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# Spatial regulation of the start repressor Whi5

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**Keywords:** start, cell cycle, β-karyopherin, Whi5, Kap95, Msn5, *S. cerevisiae*

The *Saccharomyces cerevisiae* Start repressor Whi5, the functional analogue of mammalian pRB, shuttles between the nucleus and the cytoplasm throughout the cell cycle: enters into the nucleus at the end of mitosis and remains nuclear until Start. We studied the mechanisms involved in this spatial regulation. The nuclear import depends on the β-karyopherins of the classical import pathway Kap95 and Cse1. Whi5 contains a monopartite and a bipartite classical NLS localized in its N-terminal region which are functionally redundant. A fragment of Whi5 containing these NLSs is able to constitutively accumulate a $GFP_4$ protein inside the nucleus throughout the cell cycle, which suggests that the Whi5 nuclear import is not cell cycle-regulated. The nuclear export of Whi5 is assisted by β-karyopherin Msn5. A two-hybrid assay indicates a physical interaction between Whi5 and Msn5. We identified a fragment of Whi5 with export activity from amino acids 51 to 167. Interestingly, this region drives the export of a chimeric nuclear protein in a cell cycle-regulated pattern similarly to that observed for Whi5. Moreover, the nuclear export driven by $Whi5^{51-167}$ depends on the phosphorylation of specific Ser residues. Finally, we identified Cdc14 as the phosphatase required for the nuclear accumulation of Whi5.

## Introduction

The irreversible commitment to a new cell cycle in budding yeast and metazoan cells is a finely regulated process that occurs at the end of the $G_1$ phase. Before a new cell cycle begins, *Saccharomyces cerevisiae* cells analyze external and internal conditions such as nutrients availability, presence of pheromones, DNA integrity or cell size. If cues are appropriate, cells commit to a new cell cycle by executing Start.[1,2] Start implies the coordinated transcription of a large subset of genes,[3-5] which codify for the regulators and components of the cellular machinery required for subsequent events in the cell cycle. As a result of this expression, DNA duplication, budding and spindle pole body duplication are triggered.

The periodic transcription at Start depends on two transcription factors: SBF and MBF.[3,6,7] SBF is a heterodymeric complex composed of the regulatory subunit Swi6 and the DNA binding protein Swi4. MBF comprises Swi6 and a distinct DNA binding protein, Mbp1. The genes depending on SBF are mainly implied in processes such as morphogenesis and spindle pole body duplication, and include the *CLN1* and *CLN2* cyclin genes. Most MBF targets are involved in the control and execution of DNA duplication and repair, and include the *CLB5* and *CLB6* cyclin genes. However, many genes respond to both factors since there is a clear functional redundancy between them.[8]

Although SBF and MBF are recruited to target promoters at the beginning of $G_1$, they are transcriptionally inactive.[9,10] This is due to the inhibitory action of the Whi5 repressor, although whether Whi5 affects MBF is still a controversial matter.[11,12] The activation of Start transcription depends on the cyclin dependent kinase Cdc28-Cln3.[13,14] The Cdc28-Cln3 complex is associated with the endoplasmic reticulum membrane by a mechanism that involves Whi3 and the chaperone Ssa1. In late $G_1$, the chaperone Ydj1 releases the complex to allow its entrance to the nucleus, a mechanism that links growth and the cell cycle.[15,16] Once inside the nucleus, Cdc28-Cln3 phosphorylates Whi5, SBF and MBF and promotes the dissociation of Whi5 from the transcription factors, hence activating transcription.[11,12] As a result of this transcriptional wave, kinases Cdc28-Cln1 and Cdc28-Cln2 accumulate and act on Whi5, SBF and MBF to establish a positive feedback loop that gives coherence to the $G_1$-S transition.[17] The subsequent inactivation of this transcriptional wave later in the cell cycle is differentially regulated. While SBF is inactivated by phosphorylation by Ccd28-Clb2 kinase,[18] the association of Nmr1 represses the MBF expression.[19]

The activity of SBF and MBF is regulated at additional levels. Besides the regulation by CDKs, SBF is also regulated by the DNA checkpoint kinase Rad53,[20] and the cell wall integrity kinase Slt2.[21-23] The expression of the *SWI4* and *WHI5* genes is cell cycle-regulated and shows a peak just before Start or at the S phase, respectively.[24,25] Moreover, Swi6 and Whi5 subcellular localization is regulated through the cell cycle. Swi6 is nuclear from the end of mitosis until the S-$G_2$ phases.[26-28] The import of Swi6 into the nucleus is assisted by the classical import pathway,[29] whereas the export out of the nucleus is mediated by karyopherin Msn5,[27] (see below). Changes in localization are basically due to the cell cycle regulation of the Swi6 nuclear import: phosphorylation of Ser160 by Cdc28-Clb6 block nuclear import during the S-$G_2$ phases until the end of mitosis when the Cdc14 phosphatase removes the phosphate group to once again allow the nuclear accumulation of the protein.[26,28] With regard to Whi5, is nuclear from the end of mitosis until Start, and localization is also apparently regulated by phosphorylation since some mutant

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proteins in multiple phosphorylation sites accumulates in the mucleus.[11,30]

The active transport of proteins through the nuclear pore complex is assisted by the soluble transport receptors of the β-karyopherin family that recognize specific features, the nuclear localization signals (NLS) or nuclear export signals (NES), in their cargoes (reviewed in refs. 31–37). Receptors are divided into importins and exportins depending on the transport direction. In *Saccharomyces cerevisiae,* there are 14 members of the β-karyopherin family receptors. Two pathways are relevant for this work: the classical import pathway and Msn5. The former involves the import of proteins carrying monopartite (a short cluster of basic amino acids) or bipartite (two short clusters of basic amino 10–12 residues apart) classical NLS[38] by the heterodymer composed of Kap95 (importin β1) and Kap60 (importin α).[39-41] Cse1 is another β-karyopherin involved in the classical import pathway as it is necessary for the recycling of Kap60 to the cytoplasm once the complex Kap95-Kap60-cargo has been dissociated in the nucleus.[42,43] The other relevant β-karyopherin for this work is Msn5. Msn5 is involved in the nuclear export of several proteins, among them cell cycle regulators like Swi6,[27] Cdh1,[44] Far1,[45] and HO endonuclease.[46] The minimal NES identified in some of the Msn5 cargoes often involves long protein regions, and their export activity requires the phosphorylation of critical residues.[44,46-50]

In the interest of explaining the molecular mechanism underlying the changes in the subcellular localization of repressor Whi5, we performed an exhaustive study of Whi5 spatial regulation. The nuclear import is directed by two functional redundant classical NLS and the classical nuclear import pathway, whereas the nuclear export depends on karyopherin Msn5. We characterized a region in Whi5 with phosphorylation-regulated NES activity and a role of Cdc14 phosphatase in the spatial regulation of Whi5.

## Results

**Delimiting regions of Whi5 controlling its subcellular localization.** A first approach in the study of the spatial regulation of Whi5 was conducted to identify the regions of the protein involved in its nuclear import and export. Therefore, the subcellular localization of chimeric proteins constituted by different fragments from Whi5 fused to four copies of the green fluorescence protein ($GFP_4$), to avoid the free diffusion through the nuclear pore complex, was investigated. The fusion of $GFP_4$ does not alter the spatial regulation of Whi5 since the Whi5-$GFP_4$ protein behaves as wild-type Whi5: the protein enters the nucleus at the end of mitosis and remains nuclear during $G_1$ until it is relocated to the cytoplasm after Start (**Fig. 1**). The chimeric protein composed of amino acids 1 to 100 of Whi5 and of four copies of GFP ($Whi5^{1-100}$-$GFP_4$) showed a nuclear localization throughout the cell cycle. Conversely, the fragments 101–200, 201–295 and 101–295 of Whi5 did not change the cytosolic localization of the control $GFP_4$. These results indicate that the 1–100 region of Whi5 is necessary and sufficient to mediate the nuclear import of Whi5. Moreover, the fact that $Whi5^{1-100}$ is located in the nucleus in all the cell cycle stages strongly suggests that the nuclear import of Whi5 is not cell cycle-regulated.

The constitutive nuclear localization of $Whi5^{1-100}$-$GFP_4$ indicates that additional sequences of Whi5 are required to confer cell cycle-regulated subcellular localization. Therefore, we tested the effect of a larger Whi5 fragment encompassing amino acids 1 to 200. Importantly, the $Whi5^{1-200}$-$GFP_4$ protein showed an identical localization pattern to the wild-type protein. Thus unlike the $Whi5^{1-100}$ fragment, $Whi5^{1-200}$ is able to not only enter the nucleus, but to also relocate the cytoplasm after Start, which suggests that the sequences between 100 and 200 are important for protein nuclear export.

**The nuclear import of Whi5 is mediated by the classical nuclear import pathway.** Having identified the region of Whi5 required for the nuclear import, we planned to characterize in detail the import process by identifying both the karyopherin involved and the nuclear localization signals present in the 1–100 region of the protein. In order to identify the mediator of the Whi5 nuclear import, the subcellular localization of $Whi5^{1-100}$-$GFP_4$ was analyzed in the mutant strains in the 14 β-karyopherins. The protein was localized in the nucleus in all the mutant cells except *kap95* and *cse1*, in which the GFP signal was distributed through the cytoplasm (data not shown). This result indicates that the classical import pathway is responsible for the nuclear import of $Whi5^{1-100}$-$GFP_4$. To clarify whether this was also the case for the whole Whi5 protein, distribution of Whi5-$GFP_4$ in the *tetO$_7$:KAP95* strain was analyzed in either the absence or the presence of doxycyclin. When the *KAP95* gene is inactivated, approximately the 80% of cells are arrested in $G_2$/M phase and the 20% in $G_1$ phase. Whi5 is usually nuclear in $G_1$ phase. However, no nuclear signal was observed in $G_1$ cells in the *kap95* mutant strain (**Fig. 2**), thus confirming that the import of Whi5 into the nucleus is mediated by karyopherin Kap95.

The import of proteins by Kap95 relies on the presence of classical NLS in the target proteins. These signals are constituted by a stretch of basic residues or two clusters of basic residues separated by 10–12 amino acids. Analysis of the Whi5 sequence identified two putative NLS: one (referred to as NLS1) between amino acids 6 and 10 is a relaxed version of a monopartite classical NLS, whereas a potential bipartite NLS (referred to as NLS2) was located between residues 72 to 95. In order to test whether these putative NLS are in fact functional NLS, the basic residues in both sequences were altered as indicated in **Figure 3A**, and the subcellular localization of the resulting $Whi5^{NLS1i}$-$GFP_4$ and $Whi5^{NLS2i}$-$GFP_4$ proteins was analyzed. In both cases, inactivation of the putative NLS did not significantly change the location of Whi5, which is clearly detected in the nucleus of cells from telophase to $G_1$/S (**Fig. 3B**). Thus, none of them is essential for the nuclear import of Whi5. However, it is important to note that when the both putative NLS were simultaneously mutated ($Whi5^{NLS1i,2i}$-$GFP_4$), nuclear localization was avoided and the protein was mainly located in the cytoplasm. These results demonstrate that Whi5 contains two functional and redundant NLS, which are able to mediate the nuclear import of Whi5.

**The nuclear export of Whi5 is mediated by karyopherin Msn5.** The cytosolic location of Whi5 observed in the different

stages of the cell cycle suggests the existence of a mechanism that restricts the nuclear accumulation of Whi5 at some specific periods of the cell cycle. As mentioned above, the fact that Whi5$^{1\text{-}100}$-GFP$_4$ is constitutively nuclear throughout the cell cycle suggests that the nuclear import of Whi5 is not cell cycle-regulated. Therefore, the most plausible explanation is the existence of an export mechanism responsible for relocating Whi5 to the cytoplasm during the S, G$_2$ and M phases. To confirm this hypothesis, we investigated the subcellular localization of Whi5$^{1\text{-}200}$-GFP$_4$ (the minimum region of Whi5 showing a wild-type pattern of localization) in the mutant strains in β-karyopherins. Inactivation of any of the β-karyopherins did not alter the cell cycle-regulated nuclear localization of Whi5 except for the inactivation of Msn5, which led to the nuclear localization of Whi5 in all the cell cycle stages, including cells in the S, G$_2$ and M phases (**Fig. 4**). It is noteworthy to note that the constitutive nuclear localization of Whi5 in the *msn5* mutant cells was also observed when a Whi5 protein was expressed at the endogenous level from its own promoter. In short, the changes in the localization of Whi5 throughout the cell cycle are due to nuclear export dependent on karyopherin Msn5.

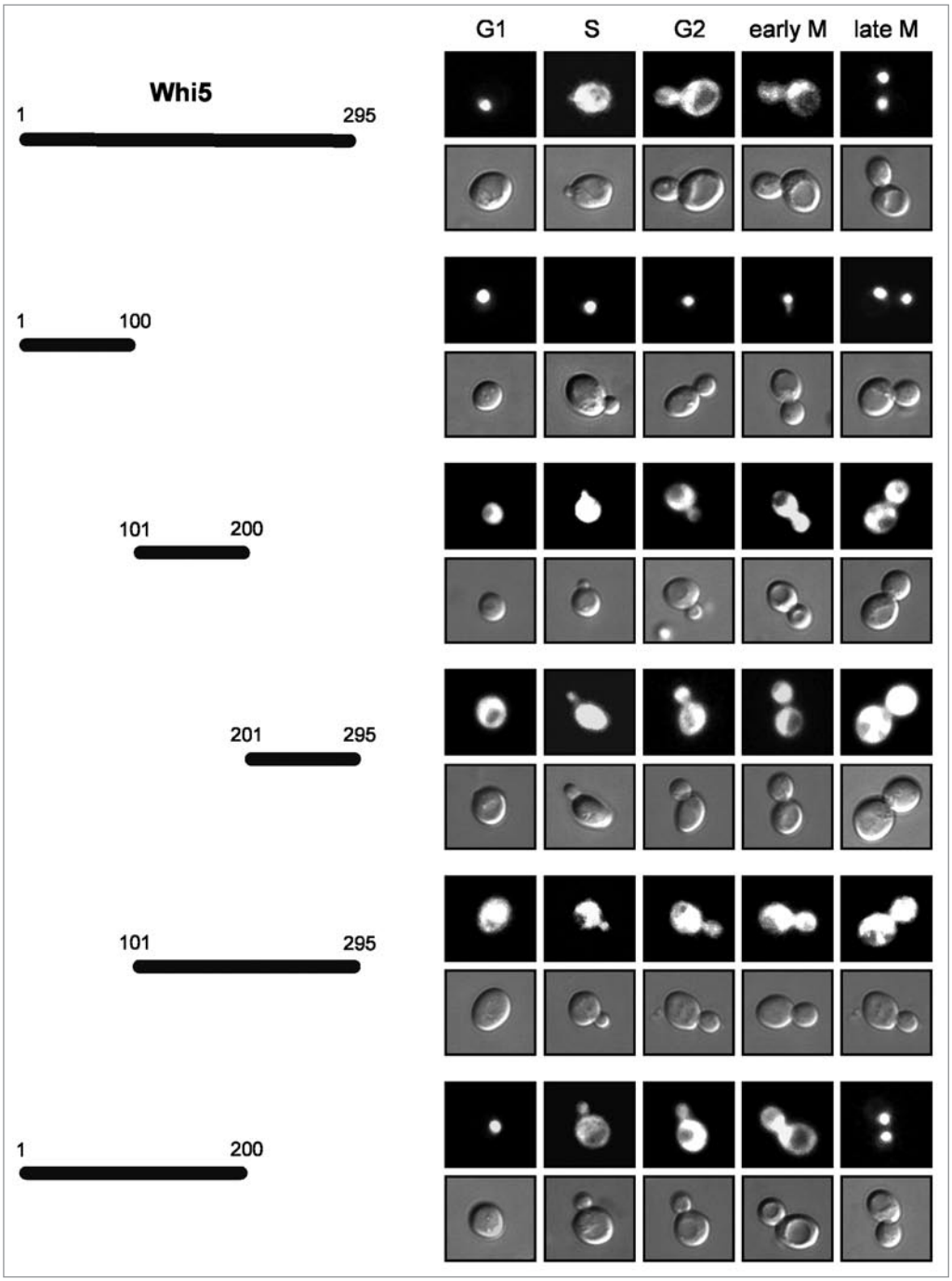


**Figure 1.** Identification of regions of Whi5 involved in the control of its subcellular localization. Exponentially growing cells of the wild type strain (BY4741) transformed with plasmids pWHI5-GFP$_4$, pWHI5$^{1\text{-}100}$-GFP$_4$, pWHI5$^{101\text{-}200}$-GFP$_4$, pWHI5$^{201\text{-}295}$-GFP$_4$, pWHI5$^{101\text{-}295}$-GFP$_4$ and pWHI5$^{1\text{-}200}$-GFP$_4$, were analyzed by fluorescence microscopy. GFP signal and DIC images for a collection of cells at the different cell cycle stages are shown.

Our results strongly suggest that Msn5 is the exportin of Whi5. To unequivocally confirm this, we tested a physical interaction between both proteins in vivo by a two-hybrid assay. The designed strategy involved the expression of the Whi5 protein fused to the Gal4 activation domain (GAD) in the presence of Msn5 fused to the Gal4 DNA-binding domain (BD), and the analysis of expression of a *GAL7:HIS3* gene. As shown in **Figure 4B**, only those cells containing both the Whi5-GAD and Msn5-BD proteins were able to grow in the absence of histidine. This result demonstrates that Msn5 interacts with Whi5 in vivo, so the requirement of Msn5 in the export of Whi5 is not an indirect effect; rather Whi5 is a direct cargo of karyopherin Msn5.

**Whi5 spatial regulation is independent of its association to SBF.** Whi5 binds to SBF on the target promoter to repress expression until phosphorylation by Cln-Cdc28 dissociates it from the complex, coincident with its nuclear export.[11] We wonder whether the spatial regulation of Whi5 could be linked to this functional

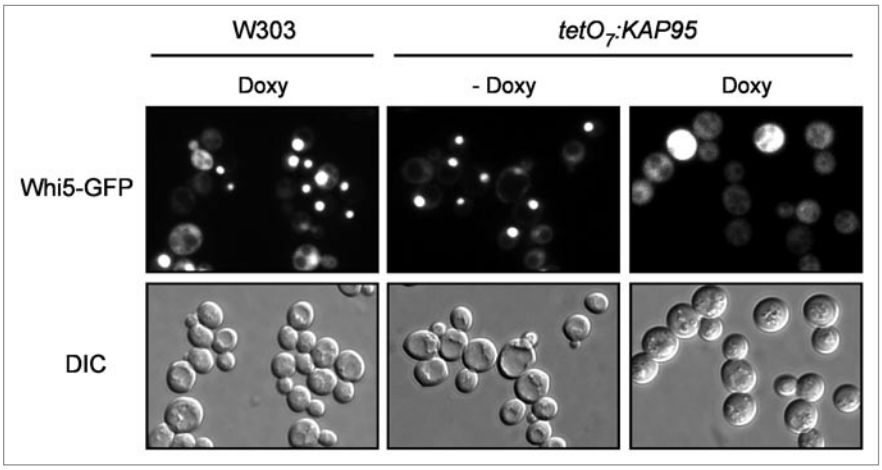


**Figure 2.** Subcellular localization of Whi5 in the *kap95* mutant strain. Exponentially growing cells of the wild type strain (W301-1a) and the *tetO$_7$:KAP95* mutant strain (JCY0635) transformed with the plasmid pWHI5-GFP$_4$, were incubated in the presence of 5 μg/mL doxycycline for 8 hours. GFP signal and DIC images are shown.

cycle (even more considering that Swi6 nuclear export is also mediated by Msn5[27]) or, on the contrary, could be independent of its association to SBF. To answer this question, the localization of Whi5 was analyzed in mutant cells in the *SWI4* or the *SWI6* gene. In both cases, cells showed the cell cycle regulated localization pattern characteristic of Whi5 (**Fig. 4C**). It is known that the inactivation of either Swi4 or Swi6 abolishes the interaction between Whi5 and the remaining SBF component,[11,12] so our result indicates that Whi5 spatial regulation is no dependent on its binding to SBF components.

**Identification of a minimum NES in Whi5.** In order to characterize the nuclear export mechanism of Whi5 in more detail, we aim to further delimit the region of Whi5 which is able to drive the Msn5-mediated nuclear export. A functional analysis of NES activity was carried out by the addition of different Whi5 fragments to a nuclear chimeric protein composed of four copies of GFP and the NLS from SV40 (NLS$^{SV40}$-GFP$_4$). A good candidate for a minimal NES is the region between 101 and 200, a region that, as described above, is necessary for the nuclear export of Whi5. However, protein NLS$^{SV40}$-Whi5$^{101-200}$-GFP$_4$ was localized in the nucleus throughout the cell cycle (**Fig. 5**), which suggests that although the fragment 101–200 of Whi5 is necessary, it is unable to mediate the nuclear export of the protein and that additional regions of Whi5 must be involved. Therefore, the nuclear export activity of a larger Whi5 fragment from 51 to 200 was investigated. In this case, protein NLS$^{SV40}$-Whi5$^{51-200}$-GFP$_4$ showed an altered localization pattern in comparison to the NLS$^{SV40}$-GFP$_4$ control. In particular, the protein was now mostly relocated in the cytoplasm in the cells from G$_1$/S to the end of mitosis (**Fig. 5**). A similar result was observed when a smaller fragment encompassing amino acids 51 to 167 was used, although the nuclear exclusion of this construct seemed slightly less efficient. Thus, the region 51–167 is able to drive the nuclear export of the NLS$^{SV40}$-GFP$_4$ chimeric protein, and more importantly, in a cell cycle-regulated manner, similarly to what occurs with Whi5. Moreover, the nuclear export of NLS$^{SV40}$-Whi5$^{51-167}$-GFP$_4$ depends on karyopherin Msn5 since the protein remains constitutively nuclear in the absence of this karyopherin (**Fig. 5**). In conclusion, the region 51–167 of Whi5 displays a cell cycle-regulated Msn5-dependent NES activity.

**NES activity of the Whi5$^{51-167}$ fragment depends on the phosphorylation of Ser$^{154}$, Ser$^{156}$ and Ser$^{161}$.** The nuclear export by Msn5 is often stimulated by the phosphorylation of target proteins.[44,46-50] Whi5 is a phosphorylated protein whose phosphorylation pattern changes during the cell cycle.[12,30] The hyperphosphorylated forms of Whi5 accumulate in G$_1$/S, which is coincident with nuclear export. In addition, a mutant version of Whi5 in which 6 Ser/Thr residues have been substituted by Ala (Whi5$^{6A}$), shows a constitutive nuclear localization throughout the cell cycle.[11] These observations suggest that the nuclear export by Msn5 could be regulated by the phosphorylation state of some residues of Whi5. Therefore, we investigated the role of phosphorylation in the NES activity of fragment 51–167. In this region, there are 8 putative CDK phosphorylation sites. Three of them (Ser$^{154}$, Ser$^{156}$ and Ser$^{161}$) are included in the nuclear Whi5$^{6A}$ variant and in the Whi5$^{7A}$ variant that show a defective Start transition,[12] which makes them good candidates to control NES activity. To test this possibility, the Ser$^{154}$, Ser$^{156}$ and Ser$^{161}$ were changed to alanine in the NLS$^{SV40}$-Whi5$^{51-167}$-GFP$_4$ protein. As described above, this protein shows cell cycle-regulated localization. However, the mutation of these Ser residues brought about a change in the localization and the protein remained nuclear throughout the cell cycle (**Fig. 5**). Thus, the mutation of these Ser residues caused the inactivation of the NES function of fragment Whi5$^{51-167}$, which strongly suggests that the phosphorylation of Ser$^{154}$, Ser$^{156}$ and/or Ser$^{161}$ is important for the nuclear export of Whi5 by Msn5.

**Cdc14 phosphatase controls localization of Whi5.** Given that the Whi5 nuclear export requires protein phosphorylation, we could expect the reaccumulation of Whi5 inside the nucleus at the end of mitosis to imply the dephosphorylation of critical residues. Cdc14 phosphatase was activated at the end of mitosis.[51] The temporal coincidence with the Whi5 nuclear entry led us to evaluate whether Cdc14 could be involved in the spatial regulation of Whi5, and more specifically in the nuclear accumulation of Whi5. To test this possibility, we analyzed the effect of the activation of Cdc14 on Whi5 localization in S phase cells, a cell cycle stage at which Whi5 shows cytosolic localization. Activation of Cdc14 in the S phase, a stage of the cell cycle when Cdc14 is normally inactive, was accomplished through the overexpression of Cdc14 from the *GAL1* promoter. Thus, cells expressing Whi5-GFP$_4$ were arrested in the S phase by incubation in the presence of hydroxyurea. Then Cdc14 activity was induced by the addition of galactose. As it can be seen in **Figure 6**, Whi5 was located in the cytoplasm when HU-arrested cells were maintained in raffinose (or glucose), as expected for S phase cells. However,

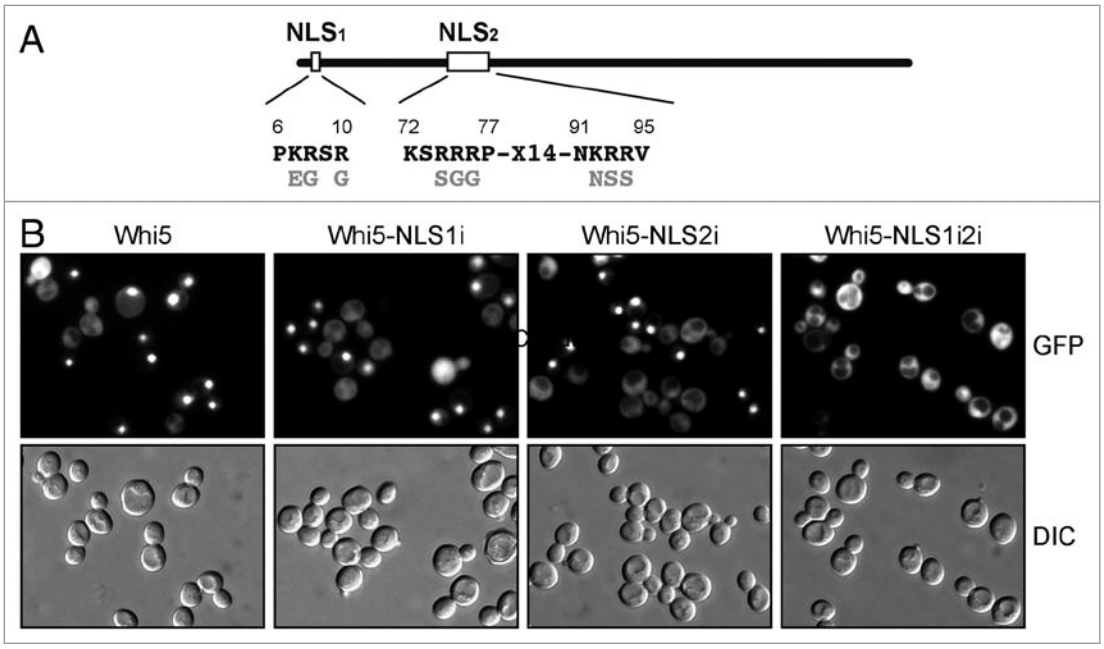


**Figure 3.** Analysis of Whi5 NLS sequences. (A) Putative NLS sequences in the Whi5 protein are indicated. Amino acids substitutions introduced to inactivate the NLSs are shown in gray. (B) Exponentially growing cells of the wild type strain (BY4741) transformed with the plasmid pWHI5-GFP$_4$, pWHI5$^{NLS1i}$-GFP$_4$, pWHI5$^{NLS2i}$-GFP$_4$ or pWHI5$^{NLS1i2i}$-GFP$_4$ were analyzed by fluorescence microscopy. GFP signal and DIC images are shown.

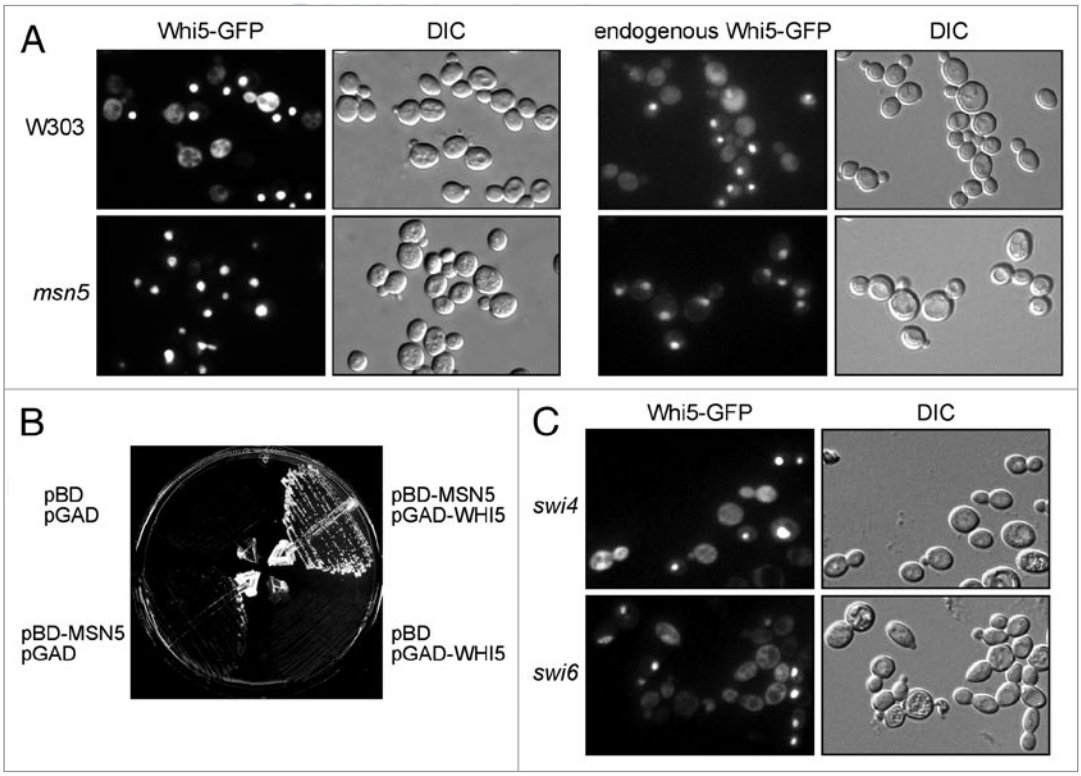


**Figure 4.** Analysis of Whi5 nuclear export by karyopherin Msn5. (A) Exponentially growing cells of the wild type (W303-1a) and the *msn5* mutant (JCY1018) strains transformed with the pWHI5-GFP$_4$ plasmid and of the wild type (JCY1277) and *msn5* mutant (JCY1451) strains carrying a GFP tagging at the *WHI5* genomic locus were analyzed by fluorescence microscopy. GFP signal and DIC images are shown. (B) The two-hybrid strain PJ69-4A transformed with plasmids expressing Whi5 fused to the Gal4 activation domain (pGAD-WHI5), Msn5 fused to the Gal4 DNA-binding domain (pBD-MSN5) and the corresponding empty vector (pGAD and pBD) as indicated, were streaked onto SD-HIS plates and incubated at 28°C for three days. (C) Exponentially growing cells of the *swi4* (BY604) and *swi6* (BY600) mutant strains transformed with the pWHI5-GFP$_4$ plasmid were analyzed by fluorescence microscopy. GFP signal and DIC images are shown.

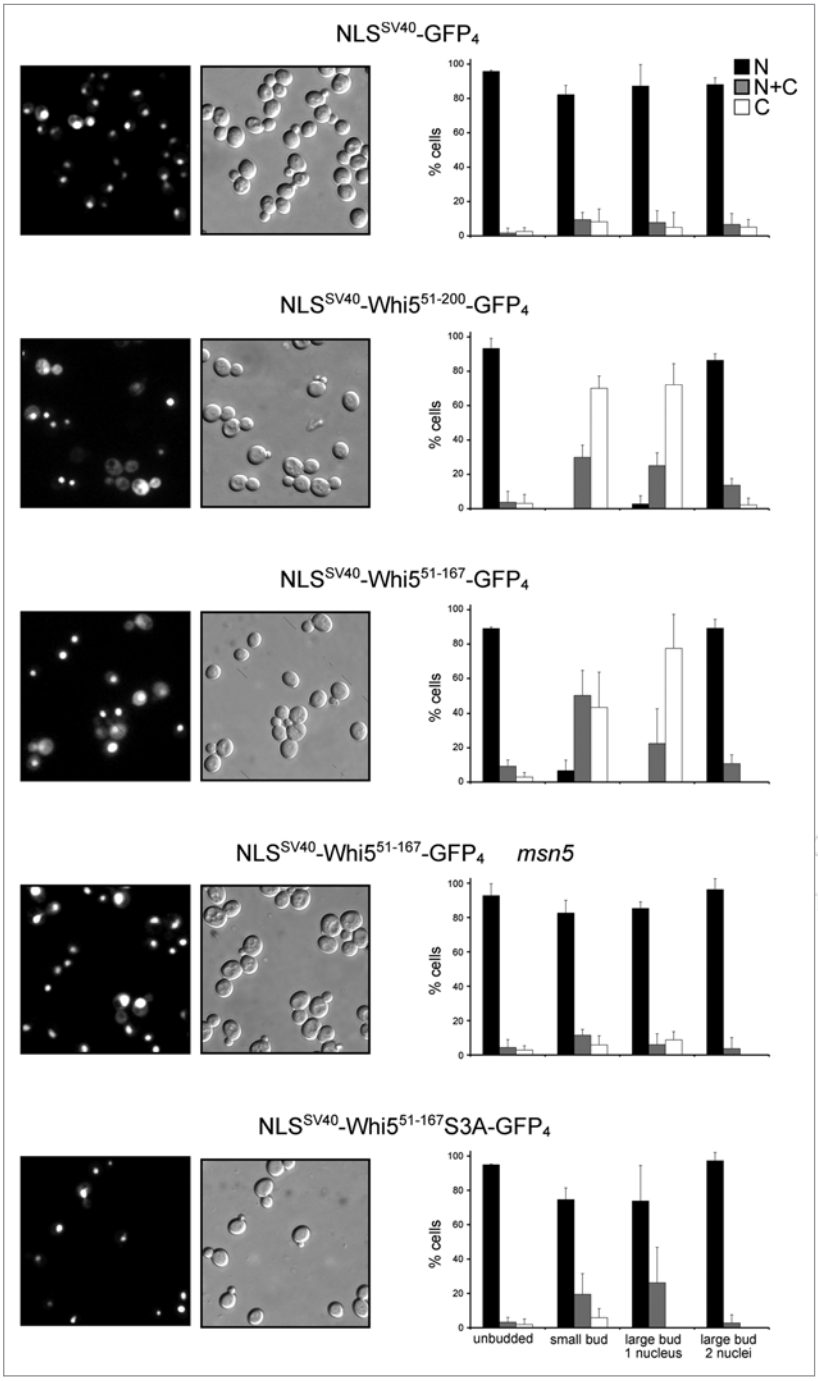


**Figure 5.** Characterization of a Whi5 fragment with NES activity. Exponentially growing cells of the wild type strain (BY4741) transformed with plasmids pNLS$^{SV40}$-GFP$_4$, pNLS$^{SV40}$-WHI5$^{51-200}$-GFP$_4$, pNLS$^{SV40}$-WHI5$^{51-167}$-GFP$_4$ or pNLS$^{SV40}$-WHI5$^{51-167}$S3A-GFP$_4$ and cells of the *msn5* mutant strain (JCY855) transformed with plasmid pNLS$^{SV40}$-WHI5$^{51-167}$-GFP$_4$ were analyzed by fluorescence microscopy. GFP signal and DIC images are shown. Graphs show protein localization at different cell cycle stages (N: fluorescence signal only in the nucleus; N + C fluorescence signal in nucleus and cytoplasm; C: fluorescence signal in cytoplasm).

Whi5 concentrated in the nucleus when HU-arrested cells were treated with galactose. This change in localization was not due to the change in the carbon source, but to the expression of Cdc14. In conclusion, this result clearly indicates that the activation of Cdc14 causes the nuclear accumulation of Whi5, which strongly supports that the reentry of Whi5 in the nucleus at the end of mitosis is mediated by Cdc14 phosphatase.

## Discussion

Spatial regulation adds a new step to the control of the protein function. With regard to transcription factors, this spatial regulation necessarily implies the existence of a nuclear import mechanism and, in some cases, the existence of an export mechanism that could contribute to the control of transcription factor activity. It is known that Whi5, the yeast analogue to mammalian pRB, shows a dynamic localization during the cell cycle: is mostly nuclear from the end of mitosis until the $G_1$/S transition, whereas it accumulates in the cytoplasm during the rest of the cell cycle.[11] We have characterized the molecular basis of this regulation. The nuclear import of Whi5 was mediated by the classical nuclear import pathway, which involves the Kap95 and Cse1 β-karyopherins. It depends on two redundant NLS signals located in the N-terminal region of Whi5 between 6–10 and 72–95. Whi5 activity is required for the correct timing of Start[11,12] and, given that it functions in the nucleus, the nuclear import of Whi5 is expected to be critical for the timely execution of Start. The existence of more than one NLS could reflect the importance of, and confer robustness to, the nuclear import process.

We have also characterized an export mechanism for Whi5. Exit to the cytoplasm was mediated by karyopherin Msn5, which is in agreement with recently published results describing that Whi5 overexpressed under the control of the *GAL1* promoter accumulates inside the nucleus in an *msn5* mutant strain.[30] We have observed that Msn5 controls Whi5 localization under endogenous conditions, thus ruling out a possible artifact due to overexpression. Moreover, we have described a physical interaction between Msn5 and Whi5, which supports a direct role of Msn5 as the exportin of Whi5. The characteristics of the export mechanism by Msn5 resemble those described for other proteins. In several Msn5 cargoes, the characterized NES are long regions of proteins of 90–120 amino acids without a sequence homology.[45,47,48] This is similar to the case of Whi5 described herein since we have shown that a region of 116 amino acids, from 51 to 167, is sufficient to mediate the nuclear export of a chimeric nuclear protein. Recently however, the binding of a short stretch of 13 amino acids from HO by Msn5 was described.[52] No homolog sequences are present in either the 51–167 fragment of Whi5 or in the rest of the protein. The structural basis

of the cargo recognition by Msn5 remains poorly known, but a common trait in the Msn5-mediated export is the phosphorylation of the cargo proteins since it has been described in the nuclear export of Pho4,[49] Crz1,[47] Mig1,[48] Aft1,[50] HO[46] and Maf1.[53] In the case of Whi5, the phosphorylation state of the protein changes in parallel to changes in the localization.[11,12] Nuclear localization of Whi5 correlates to a low CDK activity. Moreover, the mutation of 6 CDK phosphorylation sites in Whi5 renders the protein nuclear,[11] which is consistent with the requirement of phosphorylation for the nuclear export. This we have confirmed in the export activity mediated by the Whi5 fragment from 51 to 167. We have identified specific Ser residues whose substitution by Ala block nuclear export. This strongly supports that the phosphorylation of Ser 154, 156 and/or 161 is an important requirement for the export activity in the Whi5$^{51\text{-}167}$ fragment. However, other additional sequences in Whi5 must contribute to the nuclear export given the fact that the Ser 154, 156 and 161 change to Ala in the whole protein does not hinder the export of the protein (data not shown). The phosphorylation of other Ser/Thr residues outside the fragment 51–167 is expected to support the nuclear export of Whi5. In fact, Wagner et al.[30] showed recently that the mutation of Ser 262 in combination with the mutation of Ser 154, 156, 161 causes an important defect in Whi5 nuclear export and that other phosphorylation sites apart from these four Ser could contribute, to a lesser strength, to the spatial regulation of Whi5.

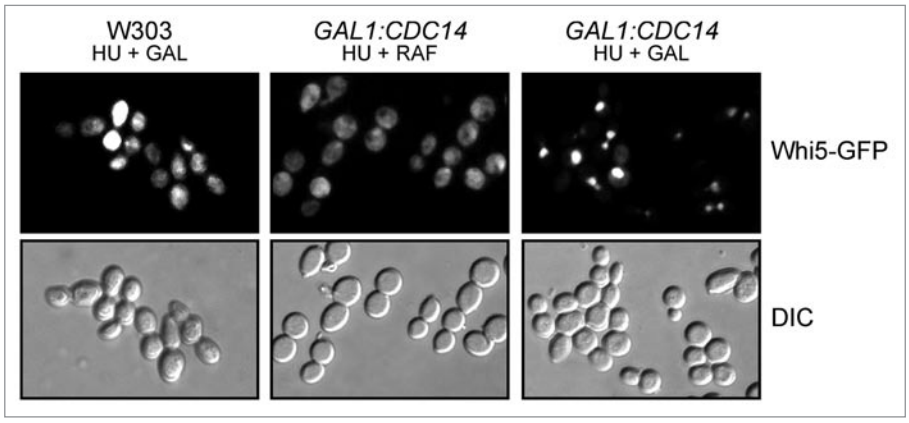


**Figure 6.** Effect of Cdc14 phosphatase activation on the subcellular localization of Whi5. Exponentially growing cells on raffinose of the wild type (W303-1a) and the *GAL1:CDC14* (JCY1205) strains transformed with the pWHI5-GFP$_4$ plasmid were arrested in the S phase by incubation in the presence of 0.2 M hidroxyurea (HU). After two hours, galactose to a final concentration of 2% was added to one half of the culture to induce overexpression of phosphatase Cdc14. Cells were incubated for four hours before Whi5 localization was monitored by fluorescence microscopy. GFP signal and DIC images are shown.

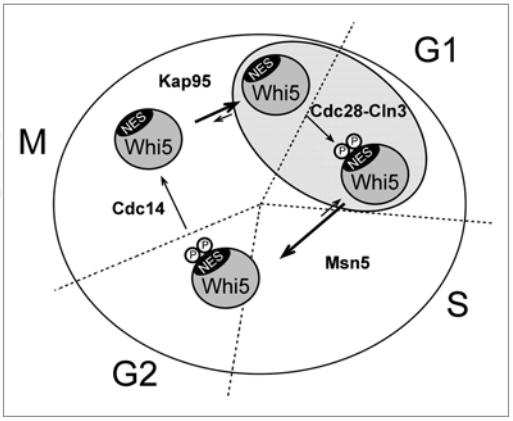


**Figure 7.** Model of the Whi5 spatial regulation.

Cell cycle changes in the nuclear/cytosolic localization of proteins can be achieved by basically controlling their nuclear import and/or their nuclear export in specific cell cycle stages. Whi5 is nuclear only in $G_1$, and this could reflect that the Whi5 nuclear import is only active in this period of the cell cycle. However, the fact that the fragment 1–100 of Whi5 containing the two NLS drives GFP into the nucleus in all the cell cycle stages argues against this possibility and strongly suggests that the Whi5 nuclear import is not cell cycle-regulated. Reinforcing this idea, Whi5 is nuclear in all the cell cycle phases in the *msn5* mutant strain. Therefore, changes in localization must be due to a cell cycle-regulated nuclear export. This is in fact the case because the fragment 51–167 of Whi5 is able to confer a cell cycle-regulated localization to the constitutive nuclear protein NLS$^{SV40}$-GFP$_4$ in a pattern which is identical to that observed for Whi5. The cell cycle regulation of the Whi5 export is due to the different temporal pattern of the activities involved in the phosphorylation of Whi5. Whi5 is phosphorylated by CDKs activities, especially by Cln3-Cdc28 and Cln1,2-Cdc28 in Start, which activate the export of Whi5. We characterized Cdc14, the phosphatase activated at the end of mitosis, as the factor responsible for deactivating Whi5 export activity.

It is a remarkable fact that Whi5 uses the same import and export pathways as Swi6,[27,29] and that Cdc14 phosphatase takes part in their spatial regulation.[26] The fact that transcriptional activators Swi6 and Swi4 (our unpublished observation) and their repressor Whi5 use the same pathways could be advantageous for the correct organization of the transcriptional system at $G_1$-S. This is especially relevant in the nuclear import process in which the concomitant import of activator and repressor could avoid the unscheduled expression of the target genes. However, differences in the spatial regulation of Whi5 and Swi6 exist. The nuclear import of Swi6, but not that of Whi5, is cell cycle-regulated.[28,29] In addition, even though both proteins are imported at the same time, they leave the nucleus at different times. Whi5 relocates to the cytoplasm at the end of $G_1$,[11] while Swi6 remains bound to the promoter of the target genes to activate transcription and leaves the nucleus later in the cell cycle.[26,27] We have observed how the transport of Whi5 is in fact independent of

Swi6 since the localization of Whi5 remains unaltered in a *swi6* mutant strain. Whi5 localization also remains unaltered in a mutant strain in Swi4, the other component of SBF. *swi6* and *swi4* mutations have been reported to abrogate the interaction of Whi5 with the remaining SBF component. Therefore, our results indicate that Whi5 transport is completely independent of its association with SBF.

The results obtained complete a model for Whi5 spatial regulation (**Fig. 7**). Whi5 enters the nucleus assisted by the classical import pathway, an import that is mediated by two redundant classical NLS located at the N-terminal part of the protein. Whi5 associates with SBF (and maybe with MBF) in the promoters of the target genes, restricting transcriptional activation until Start. Activation of the Cln3-Cdc28 kinase leads to the phosphorylation of Whi5 and to the dissociation from SBF, resulting in the activation of the gene expression. The phosphorylation of Whi5 in critical residues, among them Ser154, 156 and 161, also targets Whi5 for nuclear export by the Msn5 karyopherin. At the end of mitosis, the cytosolic localization of Whi5 will be reverted by the activation of Cdc14: the dephosphorylation of the critical residues will block the export mechanism to once again lead to the accumulation of the Whi5 protein inside the nucleus.

We still do not fully understand some aspects of the Whi5 functions. Inactivation of Whi5 is not the limiting step in the transcriptional activation at Start under certain conditions.[11,12] Recently, the *WHI5* gene has been reported to be periodically expressed with a peak in the S phase.[25] Nevertheless, the protein level seems to remain constant in the different cell cycle phases.[11] Besides, the role of phosphorylation in Whi5 activity is quite complex. Phosphorylation often controls the activity, localization or stability of proteins. Whi5 association with SBF is regulated by phosphorylation,[11,12] and the results provided herein and those of others[11,30] indicate the important function of the phosphorylation of specific residues in nuclear export. Strikingly however, the mutation of all the phosphorylation sites in Whi5 has no effect on the cell cycle.[30] The non-phosphorylation of Whi5 only has a deleterious effect for the cell cycle when combined with the expression of a mutant variant of Swi6, which suggests the existence of a supramolecular regulatory network. Finally, an open question remains, that of the functional relevance of the spatial regulation of Whi5. The fact that the localization of Whi5 is cell cycle-regulated suggests that the export of the repressor could be physiologically relevant for cells. It is conceivable that the nuclear accumulation of Whi5 could interfere with gene expresion, in fact the inactivation of Msn5 causes a defect in Start. However, the large-sized phenotype of the *msn5* mutant strain is not due to the nuclear accumulation of Whi5 since Msn5 inactivation also increases the cell size of *whi5* mutant cells (our unpublished results). So far, no defects have been reported for variants of Whi5 which are constitutively nuclear, for instance, Whi5$^{6A}$ and Whi5$^{12A}$.[11,30] Whether this behavior precludes a function for Whi5 spatial regulation or, alternatively, this function could be masked by the additional effect of Ser to Ala mutations in other aspects of Whi5 activity remains to be elucidated.

## Material and Methods

**Strains and growth conditions.** Yeast strains used are: BY4741 (*MATa leu2Δ0 his3Δ1 ura3Δ0 met15Δ0*), W303-1a (*MATa ade2-1 trp1-1 leu2-3,115 his3-11,15 ura3-52 can1-100*), BY600 (*MATα ade2-1 trp1-1 leu2-3,115 his3-11,15 ura3-52 can1-100 swi6::TRP1 ho-lacZ*), BY604 (*MATα ade2-1 trp1-1 leu2-3,115 his3-11,15 ura3-52 can1-100 swi4::LEU2 ho-lacZ*), JCY635 (*tetO$_7$:KAP95-kanMX6* in W303-1a), JCY664 (*tetO$_7$:CSE1-kanMX6* in W303-1a), JCY855 (*MATa leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 msn5::kanMX4*), JCY1018 (*msn5Δ3::HIS3* in W303-1a), JCY1205 (*ura3::URA3-GAL1:CDC14* in W303-1a), JCY1277 (*WHI5-GFP-TRP1* in W303-1a), JCY1451 (*msn5Δ3::HIS3* in JCY1277) and PJ69-4A (*Mata trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2:ADE2 LYS2::GAL1:HIS3 met2::GAL7:lacZ*). Strain containing the *msn5Δ3::HIS3* disruption cassettes was obtained by using plasmids p335-Δ3::HIS3 (from F. Estruch). The substitution of the *KAP95 or CSE1* promoter by the *tetO$_7$* promoter was obtained by integrating a DNA fragment amplified from plasmid pCM225 (from Dr. E. Herrero). JCY1205 was obtained by integrating a ApaI digested plasmid containing a *GAL1:CDC14* gene (from F. Uhlmann) at the *URA3* locus. Tagging of the Whi5 protein at the C-terminus with GFP was achieved by integrating a DNA fragment amplified from plasmid pFA6a-GFP(S65T)-TRP1 (from Dr. J.R. Pringle). Yeast cells were grown on standard yeast extract-peptone-dextrose (YPD) media or synthetic dextrose (SD) or raffinose (SR) minimal media supplemented as required. To repress the *tetO$_7$* promoter, doxycycline was added to a concentration of 5 μg/ml.

**Plasmids.** pNLS$^{SV40}$-GFP$_4$ plasmid derived from pNLS$^{SV40}$-GFP which contains, in order, the *ADH1* promoter, the SV40 NLS, one *GFP* copy and the *ADH1* terminator in YCplac33. This plasmid was constructed in a three-step process. First, *ADH1* promoter (-1473, +3) amplified from genome using a forward oligo containing an EcoRI restriction site and a reverse oligo containing a KpnI site was cloned in EcoRI-KpnI digested YCplac33. Next, the SV40 NLS and *GFP(S65T)* coding region without start and stop codons, amplified from pFA6a-GFP(S65T) using a forward oligo containing a KpnI restriction site, the SV40 NLS (PKKKRKV) coding sequence and a BamHI site and a reverse oligo containing a XbaI site, was cloned in frame by KpnI-XbaI digestion. Finally, 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. Three additional copies of the *GFP(S65T)* coding region without start and stop codons, amplified from pFA6a-GFP(S65T) using a forward oligo containing a SpeI site and a reverse oligo containing and XbaI and a SalI site and digested with SpeI and SalI, were sequentially cloned in frame in XbaI-SalI digested pNLS$^{SV40}$-GFP plasmid to render pNLS$^{SV40}$-GFP$_4$.

pWHI5-GFP$_4$, pWHI5$^{1-100}$-GFP$_4$, pWHI5$^{101-200}$-GFP$_4$, pWHI5$^{201-295}$-GFP$_4$, pWHI5$^{101-295}$-GFP$_4$, and pWHI5$^{1-200}$-GFP$_4$ plasmids were obtained removing the SV40 NLS coding region from pNLS$^{SV40}$-GFP$_4$ by KpnI-BamHI digestion and introducing the appropriate *WHI5* coding fragments amplified from genome

with forward oligos containing a KpnI restriction site and reverse oligos containing a BamHI site.

pWHI5$^{NLS1i}$-GFP$_4$ was obtained from pWHI5-GFP$_4$ by site directed mutagenesis of Whi5 NLS1 (6PKRSR10) to an inactive NLS version (PEGSG). pWHI5$^{NLS2i}$-GFP$_4$ was obtained from pWHI5-GFP$_4$ by site directed mutagenesis of Whi5 NLS2 (72KSRRRP-X$_{14}$-NKRRV95) to an inactive NLS version (KSSGGP-X$_{14}$-NNSSV). pWHI5$^{NLS1i2i}$-GFP$_4$ was obtained from pWHI5$^{NLS2i}$-GFP$_4$ by site directed mutagenesis of Whi5 NLS1 to the inactive NLS version.

pNLS$^{SV40}$-WHI5$^{101-200}$-GFP$_4$, pNLS$^{SV40}$-WHI5$^{51-200}$-GFP$_4$, pNLS$^{SV40}$-WHI5$^{51-167}$-GFP$_4$ plasmids were obtained by cloning in KpnI-BamHI digested pNLS$^{SV40}$-GFP$_4$ the appropriate *WHI5* coding fragments amplified from pWHI5$^{NLS2i}$-GFP$_4$ with forward oligos containing a KpnI restriction site and the SV40 NLS coding sequence and reverse oligos containing a BamHI site. Note that Whi5 NLS2 is inactivated in these constructs.

pNLS$^{SV40}$-WHI5$^{51-167}$S3A-GFP$_4$ was obtained from pNLS$^{SV40}$-WHI5$^{51-167}$-GFP$_4$ by site directed mutagenesis of Ser 154, 156 and 161 to Ala.

Plasmid pGAD-WHI5 used in the two-hybrid assay expressing Whi5 fused to the Gal4 activation domain was obtained by cloning the *WHI5* coding region amplified from genome with a forward oligo containing an EcoRI restriction site and a reverse oligo containing BamHI site, into EcoRI-BamHI digested pGAD-C1. pBD-MSN5 expressing Msn5 fused to the Gal4 DNA-binding domain was a gift from M. Johnston.

**Fluorescence microcospy.** To study the distribution of GFP tagged proteins, cells were grown in synthetic media and fluorescence distribution was analyzed in an *Axioskop 2* microscope (*Zeiss Inc.,*). Images were captured with a *SPOT* camera (*Diagnostic Instruments Inc.*,). Where indicated, localization of proteins was monitored by visual inspection. At least 150 cells from at least three independent experiments were scored as cells with fluorescence signal only in the nuclei (N), in the nuclei and cytoplasm (N + C) or only in the cytoplasm (C). Confirmation of GFP signal as a nuclear signal was achieved in control experiments by DAPI staining of absolute ethanol fixed cells.


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