The observed Swi5 nuclear localization in all cell cycle stages in *msn5* cells could be due to the inactivation of an export mechanism or to the constitutive nuclear import of Swi5 along the whole cell cycle. However, the fact that Swi5 remain cytosolic in nocodazole-arrested *msn5* cells ruled out the latter possibility (Supp. Fig. 1A). It has to be noted that a small percentage of nocodazole-arrested cells (13%) showed a nuclear signal, which could be due to nuclear import block leakage. In summary, the increased nuclear localization of Swi5 in the absence of Msn5 indicates that Msn5 is involved in the nuclear export of Swi5. The detected physical interaction between the two proteins described above suggests that requirement of Msn5 for Swi5 export is not an indirect effect; rather, Swi5 probably is a direct cargo of Msn5.

3.3. The N-terminal region of Swi5 mediates interaction with Msn5 and nuclear export

In a first attempt to identify the region of Swi5 responsible for its nuclear export, we did a two-hybrid assay to test the ability of different Swi5 fragments to interact with Msn5. Initially, Swi5 was divided into two complementary parts: the first including amino acids 1–325, and the second covering amino acids 326–709. β-galactosidase activity in the extracts of cells containing GAD–$Swi5^{326–709}$ was similar to that observed in control cells bearing the empty pGAD vector. This is not due to a defect in the expression of the fusion protein (Supp. Fig. 1B). However, β-galactosidase activity was significantly greater in cells expressing the GAD–$Swi5^{1–325}$ fusion product (Fig. 4A). These results indicate that amino acid region 1–325 of Swi5 is necessary and sufficient to mediate the interaction between Swi5 and Msn5.

To further delineate the Msn5-interacting region of Swi5, we also analyzed the ability of Swi5 fragments 1–285, 1–224, and 1–165 to interact with Msn5. As Fig. 4A shows, the GAD–$Swi5^{1–285}$ fusion product was still able to interact with Msn5, although β-galactosidase activity was lower than that obtained with the whole protein or with region 1–325. However, extracts from cells expressing fusion products GAD–$Swi5^{1–224}$ and GAD–$Swi5^{1–165}$ contained similar β-galactosidase activities to those observed with the control empty pGAD vector. Thus, the result indicates that region 224–285 is necessary for the interaction between Swi5 and Msn5. To determine whether this region is also sufficient for the interaction, we

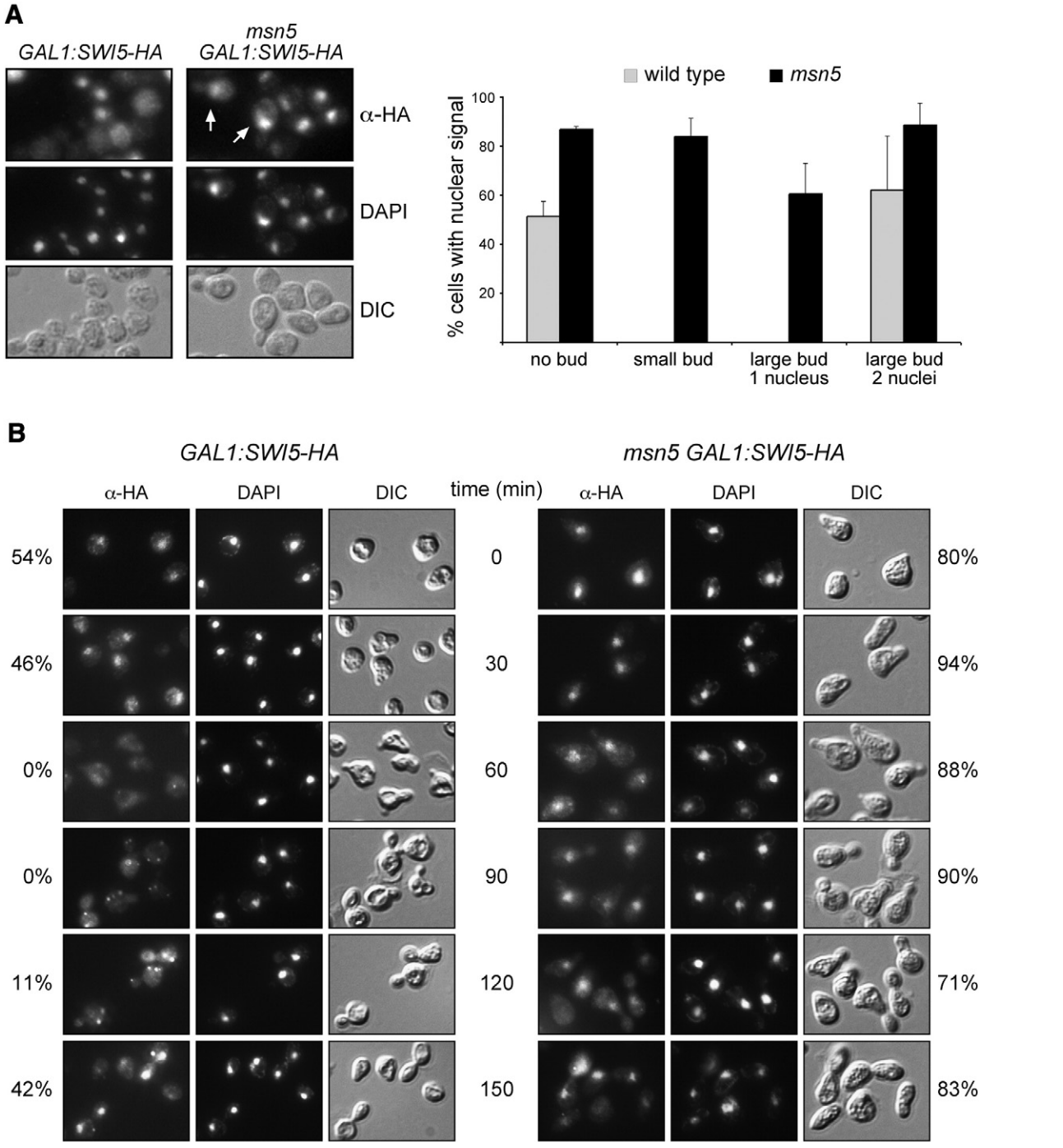

Fig. 3. Control of the subcellular localization of Swi5 by karyopherin Msn5. A) The *GAL1:SWI5-HA* (JCY1162) and the *GAL1:SWI5-HA msn5* (JCY1499) strains expressing a HA-tagged version of Swi5 under the control of the *GAL1* promoter were grown on raffinose. Galactose was added to a final concentration of 2% and after 60 min, cells were assayed by indirect immunofluorescence. The HA indirect-fluorescence signals (α-HA), the DAPI staining of DNA and the DIC images are shown. White arrows mark examples of G2/M cells with nuclear signal. No signal was detected in a control of the untagged wild type strain. Graph represents the percent of cells ($n > 500$ from three independent samples) with nuclear signal at the different stages of the cell cycle. B) Cultures of the *GAL1:SWI5-HA* (JCY1162) and the *GAL1:SWI5-HA msn5* (JCY1499) strains grown on raffinose were synchronized by the addition of α-factor. After two hours galactose was added to a final concentration of 2% and after and additional 30 min incubation cells were released from the arrest and assayed by indirect immunofluorescence at the indicated time. The percent of cells with nuclear signal is indicated.

constructed a GAD–Swi5$^{224–285}$ fusion product. However, the low β-galactosidase activity obtained with this protein demonstrates that this region did not interact with Msn5 in the two-hybrid assay. The same result was obtained with an extended region from amino acid 224 to 325. This is not due to lack of expression since both proteins are expressed at a similar level than GAD–Swi5 (Supp. Fig. 1B). Rather, these results suggest that, in addition to fragment 224–285, some element present in region 1–224 of Swi5 may play an important role in the interaction between Msn5 and Swi5.

The interaction between the N-terminal end of Swi5 and Msn5 suggests that the N-terminal region of the transcription factor acts as an NES. If this were true, the absence of this region would cause the nuclear localization of Swi5 during all cell cycle phases. To verify this prediction, we investigated the localization of a truncated version

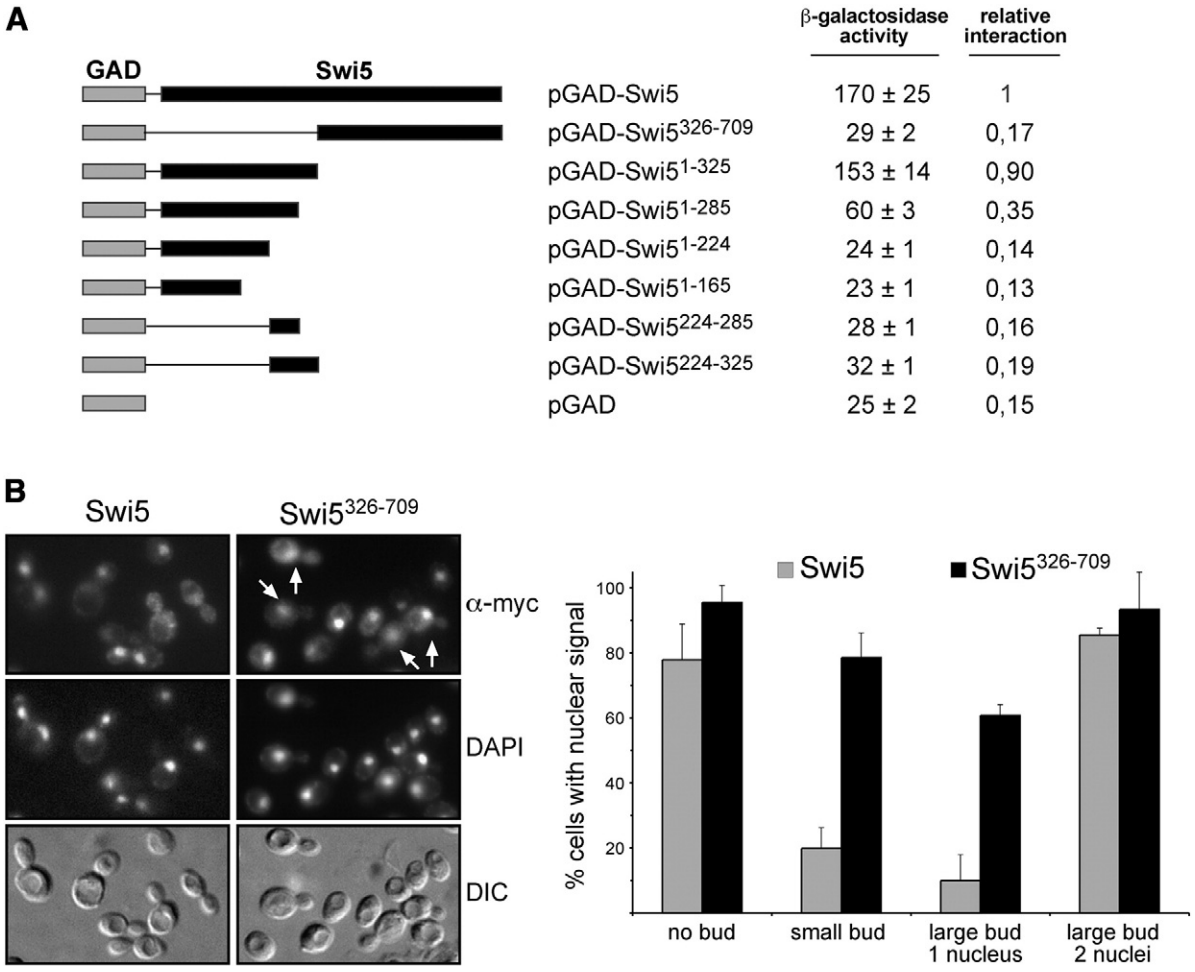

Fig. 4. Characterization of the Swi5 region interacting with Msn5. A) The interaction between Msn5 fused to the Gal4 DNA-binding domain and different truncated forms of Swi5 fused to the Gal4 activation domain was analyzed by two-hybrid analysis as described in Fig. 2. B) Cells expressing a myc-tagged version of the full length Swi5 (JCY1135) or the truncated Swi5$^{326-709}$ (JCY1138) protein under the control of the *GAL1* promoter were grown on galactose and assayed by indirect immunofluorescence. The myc indirect-fluorescence signals (α-myc), the DAPI staining of DNA and the DIC images are shown. White arrows mark examples of G2/M cells with nuclear signal. No signal was detected in a control untagged strain. Graph represents the percent of cells ($n > 250$ from two independent samples) with nuclear signal at the different stages of the cell cycle.

of the protein that lacked amino acids 1–325. While the wild-type Swi5 protein was nuclear only in telophase and G1, in the case of the Swi5$^{326-709}$ protein, nuclear signal was also observed in other cell cycle phases, although the nuclear signal was weaker in a number of large-budded cells (Fig. 4B). This result indicates that region 1–325 of Swi5 is required for proper localization of the protein throughout the cell cycle, which is consistent with the interaction detected between fragment 1–325 of Swi5 and Msn5. However, fragment 1–325 was unable to drive the nuclear export of a nuclear chimeric protein composed of four copies of the GFP protein fused to the NLS from SV40. This could suggest that additional sequences are involved in nuclear export activity. Alternatively, this result could also be caused by a stronger NLS than NES activity. Because of that we fused the 1–325 fragment to GFP in order to analyze whether 1–325 fragment could keep a protein lacking a functional NLS out of the nucleus in an Msn5 dependent manner. Surprisingly, the Swi5$^{1-325}$–GFP fusion protein showed a nuclear localization (Supp. Fig. 1C). Thus, the 1–325 region must contain a cryptic NLS. However, this cryptic NLS is not probably affecting Swi5 localization since Swi5 nuclear accumulation is dependent on the well-known C-terminal NLS since its deletion abolishes Swi5 nuclear accumulation (Supp. Fig. 2B), whereas deletion of 1–325 fragment does not impede Swi5 nuclear accumulation. We quantified the nuclear and cytosolic signal intensity in order to evaluate the NES activity of fragment Swi5$^{1-325}$ (Supp. Fig. 1C). Interestingly, nuclear/cytosolic signal intensity ratio was higher in *msn5* mutant (2.70 ± 0.21) than in wild type cells (2.28 ± 0.20), which is consistent with the presence of an Msn5-dependent NES in the Swi5$^{1-325}$ fragment.

3.4. Reduced Swi5 protein levels in msn5 mutant cells

In parallel to the experiments described above, we analyzed Swi5 protein by western blotting. As shown in Fig. 5A, the amount of Swi5 in *msn5* mutant cells is significantly lower compared to that in the wild-type cells. The effect of Msn5 inactivation on the Swi5 protein level was strikingly observed in a *GAL1:MSN5* strain: after glucose addition, the decrease in Swi5 paralleled the disappearance of Msn5 protein (Fig. 5B). Given that *SWI5* is a periodically expressed gene, the reduced Swi5 protein level in *msn5* mutant cells could be caused by a change in the cell cycle distribution of cells. However, this was not the case, because we did not observe a significant change in the cell cycle distribution of cells. Alternatively, the lower level of Swi5 in the absence of Msn5 could be attributed to a defect in *SWI5* gene expression. To investigate this possibility, we analyzed the Swi5 protein levels when the *MSN5* gene was ectopically expressed under the control of the *GAL1* promoter. Western blotting revealed that inactivation of Msn5 led to lower amounts of Swi5 (Fig. 5C). More importantly, a northern analysis done in parallel revealed that *SWI5* mRNA levels in the *msn5* mutant strain was not markedly affected (Fig. 5A, C). These results rule out the possibility of alterations in

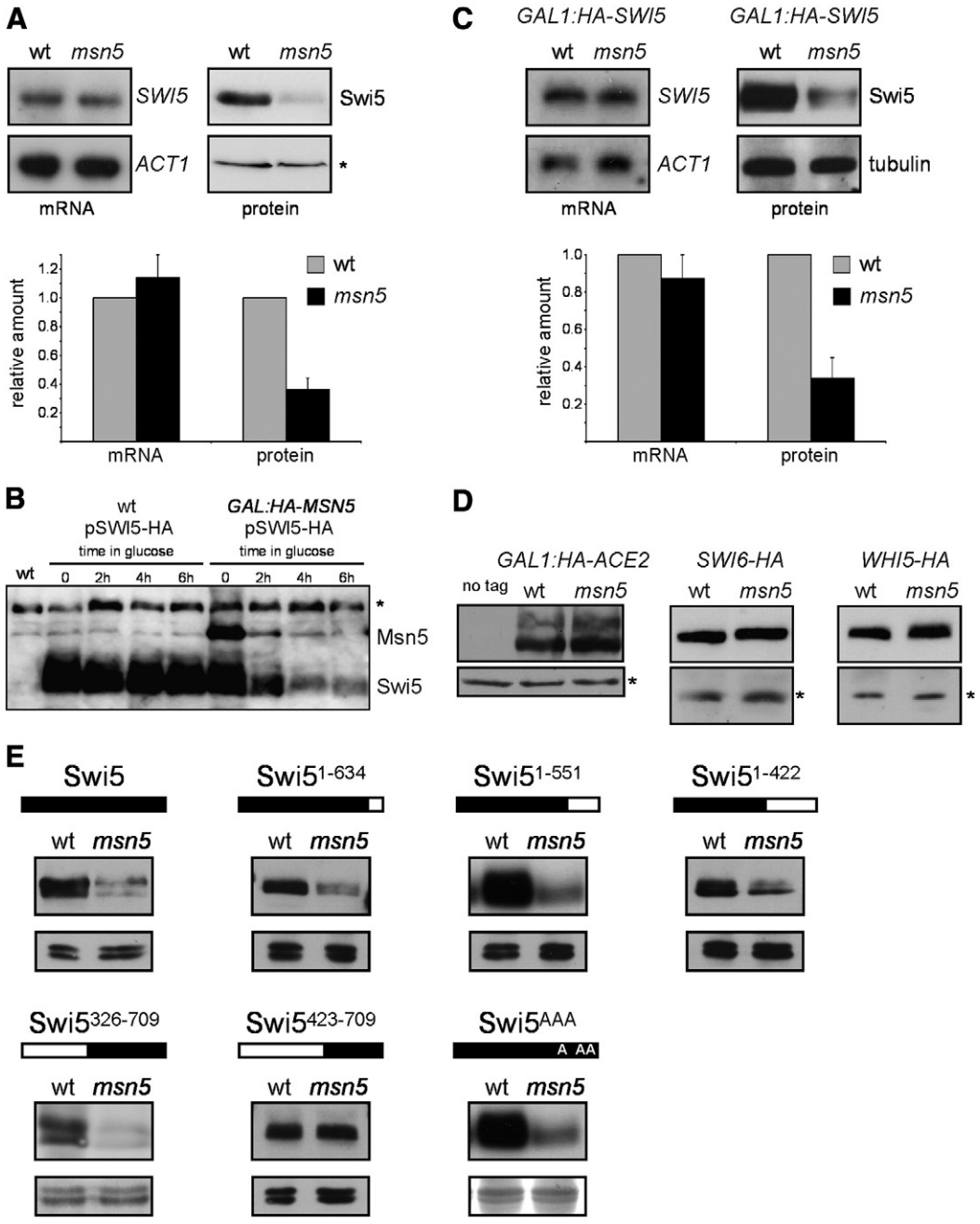

Fig. 5. Analysis of Swi5 protein level in the *msn5* mutant cells. A) The level of Swi5 protein and the *SWI5* mRNA in crude extracts of cells from exponentially growing cultures of the wild type (W303-1a) and the *msn5* mutant (JCY1018) strains was determined by western or northern analysis respectively. A non-specific band labeled with an asterisk that cross-react with the anti-Swi5 antibody and actin mRNA is shown as loading controls. Graph represents the amount of Swi5 protein (relative to the non-specific band) and *SWI5* mRNA (relative to *ACT1* mRNA) in the *msn5* mutant strain relative to the wild type strain. B) *GAL1:HA-MSN5* (JCY313) cells bearing a plasmid that express a HA-tagged version of Swi5 were grown on galactose and transferred to glucose containing medium to repress *MSN5* gene expression. The level of Msn5 and Swi5 proteins in crude extracts was determined at the indicated time by western analysis. A control extract from the wild type untagged strain (wt) and a loading control (asterisk) are shown. C) The *GAL1:HA-SWI5* (JCY1117) and the *GAL1:HA-SWI5 msn5* mutant (JCY1120) cells expressing a HA-tagged version of Swi5 under the control of the *GAL1* promoter were grown on galactose and the level of the Swi5 protein and the *SWI5* mRNA in crude extracts was determined in the same samples by western or northern analysis respectively. Tubulin and actin mRNA is shown as loading controls. Graph represents the amount of Swi5 protein (relative to tubulin) and *SWI5* mRNA (relative to *ACT1* mRNA) in the *msn5* mutant strain relative to the wild type strain. D) The level of Ace2, Swi6 or Whi5 proteins in crude extracts from cells of the *GAL1:HA-ACE2* (JCY592), *SWI6-HA* (JCY 710), *WHI5-HA* (JCY1346) and their corresponding *msn5* mutant strains (JCY590, JCY287 and JCY1348) was determined by western analysis. A non-specific band labeled with an asterisk that cross-react with the antibody is shown as loading control. E) The level of different truncated Swi5 proteins in crude extracts of cells from exponentially growing cultures was determined by western analysis. Strains used were *GAL1:SWI5-HA* (JCY1162), *GAL1:SWI5$^{1-634}$-HA* (JCY1163), *GAL1:SWI5$^{1-551}$-HA* (JCY1165), *GAL1:SWI5$^{1-422}$-HA* (JCY1491), *GAL1:HA-SWI5$^{326-709}$* (JCY1495), *GAL1:HA-SWI5$^{423-709}$* (JCY1175) and their corresponding *msn5* mutant strains (JCY1499, JCY1501, JCY1177, JCY1503 JCY1507 and JCY 1181 respectively). Also included are the wild type (W303-1a) and the *msn5* (JCY1018) strains transformed with a plasmid expressing the constitutive nuclear Swi5^{AAA} mutant protein. Lower panels for each construct show Cdc28 protein level or ponceau staining of the membrane as loading control.

Swi5 levels in *msn5* mutant cells being caused by reduced expression of the *SWI5* gene, and clearly indicate the existence of an Mns5-dependent post-transcriptional mechanism controlling the Swi5 protein level. This is a specific characteristic of the Swi5 protein, since Msn5 inactivation did not affect the protein content of either the related Ace2 transcription factor or other cell cycle transcription factors such as Swi6 and Whi5, the localization of which is regulated by Msn5 (Fig. 5D).

3.5. Lower Swi5 synthesis rate in msn5 mutant cells

A previous study of Swi5 suggested that the protein level diminishes during the G1 phase, which is coincident with its nuclear localization [23]. In addition to periodic *SWI5* gene expression, a posttranscriptional mechanism controlling the Swi5 protein level throughout the cell cycle must exist, because we observed that Swi5 levels fluctuate when the gene is expressed under the control of the constitutive *PGK1* promoter (Supp. Fig. S2A). The amount of ectopically expressed Swi5 was minimal in the G1 phase, which is the cell cycle stage in which Swi5 is nuclear. Taking these observations into account, it is possible that the reduced level of Swi5 in *msn5* mutant cells was caused by an accumulation of Swi5 in the nucleus, which would lead to a higher rate of protein degradation. Nonetheless, other results cast doubt on this hypothesis. First, the lower levels of Swi5 that we detected in *msn5* mutant cells could also be observed in a cytosolic variant of Swi5 ($Swi5^{1-634}$) in which the NLS signal was eliminated (Fig. 5E and Supp. Fig. S2B). Second, variant proteins that are mostly nuclear, like truncated $Swi5^{325-709}$ and a mutated Swi5 in which Ser 522, 646, and 664 were substituted with Ala ($Swi5^{AAA}$), also displayed reduced levels upon Msn5 inactivation (Fig. 5E). Thus, Swi5 protein levels decreased when Msn5 was inactivated irrespective of protein localization. Finally, analysis of Swi5 protein decay with either a transcriptional shut-off assay (by adding glucose to cells containing the *GAL1:SWI5* gene) or a translational shut-off assay (by adding cycloheximide) indicated that the stability of Swi5 in the *msn5* mutant strain did not differ from that observed in the wild-type strain (Fig. 6A, B and Supp. Fig. S2E). In short,

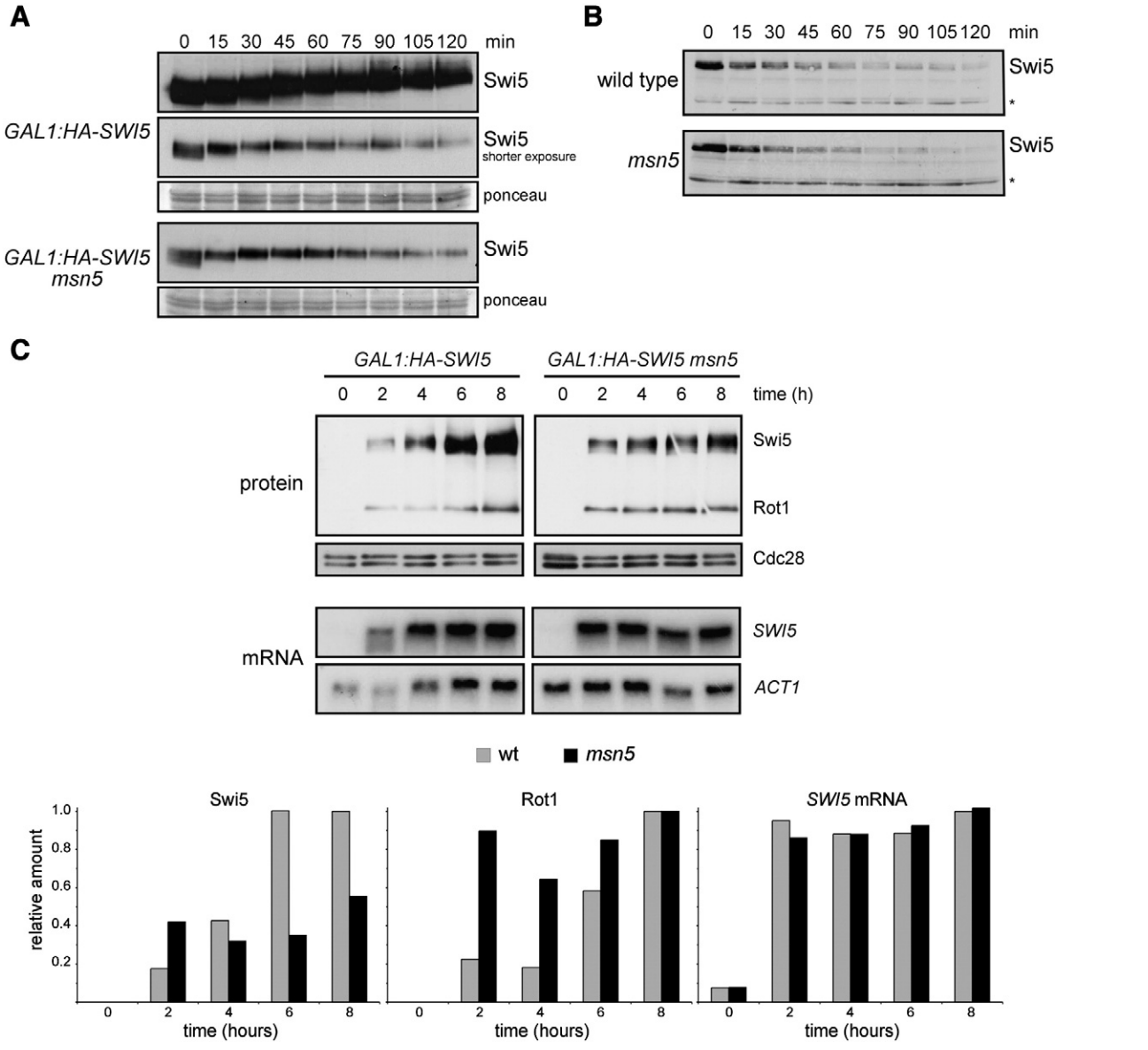

Fig. 6. Analysis of Swi5 protein stability and synthesis in the *msn5* mutant strain. A) The *GAL1:HA-SWI5* (JCY1117) and the *GAL1:HA-SWI5 msn5* (JCY1120) strains were grown on raffinose. Galactose was added to a final concentration of 2% and after 40 min cells were transferred to YPD medium. The Swi5 protein level in crude cell extracts was determined at the indicated time by western analysis. In the case of the *GAL1:HA-SWI5* strain a shorter exposure of the film is also shown. Loading controls of total protein are shown (ponceau). B) Cycloheximide to a final concentration of 50 μg/mL was added to exponentially growing cultures of the wild type (W303-1a) and the *msn5* mutant (JCY1018) strains. The Swi5 protein level in crude cell extracts was determined at the indicated time by western analysis. A non-specific band labeled with an asterisk that cross-react with the antibody is shown as loading control. C) The *GAL1:HA-SWI5* (JCY1117) and the *GAL1:HA-SWI5 msn5* (JCY1120) strains containing the pGAL1:ROT1-HA plasmid were grown on raffinose. Synthesis of Swi5 and control Rot1 protein was induced by the addition of galactose to a final concentration of 2%. The accumulation of the Swi5 and Rot1 proteins and *SWI5* mRNA was determined by western analysis (upper panel) and northern analysis (lower panel) at the indicated times. Cdc28 and actin mRNA levels are shown as loading controls. Graph represents the amount of Swi5 protein, Rot1 protein and *SWI5* mRNA relative to the 8 hours time point of the wild type strain.

Msn5 inactivation affected the Swi5 protein level irrespective of the changes in subcellular localization and by a mechanism distinct to changes in protein stability.

While performing the transcriptional shut-off experiment described above, we observed that Swi5 levels during the synthesis pulse in galactose medium was low in *msn5* mutant cells. To better characterize this finding, we analyzed the kinetics of accumulation of Swi5 in both wild-type and *msn5* cells. Cells containing the *GAL1: SWI5* gene were grown on raffinose and Swi5 synthesis was induced by adding galactose to the medium. As a control, these cells also contained a centromeric plasmid expressing the *ROT1* gene under the control of the same *GAL1* promoter. Swi5 and Rot1 protein levels were analyzed in parallel. As shown in Fig. 6C, accumulation of Swi5 in the absence of Msn5 clearly lagged behind the accumulation of Swi5 in wild-type cells. The defect in Swi5 accumulation was not due to defects in *GAL1* driven expression, because the control protein Rot1 accumulated properly in the absence of Msn5. Moreover, northern analysis confirmed that *SWI5* mRNA levels were roughly similar in both the presence or absence of Msn5. The steady-state level of a protein is the result of the balance between the synthesis and degradation rates. Once we had ruled out protein degradation as the source of the difference in the Swi5 level between wild-type and *msn5* strains, we were able to conclude that Swi5 synthesis takes place at a reduced rate in *msn5* mutant cells.

After establishing that Msn5 controls Swi5 synthesis, we decided to analyze whether a specific region of Swi5 mediated this effect. Thus, levels of various truncated forms of Swi5 were analyzed in both wild-type and *msn5* mutant strains (Fig. 5E). Truncated Swi5 forms encompassing fragments 1–635, 1–551 and 1–422 showed the characteristic differences between wild-type and *msn5* cells. Thus, the C-terminal part of Swi5 was not required for regulation by Msn5 and, reciprocally, region 1–422 was sufficient to mediate control by Msn5. Similarly, the truncated form $Swi5^{326–709}$ also manifested the reduction in Swi5 levels when Msn5 was inactivated. However, $Swi5^{422–709}$ lost this reduction, so that the protein concentration was the same in both *msn5* and wild-type cells. These observations indicate that while neither the N-terminal region 1–325 nor the C-terminal region 422–709 of Swi5 was required for regulation of Swi5 synthesis by Msn5, fragment 325–422 was important for Msn5 control of Swi5 protein levels. Nevertheless, when fragment 325–422 was fused to a GFP protein, it did not confer Msn5-dependent regulation of protein levels. This suggests that, although this region was necessary, it was not sufficient to mediate the reduction in Swi5 levels in *msn5* cells; thus, other sequence fragments are required.

3.6. Swi5 is functional in the msn5 mutant strain

Having determined that the *msn5* mutation alters the localization and concentration of Swi5, we wondered if it also affected the transcriptional activity of the protein. We initially assessed the functionality of Swi5 using the reporter gene fusion *HO(31): lacZ*, which comprised the coding region of the *lacZ* gene and the region from the *HO* gene promoter necessary for its regulation by Swi5 [18]. The β-galactosidase activity present in extracts from wild-type and *msn5* strains bearing the *HO(31): lacZ* gene indicated that, although the activity achieved in the absence of Msn5 was slightly lower than that observed in the wild-type strain (probably due to the reduced amount of protein), Swi5 retained its functionality as a transcription factor (Fig. 7A).

To further test Swi5 activity, we analyzed *SIC1* gene expression. Inactivation of Swi5 strongly affects periodic expression of the *SIC1* gene in late mitosis [7,8]. However, inactivation of Msn5 did not alter global levels or cell-cycle regulation of *SIC1* gene expression (Fig. 7B, C). This is in agreement with the previous result and confirms that Swi5 was functional in the *msn5* mutant strain.

3.7. msn5 mutant cells are sensitive to overexpression of Swi5

We envisaged the possibility that *msn5* cells were sensitive to high levels of Swi5. To explore this possibility in detail, we analyzed growth of different *GAL1: HA-SWI5* transformants that differed in

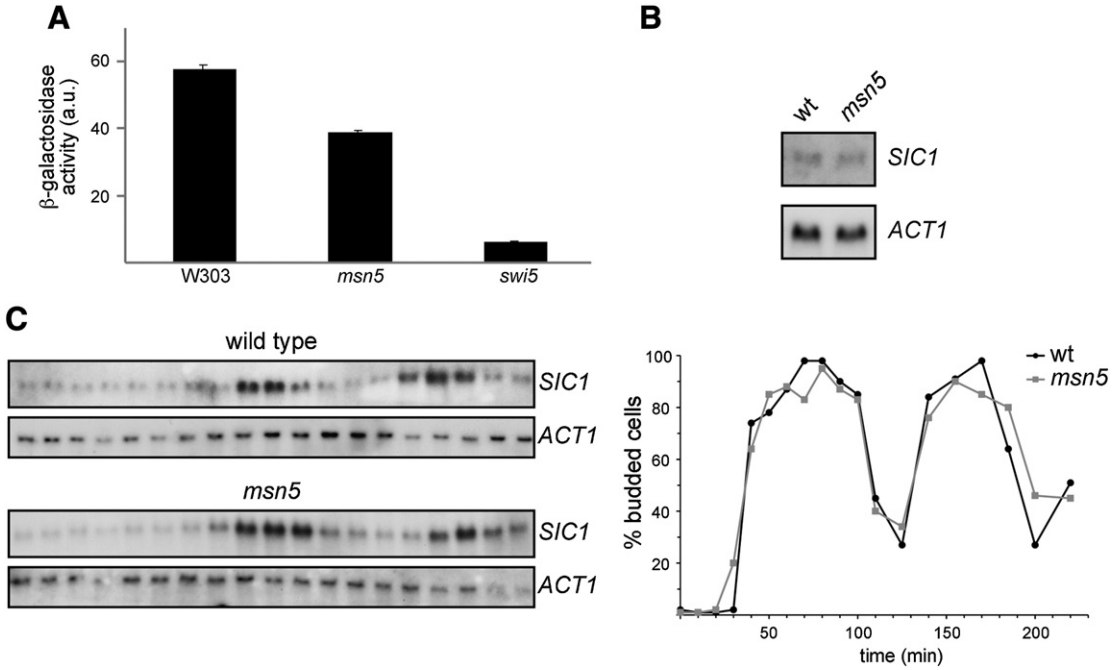

Fig. 7. Analysis of Swi5 functionality in the *msn5* mutant strain. A) β-galactosidase activity in extracts from wild type (W303-1A), *msn5* (JCY1018) and *swi5* (JCY920) strains transformed with the pHO(31):lacZ plasmid containing the *lacZ* reporter gene under the control of a fragment from *HO* promoter regulated by Swi5. Values are derived from extracts from at least 3 independent transformants. B) Expression of *SIC1* gene in mid-log cultures of wild type (W303-1A) and *msn5* (JCY1018) strains was studied by Northern analysis. *ACT1* is shown as loading control. C) Expression of *SIC1* gene in α-factor synchronized cultures. Samples were collected at 0, 10, 20 30, 40 50, 60, 70, 80, 90, 100, 110, 125, 140, 155, 170, 185, 200 and 220 min after the release. *ACT1* is shown as loading control. Graph represents the budding index as a control of synchronization.

the amounts of Swi5 expressed in cells and their respective *msn5*-derived mutants in medium with galactose as the sole carbon source. While all of the *GAL1: HA-SWI5* strains grew in galactose medium, the *msn5* mutant strains displayed heterogeneous behavior ranging from no growth, poor growth and normal growth (Fig. 8A). Strikingly, the strength of the growth defect in the *msn5* mutant strains correlated perfectly with the amount of Swi5 in the cell extracts (Fig. 8B). Thus, the higher the Swi5 content, the lower the growth rate of the *GAL1: HA-SWI5 msn5* strains. This result strongly suggests that the *msn5* mutant was sensitive to Swi5 in a dose-dependent manner. No accumulation of cells in a specific cell cycle stage was observed, which suggest that cells were affected at several cell cycle points or at an essential function no related to a specific cell-cycle stage (F.J.T. unpublished data).

Considering the nuclear localization of Swi5 protein in *msn5* mutants, the observation above suggests that abnormal accumulation of high levels of Swi5 inside the nucleus could prove toxic for cells. To investigate this possibility, we studied whether the growth defect on galactose of *GAL1: HA-SWI5 msn5* strain could be reverted by restricting Swi5 localization to the cytoplasm. To do this, we removed the C-terminal end (amino acids 635–709) containing the NLS from the *GAL1: HA-SWI5 msn5* strain. Elimination of this fragment completely suppressed the growth defect in this strain (Fig. 8C). Growth recovery was not due to reduced levels of Swi5, since Swi5 and Swi5$^{1-634}$ levels were equivalent (Fig. 8D). This result strongly suggests that the toxicity of high amounts of Swi5 in the absence of karyopherin Msn5 was associated with its accumulation inside the nucleus.

4. Discussion

The genetic interactions between the *msn5* mutant and the mitotic exit machinery suggest a role for Msn5 at this point in the cell cycle. Previous results reported by other authors showed that Msn5 interacted with proteins involved in exit from mitosis. For example, Msn5 was reported to be involved in the regulation of the APC substrate-recognition subunit Cdh1 [35]. Cdh1 participates in the degradation of Clb cyclins, a key process in the inactivation of CDKs at the end of mitosis. Cdh1 is activated at the end of mitosis by phosphatase Cdc14 and is inactivated at the beginning of the next cell cycle by Cln-Cdc28 kinases. Msn5 may contribute to the inactivation of Cdh1 by assisting in its export from the nucleus. However, this possibility is not in line with the observed sensitivity of *msn5* mutant to high levels of Clb2. In addition, the genetic interaction detected between *msn5* and a null mutant of *CDH1* suggests that Msn5 carries out other functions in the mitotic exit network irrespective of Cdh1. In this study, we identified another protein involved in mitotic exit that is regulated by Msn5. Msn5 is involved in the regulation of both the subcellular localization and the cellular concentration of the transcription factor Swi5 (Supp. Fig. S3).

Control of the subcellular localization of Swi5 is an example of the spatial regulation mechanisms in cell cycle control. Swi5 is one of the first proteins for which a change in subcellular localization during the cell cycle was described. It contains a classic NLS at the C-terminal end of the protein that is regulated by the phosphorylation status of nearby Ser residues [24]. Once *SWI5* gene expression is activated in G2, Swi5 is restricted to the cytoplasm by phosphorylation of Ser 522,

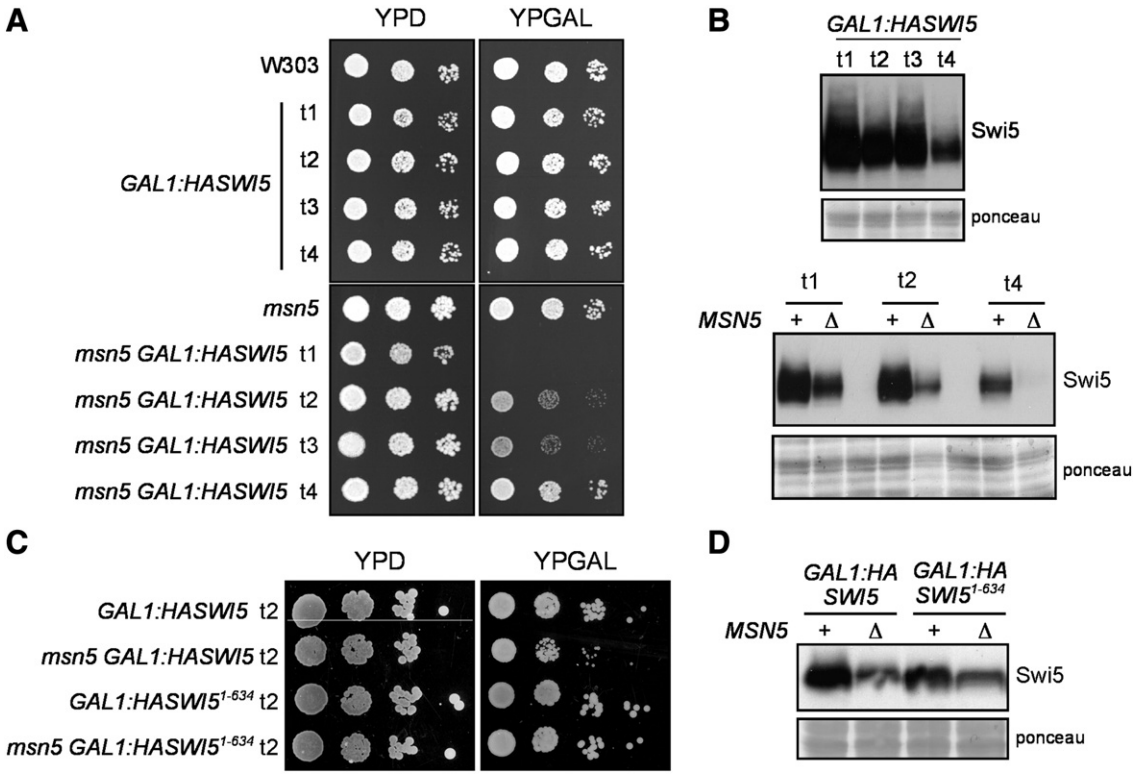

Fig. 8. Effect of Swi5 misregulation on cell growth. A) 10-fold serial dilutions from exponentially growing cultures of wild type (W303-1a), *msn5* (JCY1018), four different clones of the *GAL1:HA-SWI5* strain (JCY1117) and their *msn5* derived mutant strains were spotted onto YPD or YPGal medium and incubated at 28° for 3 days. B) The level of Swi5 protein in crude extracts of cells from exponentially growing cultures of the *GAL1:HA-SWI5* and their *msn5* derived strains used in A was determined by western analysis. A loading control of total protein is shown (ponceau). C) 10-fold serial dilutions from exponentially growing cultures of the *GAL1:HA-SWI5* (JCY1117), its *msn5* derived mutant (JCY1120) and the corresponding *GAL1:HA-SWI5*$^{1-634}$ (JCY1235) and *GAL1:HA-SWI5*$^{1-634}$ *msn5* (JCY1237) strains expressing a Swi5 truncated version of the proteins lacking the NLS sequence, were spotted onto YPD or YPGal medium and incubated at 28° for 3 days. D) The level of Swi5 protein in crude extracts of cells from exponentially growing cultures of strains used in C was analyzed by western analysis. A loading control of total protein is shown (ponceau).

646, and 664 by Clb2-Cdc28 until late mitosis, when Swi5 enters the nucleus after Cdc14 phosphatase-mediated dephosphorylation. Our results provide additional information about the spatial regulation of Swi5, because they show that yeast cells also control subcellular localization of Swi5 by an Msn5-mediated export mechanism.

Several observations call into question the importance of control of Swi5 by Msn5, and whether Msn5 itself is important during the M-G1 transition. First, transcriptional activation by Swi5 was not substantially affected in *msn5* mutants. Second, inactivation of Msn5 was deleterious for growth in mitotic exit mutants that expressed the Swi5$^{1-634}$ truncated protein lacking the NLS (Supp. Fig. S4), which suggests that the detected genetic interactions are not related to the misregulation of Swi5 localization in the *msn5* mutant. Moreover, we observed greater sensitivity to overexpression of Clb2 in the *msn5 swi5* double-mutant strain compared to single mutants (Supp. Fig. S4); this indicates that the effect of the *msn5* mutation was not mediated through regulation of Swi5 alone. Third, despite numerous genetic interactions with mitotic exit genes or control of the subcellular localization of Swi5 and Cdh1, Msn5 inactivation had no apparent effect on the kinetics of the telophase/G1 transition, as deduced from analysis of the cell cycle progression of *msn5 cdc15* double mutants compared to *cdc15* mutants after release from telophase arrest (F.J.T. unpublished data). This observation suggests that the role played by Msn5 in mitotic exit may be dispensable, unless the robustness of the transition is weakened by mutations in a component of this regulatory system. However, the export of Swi5 by Msn5 is indeed relevant for cellular physiology. A stable variant of Swi5 was recently reported to cause defects in the G1-S transition [27]. This observation suggests that high levels of Swi5 may have a deleterious effect on cells. More importantly, the toxic effect of high Swi5 activity was strongly exacerbated in the absence of Msn5, regardless of the reduced amount of the Swi5 protein. This could be because the toxicity of Swi5 was due to its nuclear accumulation, which was supported by the finding that restricting nuclear import completely alleviated Swi5 toxicity in *msn5* mutant cells. If we consider that Swi5 is active as a transcriptional activator in the absence of Msn5, we can speculate that unscheduled Swi5-dependent transcription is toxic and that Swi5 export by Msn5 helps to restrict the period during which this activity takes place. For example, Swi5 export by Msn5 could contribute, together with degradation of the protein, to the inactivation of Swi5 during G1/S. Additionally, export of Swi5 may be important for avoiding premature activation of target genes. Swi5 and Clb2 are synthesized in the G2 phase at the same time; therefore, it is conceivable that some Swi5 molecules could enter the nucleus before Clb2 blocks its NLS, even more so if we consider that Clb2 is mostly nuclear. Thus, Swi5 export by Msn5 could act as a security mechanism by excluding those Swi5 molecules that eluded Clb2-mediated nuclear import inhibition from the nucleus in order to avoid premature activation of Swi5 target genes.

In addition to the spatial regulation of Swi5, Msn5 also participates in regulating Swi5 protein levels. *msn5* mutant cells had lower Swi5 levels compared to wild-type cells. The fact that this effect was observed in *msn5* mutant strains with no growth defect and that Swi5 level diminished quickly in parallel to Msn5 decay clearly indicate that detection of low amounts of Swi5 is not correlated to grow defect. The decay in Swi5 level was not due to alterations in transcription of the *SWI5* gene; rather, it occurred at the posttranscriptional level. Previous work on Swi5 suggested a model whereby Swi5 was gradually degraded inside the nucleus during G1 until it disappeared, which would explain the drop in Swi5 protein levels in this phase [23,24]. Therefore, it is conceivable, in principle, that nuclear accumulation of Swi5 in *msn5* mutant cells could lead to greater protein instability and, consequently, to lower Swi5 cellular levels. However, several results have contradicted this model, particularly with regard to Swi5 degradation being due to its nuclear import during G1. For example, we observed that a truncated version of Swi5 that is mainly localized in the cytoplasm (Swi5$^{1-634}$) had reduced protein levels during G1 (Supp. Fig. S2C). This suggests that Swi5 instability during G1 is not dependent, or at least not totally, on its nuclear location. Accordingly, blocking nuclear import of Swi5 in the *cse1* mutant strain (Cse1 is a component of the classical import pathway) does not stabilize Swi5 [49]. In fact, we observed that deletion of the NLS had no effect on the protein's half-life (Supp. Fig. S2D). Furthermore, we describe how the drop in Swi5 protein levels caused by Msn5 inactivation also occurred in the nuclear and cytosolic versions of the protein, which suggests that the effect of Msn5 on the Swi5 level is independent of the control of subcellular localization. The findings regarding Swi5$^{326-709}$ are particularly interesting, since this truncated version of Swi5 did not interact with Msn5 in the two-hybrid assay, which supports the conclusion that protein level control is independent of subcellular localization control. Finally, the shut-off experiments demonstrated that the stability of Swi5 was not affected in the *msn5* strain. All of these observations led us to rule out the possibility that an increased rate of degradation as the result of altered localization was the cause of the reduced amount of Swi5 in *msn5* cells. Instead, the results indicate that problems in Swi5 protein synthesis arose when Msn5 was absent. If excess nuclear Swi5 is indeed toxic for cells, the concomitant protein level reduction accompanying its nuclear accumulation when exportin Msn5 is absent may be an evolutionary advantage.

Typically, mRNA is regulated by sequences present in the 5′- or 3′-unstranslated regions. For Swi5 regulation by Msn5, however, neither substitution of the 5′-UTR (*GAL1* constructs) nor the 3′-UTR (C-terminal epitope-tagged proteins) eliminated the drop in Swi5 levels in the *msn5* mutant strain. Thus, the effect of Msn5 on the Swi5 synthesis rate must be mediated by sequences in the coding region. In support of this, we show that the region coding for amino acids 326–422 was required for control of Swi5 synthesis. Different scenarios can be envisaged as to how Msn5 could control Swi5 synthesis (Supp. Fig. S3B). Absence of exportin Msn5 could prevent the nuclear exit of specific factors required for either proper *SWI5* mRNA processing or translation in the cytoplasm. A physical interaction has been reported between Msn5 and Vts1 [48], a protein that can interact with RNA [50]. However, inactivation of *msn5* decreased Swi5 protein levels even in the absence of Vts1 (Supp. Fig. S5). Alternatively, Msn5 could directly affect *SWI5* mRNA nuclear export. Although the mRNA export pathway is dependent on receptor Mex67-Mtr2 (reviewed in [51], Msn5 has been shown to bind RNA molecules *in vitro* [45]. Therefore, a direct role for Msn5 in the export of specific mRNAs cannot be ruled out. In addition, Msn5 may affect tRNA so that the translation rate of specific messengers decreases in its absence. A good candidate for mediation of tRNA control is Maf1, a repressor of RNApol III, the nuclear export of which is mediated by Msn5 [43]. However, *msn5* affected Swi5 protein levels even in the absence of Maf1 (Supp. Fig. S5). Msn5 was reported to bind tRNA and to play a role in tRNA export [44,45]. Inactivation of Msn5 could alter the cytosolic pools of tRNA and affect the translation of specific sequences in *SWI5* and other mRNAs. Future studies could help to elucidate how Msn5 affects Swi5 synthesis and to identify additional targets under Msn5 control.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2012.02.009.

Acknowledgments

We are very grateful to Dr. D. Stillman for kindly provide anti-Swi5 antibody. This work was supported by Grants BFU2007-67929-C02-02 and BFU2010-20927 from Ministerio de Ciencia e Innovación of the Spanish Government (co-financed by ERDF from European Union) and ACOMP/2010/134 from Generalitat Valenciana. F.T., I.Q. and J.S. were recipient of a Predoctoral Fellowship from the Spanish Government.

References

[1] D.O. Morgan, Cyclin-dependent kinases: engines, clocks, and microprocessors, Annu. Rev. Cell Dev. Biol. 13 (1997) 261–291.
[2] W.J. Bosl, R. Li, Mitotic-exit control as an evolved complex system, Cell 121 (2005) 325–333.
[3] J.M. Peters, The anaphase-promoting complex: proteolysis in mitosis and beyond, Mol. Cell 9 (2002) 931–943.
[4] F. Stegmeier, A. Amon, Closing mitosis: the functions of the Cdc14 phosphatase and its regulation, Annu. Rev. Genet. 38 (2004) 203–232.
[5] W. Zachariae, K. Nasmyth, Whose end is destruction: cell division and the anaphase-promoting complex, Genes Dev. 13 (1999) 2039–2058.
[6] M.D. Mendenhall, A.E. Hodge, Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast *Saccharomyces cerevisiae*, Microbiol. Mol. Biol. Rev. 62 (1998) 1191–1243.
[7] D. Knapp, L. Bhoite, D.J. Stillman, K. Nasmyth, The transcription factor Swi5 regulates expression of the cyclin kinase inhibitor p40SIC1, Mol. Cell. Biol. 16 (1996) 5701–5707.
[8] J.H. Toyn, A.L. Johnson, J.D. Donovan, W.M. Toone, L.H. Johnston, The Swi5 transcription factor of *Saccharomyces cerevisiae* has a role in exit from mitosis through induction of the cdk-inhibitor Sic1 in telophase, Genetics 145 (1997) 85–96.
[9] M. Stern, R. Jensen, I. Herskowitz, Five SWI genes are required for expression of the HO gene in yeast, J. Mol. Biol. 178 (1984) 853–868.
[10] C. Wittenberg, S.I. Reed, Cell cycle-dependent transcription in yeast: promoters, transcription factors, and transcriptomes, Oncogene 24 (2005) 2746–2755.
[11] P.R. Dohrmann, G. Butler, K. Tamai, S. Dorland, J.R. Greene, D.J. Thiele, D.J. Stillman, Parallel pathways of gene regulation: homologous regulators SWI5 and ACE2 differentially control transcription of HO and chitinase, Genes Dev. 6 (1992) 93–104.
[12] P.R. Dohrmann, W.P. Voth, D.J. Stillman, Role of negative regulation in promoter specificity of the homologous transcriptional activators Ace2p and Swi5p, Mol. Cell. Biol. 16 (1996) 1746–1758.
[13] B.L. Aerne, A.L. Johnson, J.H. Toyn, L.H. Johnston, Swi5 controls a novel wave of cyclin synthesis in late mitosis, Mol. Biol. Cell 9 (1998) 945–956.
[14] D.J. Stillman, A.T. Bankier, A. Seddon, E.G. Groenhout, K.A. Nasmyth, Characterization of a transcription factor involved in mother cell specific transcription of the yeast HO gene, EMBO J. 7 (1988) 485–494.
[15] A. Colman-Lerner, T.E. Chin, R. Brent, Yeast Cbk1 and Mob2 activate daughter-specific genetic programs to induce asymmetric cell fates, Cell 107 (2001) 739–750.
[16] M. Sbia, E.J. Parnell, Y. Yu, A.E. Olsen, K.L. Kretschmann, W.P. Voth, D.J. Stillman, Regulation of the yeast Ace2 transcription factor during the cell cycle, J. Biol. Chem. 283 (2008) 11135–11145.
[17] E.L. Weiss, C. Kurischko, C. Zhang, K. Shokat, D.G. Drubin, F.C. Luca, The *Saccharomyces cerevisiae* Mob2p-Cbk1p kinase complex promotes polarized growth and acts with the mitotic exit network to facilitate daughter cell-specific localization of Ace2p transcription factor, J. Cell Biol. 158 (2002) 885–900.
[18] R.M. Brazas, L.T. Bhoite, M.D. Murphy, Y. Yu, Y. Chen, D.W. Neklason, D.J. Stillman, Determining the requirements for cooperative DNA binding by Swi5p and Pho2p (Grf10p/Bas2p) at the HO promoter, J. Biol. Chem. 270 (1995) 29151–29161.
[19] G. Tebb, T. Moll, C. Dowzer, K. Nasmyth, SWI5 instability may be necessary but is not sufficient for asymmetric HO expression in yeast, Genes Dev. 7 (1993) 517–528.
[20] L.T. Bhoite, D.J. Stillman, Residues in the Swi5 zinc finger protein that mediate cooperative DNA binding with the Pho2 homeodomain protein, Mol. Cell. Biol. 18 (1998) 6436–6446.
[21] V. Measday, H. McBride, J. Moffat, D. Stillman, B. Andrews, Interactions between Pho85 cyclin-dependent kinase complexes and the Swi5 transcription factor in budding yeast, Mol. Microbiol. 35 (2000) 825–834.
[22] K. Nasmyth, A. Seddon, G. Ammerer, Cell cycle regulation of SW15 is required for mother-cell-specific HO transcription in yeast, Cell 49 (1987) 549–558.
[23] K. Nasmyth, G. Adolf, D. Lydall, A. Seddon, The identification of a second cell cycle control on the HO promoter in yeast: cell cycle regulation of SW15 nuclear entry, Cell 62 (1990) 631–647.
[24] T. Moll, G. Tebb, U. Surana, H. Robitsch, K. Nasmyth, The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the *S. cerevisiae* transcription factor SWI5, Cell 66 (1991) 743–758.
[25] S. Hahn, P. Maurer, S. Caesar, G. Schlenstedt, Classical NLS proteins from *Saccharomyces cerevisiae*, J. Mol. Biol. 379 (2008) 678–694.
[26] R. Visintin, K. Craig, E.S. Hwang, S. Prinz, M. Tyers, A. Amon, The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation, Mol. Cell 2 (1998) 709–718.
[27] T. Kishi, A. Ikeda, N. Koyama, J. Fukada, R. Nagao, A refined two-hybrid system reveals that SCF(Cdc4)-dependent degradation of Swi5 contributes to the regulatory mechanism of S-phase entry, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 14497–14502.
[28] A. Cook, F. Bono, M. Jinek, E. Conti, Structural biology of nucleocytoplasmic transport, Annu. Rev. Biochem. 76 (2007) 647–671.
[29] H. Fried, U. Kutay, Nucleocytoplasmic transport: taking an inventory, Cell. Mol. Life Sci. 60 (2003) 1659–1688.
[30] L.F. Pemberton, B.M. Paschal, Mechanisms of receptor-mediated nuclear import and nuclear export, Traffic 6 (2005) 187–198.
[31] S. Kuersten, M. Ohno, I.W. Mattaj, Nucleocytoplasmic transport: ran, beta and beyond, Trends Cell Biol. 11 (2001) 497–503.
[32] E. Queralt, J.C. Igual, Cell cycle activation of the Swi6p transcription factor is linked to nucleocytoplasmic shuttling, Mol. Cell. Biol. 23 (2003) 3126–3140.
[33] F.J. Taberner, I. Quilis, J.C. Igual, Spatial regulation of the start repressor Whi5, Cell Cycle 8 (2009) 3010–3018.
[34] M. Blondel, P.M. Alepuz, L.S. Huang, S. Shaham, G. Ammerer, M. Peter, Nuclear export of Far1p in reesponse to pheromones requires the export receptor Msn5p/Ste21p, Genes Dev. 13 (1999) 2284–2300.
[35] M. Jaquenoud, F. van Drogen, M. Peter, Cell cycle-dependent nuclear export of Cdh1p may contribute to the inactivation of APC/C(Cdh1), EMBO J. 21 (2002) 6515–6526.
[36] A. Bakhrat, K. Baranes-Bachar, D. Reshef, O. Voloshin, O. Krichevsky, D. Raveh, Nuclear export of Ho endonuclease of yeast via Msn5, Curr. Genet. 54 (2008) 271–281.
[37] A. Kaffman, N.M. Rank, E.M. O'Neill, L.S. Huang, E.K. O'Shea, The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of tge nucleus, Nature 396 (1998) 482–486.
[38] L.M. Boustany, M.S. Cyert, Calcineurin-dependent regulation of Crz1p nuclear export requires Msn5p and a conserved calcineurin docking site, Genes Dev. 16 (2002) 608–619.
[39] M.J. DeVit, M. Johnston, The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of *Saccharomyces cerevisiae*, Curr. Biol. 9 (1999) 1231–1241.
[40] W. Gorner, E. Durchschlag, J. Wolf, E.L. Brown, G. Ammerer, H. Ruis, C. Schuller, Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor, EMBO J. 21 (2002) 135–144.
[41] A. Komeili, K.P. Wedaman, E.K. O'Shea, T. Powers, Mechanism of metabolic control. Target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors, J. Cell Biol. 151 (2000) 863–878.
[42] R. Ueta, N. Fujiwara, K. Iwai, Y. Yamaguchi-Iwai, Mechanism underlying the iron-dependent nuclear export of the iron-responsive transcription factor Aft1p in *Saccharomyces cerevisiae*, Mol. Biol. Cell 18 (2007) 2980–2990.
[43] J. Towpik, D. Graczyk, A. Gajda, O. Lefebvre, M. Boguta, Derepression of RNA polymerase III transcription by phosphorylation and nuclear export of its negative regulator, Maf1, J. Biol. Chem. 283 (2008) 17168–17174.
[44] A. Takano, T. Endo, T. Yoshihisa, tRNA actively shuttles between the nucleus and cytosol in yeast, Science 309 (2005) 140–142.
[45] S. Shibata, M. Sasaki, T. Miki, A. Shimamoto, Y. Furuichi, J. Katahira, Y. Yoneda, Exportin-5 orthologues are functionally divergent among species, Nucleic Acids Res. 34 (2006) 4711–4721.
[46] B. Martinez-Bono, I. Quilis, E. Zalve, J.C. Igual, Yeast karyopherins Kap123 and Kap95 are related to the function of the cell integrity pathway, FEMS Yeast Res 10 (2010) 28–37.
[47] S.L. Jaspersen, J.F. Charles, R.L. Tinker-Kulberg, D.O. Morgan, A late mitotic regulatory network controlling cyclin destruction in *Saccharomyces cerevisiae*, Mol. Biol. Cell 9 (1998) 2803–2817.
[48] P. Uetz, L. Giot, G. Cagney, T.A. Mansfield, R.S. Judson, J.R. Knight, D. Lockshon, V. Narayan, M. Srinivasan, P. Pochart, A. Qureshi-Emili, Y. Li, B. Godwin, D. Conover, T. Kalbfleisch, G. Vijayadamodar, M. Yang, M. Johnston, S. Fields, J.M. Rothberg, A comprehensive analysis of protein–protein interactions in *Saccharomyces cerevisiae*, Nature 403 (2000) 623–627.
[49] S. Irniger, S. Piatti, C. Michaelis, K. Nasmyth, Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast, Cell 81 (1995) 269–278.
[50] T. Aviv, Z. Lin, S. Lau, L.M. Rendl, F. Sicheri, C.A. Smibert, The RNA-binding SAM domain of Smaug defines a new family of post-transcriptional regulators, Nat. Struct. Biol. 10 (2003) 614–621.
[51] M. Stewart, Ratcheting mRNA out of the nucleus, Mol. Cell 25 (2007) 327–330.