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The budding yeast RasGEF Cdc25 reveals an unexpected nuclear localization

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ABSTRACT

The mechanisms regulating the activity of *Saccharomyces cerevisiae* Ras-GEF Cdc25 are still largely unknown. While the catalytical function of the C-terminal domain has been thoroughly studied, only recently a role of negative control on the protein activity has been suggested for the dispensable N-terminal domain. In order to investigate Cdc25 localization and the role of its different domains, several fusion proteins were constructed using the full length Cdc25 or different fragments of the protein with the green fluorescent protein. Unexpectedly, even if only slightly overexpressed, the full protein was not located in the cell plasma membrane, but accumulates inside the cell and also into the nucleus. Moreover, the endogenous Cdc25, tagged with HA, was also found in purified nuclear extracts. The fusions spanning aa 353–875, aa 876–1100 or aa 353–1100 localize heavily in the nucleus, concentrating in the nuclear peripheral area, in a region distinct from the nucleolus. This could be related to the presence of two predicted nuclear localization signals (NLS) in positions 547 and 806, but also to the contribution of another region, spanning residues 876–1100. This localization is likely to be physiological, since the fusion proteins can be efficiently exported and then imported back into the nucleus.

1. Introduction

In the yeast *S. cerevisiae* the Cdc25/Ras/cAMP pathway plays a role in the regulation of cell growth and proliferation in response to the availability of nutrients [1] and in the adaptation to glucose. The increase in cytosolic cAMP levels activates the cAMP-dependent protein kinase (pKA), which has an essential role in the progression from the G1 to S phase of the cell cycle [2]. The Ras proteins are, in addition, necessary for completing mitosis [3] and regulating filamentous growth [4].

Due to their intrinsically slow GTPase and GTP/GDP exchange activities, Ras proteins are strictly controlled by two classes of regulatory proteins: GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). In yeast, RasGAPs are represented by Ira1 and Ira2 proteins, while RasGEFs are Cdc25 and Sdc25 [2]. While *CDC25* is an essential gene in most genetic backgrounds [5], *SDC25* is dispensable and is normally expressed only during nutrient depletion or on non-fermentable carbon sources [6]. Thus, the main positive regulator of yeast Ras proteins is *CDC25* gene product.

Cdc25 protein is a ~180 kDa polypeptide with a C-terminal highly conserved Ras-GEF catalytic domain [7,8], which seems sufficient for full biological activity in yeast [9,10]. The large N-terminal portion (residues 1–1121) contains an SH3 domain (residues 65–134), involved in protein–protein interactions with adenylyl cyclase [11], and a cyclin destruction box (CDB, position 149) which is important for the short half-life of the protein [12]. Despite many studies on this subject, some Cdc25 features remain obscure: although its catalytical activity is required for the glucose-activation of adenylyl cyclase [9,13–15], the N-terminal domain seems to be completely dispensable for this response [16]. In addition there are some evidences that the N-terminal region could have a negative regulatory role [17–19].

Cdc25 protein is tightly bound to a crude membrane fraction even after treatment with reagents able to release membrane peripheral proteins [4,8,20]. The mechanism of this membrane attachment is still unclear, since Cdc25 does not contain a well defined transmembrane domain or a post-translational modification responsible for membrane attachment, and can be released from membranes only in denaturing conditions [20]. It was proposed that the phosphorylation of several Cdc25 residues within the 114–348 region could influence this membrane attachment, making the protein more soluble when hyperphosphorylated [17]; nevertheless, the most C-terminal of the three highly conserved boxes in the catalytical domain (aa 1454–1475) is hydrophobic and was included in the minimal region identified as sufficient for insolubility, spanning residues 1141–1552 [19]. Immunofluorescence against overexpressed Cdc25 showed a particulate localization in distinct patches [10,19] both for the full-length protein, for the catalytic fragment (residues 987–1589) or for the shortest insoluble fragment (1441–1552). On the contrary, the 411 aa N-terminal domain was reported as at least partially soluble but its role in Cdc25 localization has never been further investigated.

In this work, Cdc25 localization in growing cells was studied using protein fusions with the green fluorescent protein (eGFP). Unfortunately, the level of endogenous protein expression makes it very hard to

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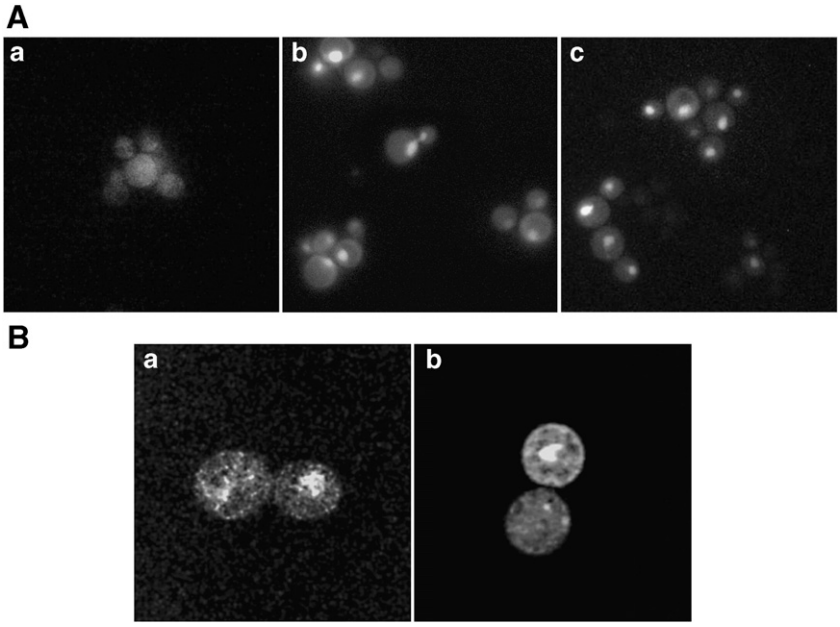


Fig. 1. Cdc25 protein as an eGFP fusion reveals a nuclear and cytosolic localization. (A) Cells transformed whether with YICDC25eGFP (a), YEpCDC25eGFP (b) or YEpeGFPCDC25 (c) were grown in SD-ura medium at 30 °C until exponential phase and then eGFP fluorescence was photographed on living cells with a Nikon Eclipse E600 microscope. (B) Analysis of living cells expressing YEpCDC25eGFP at the confocal microscope shows a punctuate pattern. Setting is described in Materials and methods. (a) Single optical section. (b) Image generated by the average of a stack of 5 optical sections.

observe the localization as an eGFP fusion. However, when over-expressed, the fusion protein accumulates in both the cytosol and the nucleus, but not in the plasma membrane. The region responsible for nuclear localization was identified as the central uncharacterized region (residues 353–1100). In agreement, the endogenous Cdc25 (tagged with HA) was also found in purified nuclei. The effect of cell stress, nutrient starvation or unbalanced PKA activity, on Cdc25 localization, was investigated.

2. Materials and methods

2.1. Yeast strains and media

Plasmids were introduced in the W303-1a wild-type strain (*Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) by lithium/acetate standard method. KT98 (a kind gift from K. Thorn, Harvard University, USA), isogenic to W303-1a, expresses a fusion of Nup49 protein with the fluorescent protein tdimer [22]. The haploid OL568-1C strain (Mat*α ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 3xHA::CDC25*) [23], kindly given by M. Jacquet (Université Paris-Sud, Orsay Cedex, France), was used for nuclei preparation. The OL97-1-11B strain (*Mata cdc25-5 his3 his7 leu2 ura3*) was used for complementation assay. W303 [YepTPK1] was previously described [15]. The CDC25^{NES} yeast strain (OL568-1C except for *CDC25::3xHAcdc25NES::Sphis5*$^{+}$) construction is described below.

YPD media contained 1% yeast extract and 2% peptone (Biolife) and was supplemented with 2% glucose; synthetic complete media (SD) contained 2% glucose, 6.7 g/l YNB (Difco) w/o aminoacids, and the proper selective drop-out CSM (Complete Synthetic Medium, supplied by BIO101, California, USA); synthetic media (SM) contained 2% glucose, 6.7 g/l YNB (Difco) w/o aminoacids, and 50 mg/l of the essential aminoacids and bases.

2.2. Plasmids

eGFP *ORF* was sub-cloned from pEGFP plasmid (Clontech) cutting with EcoRI (filled with Klenow fragment) and HindIII into HindIII–EcoRV digested pYX212 (Novagen), as a control for eGFP localization in yeast.

CDC25-eGFP fusion was made by utilizing the SphI site in *CDC25 ORF* (position 4650) and the SphI site in the pEGFP plasmid. First, the fusion was obtained in a N-terminal deletion construct, Yep13-CDC25 (907–1589), previously described [19], by sub-cloning the PvuII–SphI fragment in PvuII–SphI digested pEGFP, obtaining pCAT-eGFP. Then, the fusion was excised by cutting with PvuII and NotI (filled with Klenow fragment), and inserted into the SmaI site of a multicopy plasmid, YEplac195 [24], to generate YEp(907–1551)eGFP.

The 1–1551 aa fusion construct was realized by sub-cloning the HindIII fragment from pCDC25(LEU2)-2 [25] in the HindIII cut pCAT-eGFP, creating pCDC25eGFP. The multicopy plasmid pYX(1–1551) eGFP, with the 1–1551 aa fusion protein expression driven by a strong

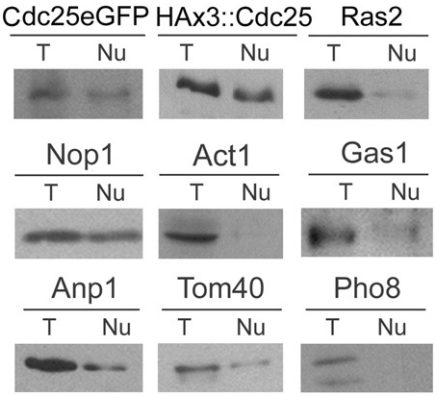


Fig. 2. Endogenous Cdc25 protein co-purifies with nuclei. Nuclei were prepared from exponentially growing cells of OL568-1C strain or from W303-1a strain carrying YEpCDC25eGFP (for Cdc25eGFP) as described in Materials and methods and the indicated proteins were revealed as internal membrane markers: Act1 (cytosolic and cytoskeleton protein), Gas1 (plasma membrane, ER and Golgi), Anp1 (Golgi), Tom40 (mitochondrial membrane), Pho8 (vacuolar membranes), while Nop1 was assayed as an intact nuclei marker. Total extract (30 μg of proteins for HAx3::Cdc25, Cdc25eGFP, Ras2, Pho8 and Nop1; 10 μg for Act1, Gas1, Anp1, Tom40) and the corresponding amount of nuclei fraction (1/6th of the proteins loaded for total extract) were loaded on SDS-PAGE and the indicated proteins were visualized by western blot.

constitutive promoter, the *TPI1* promoter, was obtained by a two-step strategy: first, the HindIII fragment from Yep13-CDC25 was subcloned in HindIII site of pYXeGFP, giving pYX(1–1501)eGFP; then, the PvuII–StuI fragment from pCDC25eGFP was rearranged by homologous recombination in yeast with the SalI digested pYX(1–1501)eGFP, giving pYX(1–1551)eGFP.

An episomic construct, YEpCDC25eGFP, containing the 1–1551 aa fusion *ORF* under *CDC25* own promoter was obtained by homologous recombination in yeast of the HindIII fragment from pCDC25(LEU2)-2 and the BglII digested YEp(907–1551)eGFP. An integrative vector, YI CDC25eGFP, was obtained by ligating a ClaI digested un-methylated preparation of the pYX(1–1551)eGFP plasmid.

pYX(1–1100)eGFP was constructed by ligating the HindIII fragment from pYX212-CDC25(1–1100) [19] in the HindIII site of pYXeGFP. pYX(354–1100)eGFP and pYX(876–1100)eGFP were obtained by subcloning the HindIII–PvuI fragment from pYX(1–1100)eGFP in the HindIII–SmaI digested pYX212-CDC25(353–1100) or pYX212-CDC25(876–1100) [19], respectively. pYX(353–875)eGFP and pYX(1–353)eGFP were prepared by ligating respectively the 1600 bp BglII-fragment or the 1400 bp BamHI–BglII fragment from pYX212-CDC25(1–1100) in the BamHI digested pYX(1–1100)eGFP. pYX(770–875)eGFP was obtained by EcoRI–EcoRI deletion in pYX(353–875)eGFP. pYXeGFP(494–685) was prepared by first deleting SacI–SacI fragment from pYX212-CDC25(353–875), giving pYX212-CDC25(354–685), then subcloning NcoI–SmaI 777 bp fragment from pEGFP-C1 (Clontech) in NcoI–EcoRV of pYX212-CDC25(354–685). An eGFP dimer was created by modifying by PCR the eGFP ORF from pEGFP-C1 (Clontech), inserting an upstream SmaI site and deleting a nucleotide before the BglII site. Then the fragment was digested with SmaI and BglII and inserted in the HincII–BamHI sites of pEGFP, giving pEGFP2. An NcoI fragment containing the eGFP ORF was then inserted in the NcoI site of pYX(770–875)eGFP and pYXeGFP(494–685), giving respectively pYX(770–875)eGFP2 and pYXeGFP2(494–685). The fragment was also inserted twice, giving pYX(770–875)eGFP3. As a control for eGFP dimer localization, the NcoI eGFP fragment was also inserted in NcoI digested pYXeGFP, giving pYXeGFP2.

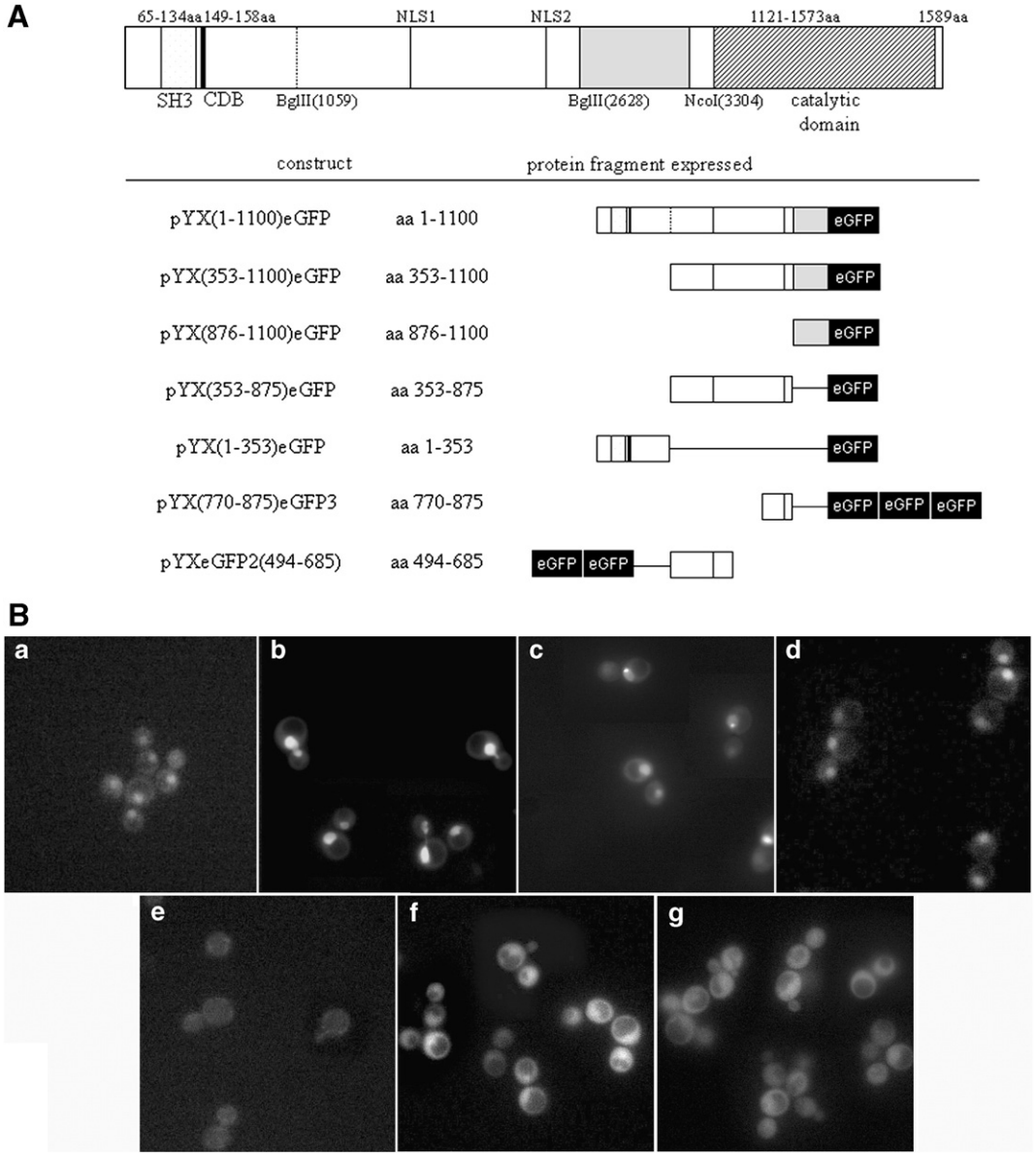


Fig. 3. Overexpression of Cdc25 N-terminal region sub-fragments identifies two domains that lead the protein in the nucleus, spanning aa 353–875 and aa 876–1100. (A) Schematic representation of the constructs utilized to overexpress different eGFP fusions with the indicated fragments of the putative regulatory N-terminal region of Cdc25. (B) W303-1a cells transformed with pYX(1–1100)eGFP (a), pYX(353–1100)eGFP (b), pYX(876–1100)eGFP (c), pYX(353–875)eGFP (d), pYX(1–353)eGFP (e), pYX(770–875)eGFP3 (f) or pYX eGFP2 (494–685) (g) were grown in SD-ura medium at 30 °C until exponential phase and then eGFP fluorescence was photographed with a Nikon Eclipse E600 microscope.

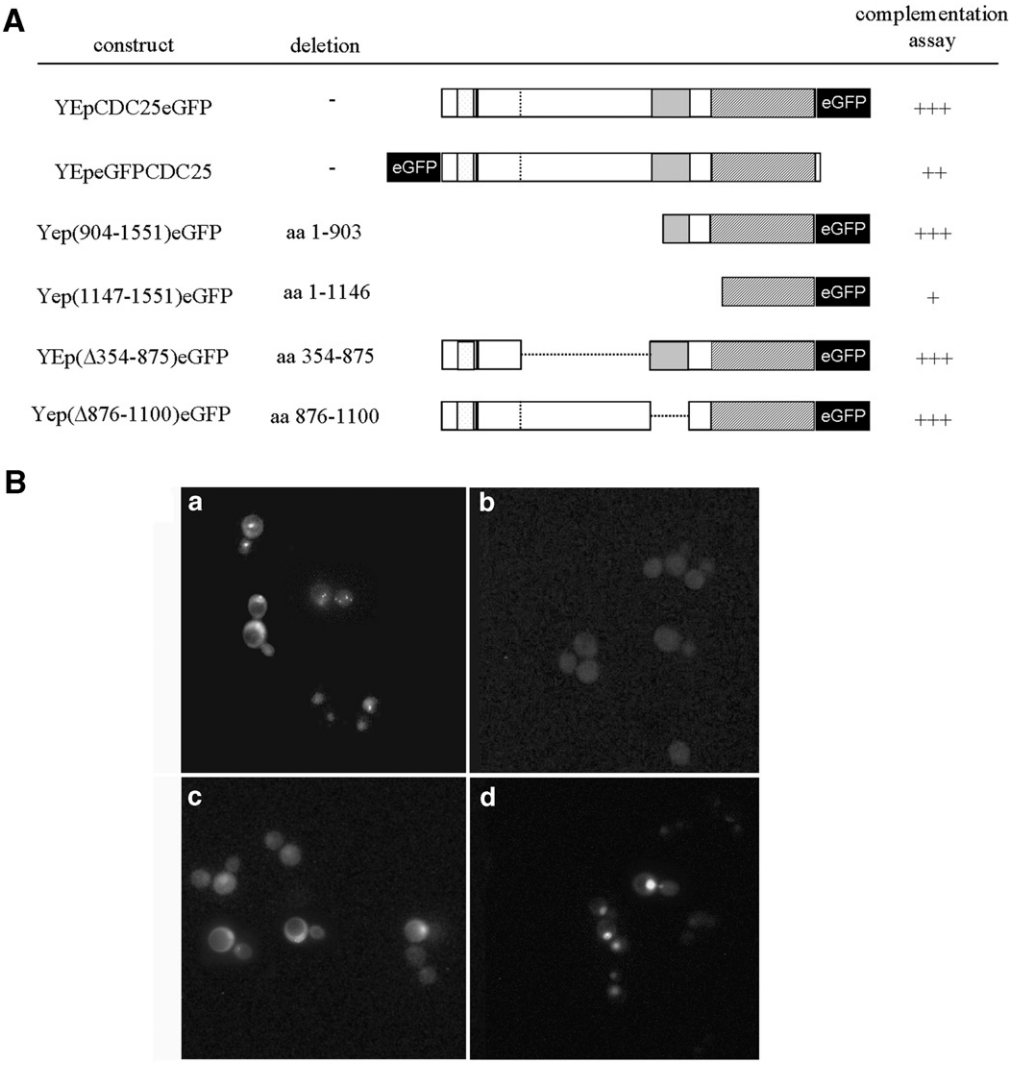


Fig. 4. Localization of a set of Cdc25 deletion proteins as eGFP fusions confirms that either aa 353–875 or aa 876–1100 regions are sufficient to drive the protein to the nucleus. (A) Schematic representation of the multicopy plasmids utilized to express different eGFP fusions, under *CDC25* promoter, with the indicated deletion constructs of the Cdc25 protein, all spanning the catalytic domain. Efficiency of each construct in complementation of the *cdc25-5 ts* defect is indicated. (B) W303-1a cells transformed with YEp(904–1551)eGFP (a), YEp (1147–1551)eGFP (b), YEp(Δ354–875)eGFP (c) or YEp(Δ876–1100)eGFP (d) were grown in the proper selective SD medium at 30 °C until exponential phase and then eGFP fluorescence was photographed with a Nikon Eclipse E600 microscope.

The deletion construct YEp(1147–1551)eGFP was generated by using the deletion construct already described [19] and making it recombine in yeast with the BglII digested YEp(904–1551)eGFP.

The YEplac195-CDC25(Δ876–1100) construct was obtained by homologous recombination in yeast between the NcoI digested pCDC25(LEU2)-2 plasmid and the 600 bp PCR fragment obtained with RedAccuTaq enzyme (SIGMA) with the primers cdc25-9 (AAATCAGAATGTTTTACTATTGGAGATACTGGAGAATTTAAACCTTc-catggTTTTTAACTTCAGATTATG) and cdc25-6 (GTCTGT ACGACTGCCGA-TACCG) using the same plasmid as a template. The BamHI fragment from the resulting YEplac195-CDC25(Δ876–1100) was recombined in yeast with BglII-digested YEpCDC25eGFP, giving YEp(Δ876–1100) eGFP. YEp(Δ354–875)eGFP plasmid was created by BglII fragment deletion in YEpCDC25eGFP.

NES-GFP sequence was obtained from the pREV(1.4)NESGFP plasmid [26] carrying the HIV Rev protein nuclear export sequence (PVPLQLPPLERLTLDCNE) fused to the GFP protein, and subcloned as a BamHI NotI fragment in pEGFP, giving pNES3-eGFP. The SphI NotI fragment was then subcloned in SphI NotI cut pCAT-eGFP. The NES sequence was then inserted between Cdc25 coding sequence and eGFP ORF by homologous recombination between the BglII NotI fragment from this plasmid and YEpCDC25eGFP, giving YEpCDC25-NES-eGFP. To generate the CDC25^{NES} yeast strain, a construct was prepared by inserting the NcoI (Klenow filled)–PvuII fragment from pNES3-eGFP into the EcoRV site of pDH5 plasmid (from the National Center for Research Resources at the NIH, University of Washington, USA), containing the *S. pombe his5*$^{+}$ gene, and NES was then fused to *CDC25* coding sequence by amplifying the NES and *Sphis5*$^{+}$ sequence by PCR, with the primers GAAGCTAGGTAAGAAAAAACCTCCTTCTAGGT-TATTTCGAAGGTCGACTCTAGAGGATCCA and GCTAAGGTTCTTCTTGAT-TAGCAAATTGTATAAACT-TTTACTGTTTAGCTTGCCTCGTCCCC.

Table 1
Both the 354–876 aa and the 876–1100 aa regions contribute to the accumulation in the nucleus of the Cdc25 protein, and their effect is counteracted by Tpk1 overexpression

	W303-1A	W303-1A [YEpTPK1]
YEpCDC25eGFP	33.6±0.8	23.9±1.0
Yep(Δ354–875)eGFP	23.9±1.4	24.5±1.2
Yep(Δ876–1100)eGFP	22.1±1.5	25.1±0.9

The percentage of fluorescence in the nucleus was determined as described in Materials and methods for the indicated constructs expressed in the indicated strains (n=30, ±SD).

2.3. PSORT II analysis

The Cdc25 protein sequence was analyzed by the on-line available tool PSORT II [27], for prediction of protein sorting signals and localization sites in aminoacid sequences (http://psort.ims.u-tokyo.ac.jp/).

2.4. Epifluorescent microscopy

Cells were grown at 30 °C in the proper selective SD medium to early exponential phase and observed with a Nikon Eclipse E600 microscope, fitted with either a 40× or a 60× immersion objective and a standard fluorescein isothiocyanate filter set (Nikon, EX 450-490, DM 505, BA 520) for GFP-fluorescence, and a cy3 filter set for tdimer2 fluorescence. Images were recorded digitally using a Nikon FDX-35 camera and processed using MetaMorph 6.3r1 (Molecular Devices, Sunnyvale, CA) and Adobe Photoshop (Adobe Systems, Inc.).

Confocal microscopy images were collected using the Leica TCS SP2 confocal microscope equipped with an inverted Leica DMIRE2 microscope and a PL APO 63× oil immersion objective (numerical aperture: 1.4). An average of 10 optical sections was acquired for every single cell, and a representative single optical section or an average of the most significant ones is shown.

In vivo DAPI staining was performed by incubating exponentially growing cells with 1.6 μg/ml DAPI for 2–3 h at 30 °C. Propidium iodide (PI, Sigma Aldrich) staining was utilized to visualize DNA with the confocal microscope. Briefly, cells were fixed in cold 70% (v/v) ethanol, then washed with phosphate buffer saline (PBS, containing Na_2HPO_4/NaH_2PO_4 10 mM, pH 7.2, 130 mM NaCl, 0.2 mM EDTA) three times, treated with 2 mg/ml RNase in PBS for at least 2 h at 37 °C, washed with PBS and incubated in propidium iodide 50 μM in Tris 50 mM pH 7.7, $MgCl_2$ 15 mM for 30 min on ice.

Fluorescence measurements were performed by using MetaMorph fluorescence Integrated Intensity tools, and the percentage of nuclear fluorescence was calculated on at least thirty cells per strain as the average ratio of the Integrated Intensity measured in the nucleus over the Integrated Intensity of the whole cell.

For observation after hyperosmotic shock, hydrogen peroxide or ethanol treatment, cells were grown at 30 °C in the proper selective SD medium to early exponential phase. Some images were taken as time zero, then a NaCl, hydrogen peroxide, or ethanol stock solution was added to the culture, giving a final concentration of 0.5 M NaCl, 2 mM hydrogen peroxide or 10% ethanol respectively. At different times, samples were taken and observed at the microscope, and images were recorded of representative cells. For carbon and/or nitrogen starvation treatments, cells exponentially growing in SD medium were collected by filtration and resuspended either in Mes/Tris 0.1 M pH 7, or in YNB (Difco) 0.67 g/l or in YNB w/o ammonium sulphate (Difco) 0.17 g/l for 3 h. Rapamycin (Calbiochem) was added to cells growing in SD medium to a final concentration from 200 μg/l up to 10 mg/l.

2.5. Preparation of nuclear extracts

Nuclear extracts were prepared as described on the Hahn Laboratory website (www.fhcrc.org/labs/hahn) with minor modifications to scale-down the protocol. In order to evaluate the contamination of the purified nuclei from the cellular membranes, the following proteins were tested by immuno-blotting as membrane markers: actin for proteins not associated to membranes, Gas1 for plasma membrane, ER and Golgi [28], Anp1 for Golgi apparatus [29], Tom 40 for mitochondria [30], Pho8 for vacuolar membranes [31] and Nop1 for nuclei [32]. Primary antibodies for Gas1 (kind gift from M. Vai, University of Milano-Bicocca), Anp1 (kind gift from S. Munro, MRC Lab of Molecular Biology, Cambridge, UK), Tom40 (kind gift from T. Endo, Nagoya University, Japan) and Act1 (Sigma) were raised in rabbits, antibodies against Pho8 (kind gift from J. Winderickx, K.U. Leuven, Belgium) and Nop1 (Encor Biotechnology) were from mouse.

Nuclear extracts were prepared from OL568-1C strain, W303-1a strain carrying YEpCDC25eGFP and $CDC25^{NES}$ strain, giving similar results as far as membrane markers or Ras2 detection in nuclear extracts is concerned.

2.6. cdc25-5 defect complementation assay

The OL97-1-11B strain (*Mata leu2 ura3 his3 his7 cdc25-5*), which has a temperature sensitive growth defect, was transformed with the plasmids driving the expression of different GFP fusion proteins and the rescue of growth at the restrictive temperature of 37 °C was evaluated as the percentage of colonies growing at restrictive temperature with respect to the colonies growing at permissive temperature.

2.7. Solubility assay

Cells expressing the different fusion proteins were grown in the proper selective SD media at 30 °C to the density of 0.5–0.8 OD_{600}, then collected by centrifugation and frozen. Extracts were prepared by breaking cells with glass beads in a FastPrep (Savant) in HB buffer (25 mM MOPS pH 7.2, 15 mM $MgCl_2$, 15 mM EGTA, 1 mM PMSF, 10 mg/ml pepstatin A, Complete EDTA-free Protease Inhibitor Mixture from Roche Applied Science). Fractionation was performed by sequential centrifugation at 5000 ×g at 4 °C, which gave the first pellet (P5), and then at 16,000 ×g, which gave the second pellet (P16) and the supernatant (S). The extracts, P5, P16 and S were loaded on SDS-PAGE, transferred to nitrocellulose and decorated with eGFP directed BD Living Colors A.v. Antibodies (BD Biosciences Clontech) and α-rabbit antibodies conjugated to peroxidase (Jackson Immunoresearch). The extract equivalent to 6×10^7 cells was loaded for total extract and S, a triple amount was loaded for P5 and P16.

2.8. Anti-Nop1 immunofluorescence

Cells harbouring pYX212-CDC25(353–1100)-eGFP were grown in SD-ura medium at 30 °C until early exponential phase, then the culture was buffered with a stock phosphate buffer, leading to a final concentration of K_2HPO_4/KH_2PO_4 100 mM, pH 6.5, and fixed with 3.7% formaldehyde for 30 min at room temperature. Then, cells were recovered by filtration and fixed again for an additional 1 h at room temperature in 100 mM phosphate buffer pH 6.5, 3.7% formaldehyde. Cells were collected by filtration and washed three times with phosphate buffer, then once with digestion buffer (1.2 M sorbitol,

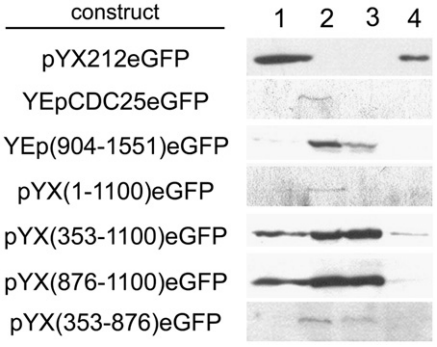
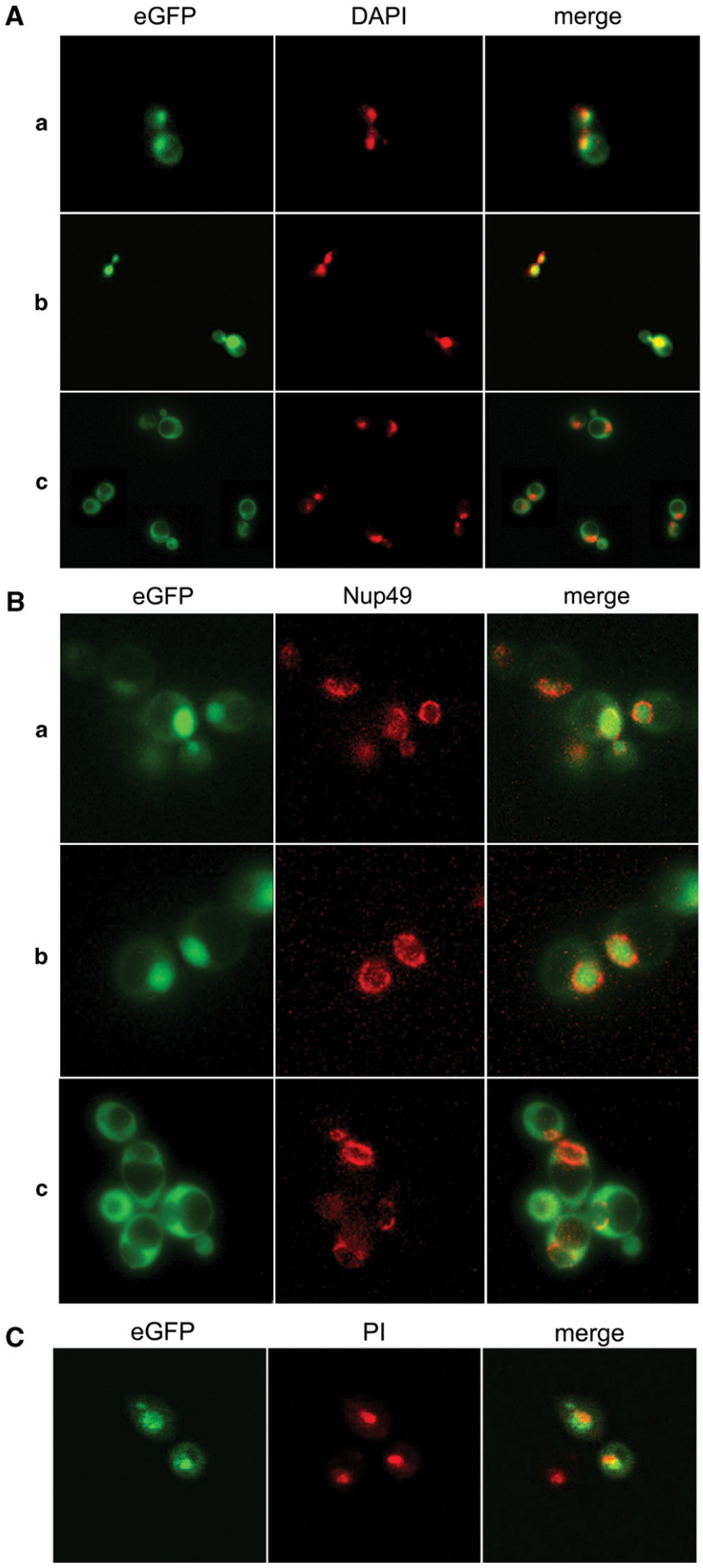


Fig. 5. The fusion proteins spanning the central region of Cdc25 are highly insoluble. Total proteins were extracted and fractionated by sequential centrifugation. The total extract and supernatant equivalent to 6×10^7 cells, the triple for pellets at 5000 ×g and 16,000 ×g, were loaded on SDS-PAGE and immunoblotted with α-eGFP antibodies. Lane 1, total extract. Lane 2, pellet at 5000 ×g. Lane 3, pellet at 16,000 ×g. Lane 4, supernatant.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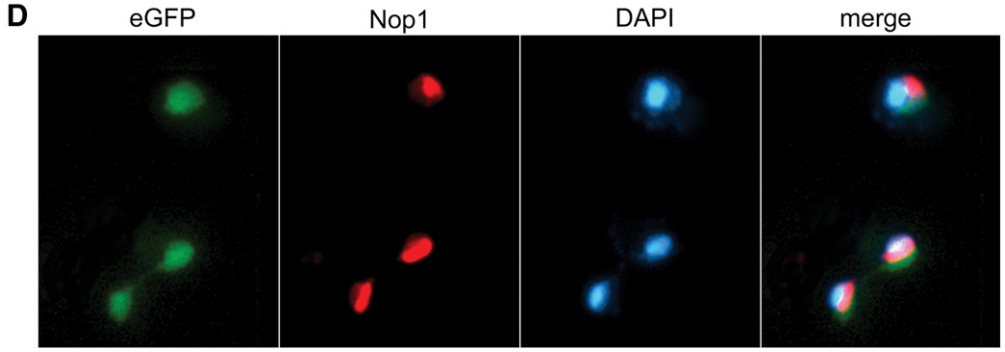


Fig. 6. Cdc25 1–1551 aa and 353–1100 aa eGFP fusions localize inside the nucleus: the introduction of a heterologous nuclear export signal can exclude the protein from the nucleus. In detail, Cdc25 localize to the non-nucleolar region of the nucleus. Unless differently indicated, photographs were obtained with Nikon Eclipse microscope as described in Materials and methods section. (A) Co-localization of eGFP fluorescence and DNA (DAPI stained) in W303-1a cells transformed with YEpCDC25eGFP (a), pYX(353–1100)eGFP (b) or YEpCDC25-NES-eGFP (c). (B) Co-localization of eGFP fluorescence and Nup49, a protein of the nuclear envelope, as a tdimer2 fluorescent protein fusion, in KT98 cells transformed with YEpCDC25eGFP (a), pYX(353–1100)eGFP (b) or YEpCDC25-NES-eGFP (c). (C) Single optical section obtained by confocal microscopy analysis of W303-1a cells transformed with pYX (353–1100)eGFP and stained with propidium iodide to visualize DNA. (D) Co-localization of eGFP fluorescence and the nucleolar protein Nop1, immuno-detected as described in Materials and methods, in W303-1a cells transformed with pYX(353–1100)eGFP. DNA was stained with DAPI.

100 mM KH_2PO_4/K_2HPO_4 pH 5.9), resuspended in digestion buffer with 0.25 g/l zymolyase 20 T (ICN), 200 μM β-mercaptoethanol and incubated for up to 1 h at 37 °C. In the meanwhile, a multiwell slide (ICN) was coated with poly-L-lysine (SIGMA) 1 mg/ml for 10 s, then washed three times with water and let dry. After digestion of the cell wall, cells were collected at 2000 rpm for 10 min in a microfuge, resuspended in digestion buffer and placed on the slide for 10 s; unattached cells were aspirated off and the slides were immersed in −20 °C pre-cooled methanol for 6 min and then in cold acetone for 30 s to permeabilize cells.

To immunodecorate cells, the wells were washed 3 times with phosphate buffer saline (PBS, containing Na_2HPO_4/NaH_2PO_4 10 mM, pH 7.2, 130 mM NaCl, 0.2 mM EDTA), incubated 5 min with PBS and 0.1% Triton X-100 to reduce autofluorescence, then washed again 3 times with PBS. Staining with antibody α-Nop1 (EnCor Biotechnology) 1:100 in PBS 1% BSA was conducted for 1 h in a humid chamber, then wells were washed 10-times with PBS 0.1% BSA and covered with secondary antibody (Jackson Immunoresearch, goat α-mouse-Cy3 conjugated, 1:400 in PBS 1% BSA), incubated for 1 h at room temperature in a dark humid chamber, then 10-times washed before incubation for 10 min with DAPI 200 mg/l. After 5 washes with PBS 0.1% BSA, slides were mounted with 75% glycerol in PBS.

3. Results

3.1. Mild overexpression of Cdc25 full protein eGFP fusion results in its import into the nucleus

Previous studies on Cdc25 localization revealed that the protein co-segregates with a crude membrane fraction after centrifugation [8,17,20,21]. However, more accurate cell fractionation experiments have never been reported that could indicate the cellular compartment where Cdc25 is located. An immunolocalization experiment of the overproduced catalytic domain or the full-length protein [21] showed a labelling in patches near the cell periphery, distinct from actin patches.

With the aim of investigating Cdc25 localization in physiological conditions, a fusion was made between the Cdc25 protein and the green fluorescent protein (eGFP). As a single copy integrated in the *CDC25 locus*, this fusion was expressed at a very low level, as it is the case for the wild type protein, mainly due to its short half-life [12]. The fluorescent signal from the fusion protein was very low, giving a diffuse but not uniform labelling of the cells (Fig. 1A, subpanel a), possibly suggesting an association with the internal membrane system, but in no case labelling of the cell surface was observed.

In order to increase the amount of protein within the cell, the Cdc25 (1–1551)eGFP fusion was also inserted in a multicopy plasmid, under the control of the *CDC25* promoter. This kind of overexpression did not give any phenotypical defect in the W303-1a strain (heat shock sensitivity, iodine staining, growth on different carbon sources, cell size, percentage of budded cells were tested), but raised the level of the protein enough to be faintly detectable on anti-GFP Western blot (data not shown): this was enough to produce a remarkable effect on the fluorescence distribution inside the cells. Instead of accumulating in cell membrane compartments, as suggested by solubility assays in literature, the fusion protein showed an evident fluorescence pattern that suggested an accumulation in the nucleus (Fig. 1A, subpanel b).

Since the C-terminal 38 residues were removed in the Cdc25 (1–1551)eGFP fusion and the presence of the eGFP protein at the C-terminal could modify the normal localization of the protein, another fusion was made with eGFP in the N-terminal position, immediately before the first residue of the Cdc25 protein, and expressed under the control of *CDC25* promoter on an episomic plasmid. This fusion protein showed exactly the same pattern as the C-terminal eGFP fusion (Fig. 1A, subpanel c), showing that GFP tag was not affecting Cdc25 localization.

No correlation between bud morphology and Cdc25 distribution was seen, suggesting that localization was not regulated by the cell cycle, nor the localization was different during growth on carbon sources different than glucose, such as galactose or not fermentable carbon sources as glycerol.

In order to better observe the presence of the full protein in membrane compartments, the strain expressing the Cdc25(1–1551) eGFP fusion was observed with a confocal microscope. The fluorescence was revealed not only in the central region of the cell, but also in the cytosol, with a pattern suggesting association to the internal membranes (Fig. 1B).

3.2. Cdc25 protein co-purifies with nuclei even if not overexpressed

The hypothesis of a nuclear localization was further investigated through a purification of yeast nuclei as described in the Materials and methods section. The nuclear protein Nop1 was used as a nuclear marker, and it was enriched several times (from 3 to 6 times) in the nuclear extract, as expected. Cdc25 was also enriched in the nuclear extract, to a similar extent than Nop1, in the strain expressing Cdc25eGFP fusion on a multicopy plasmid (Fig. 2).

The eGFP fusions would have allowed to rapidly test the Cdc25 protein fragments required for nuclear localization but, in order to obtain a detectable signal, overexpression was required. This leads to

an accumulation in the nuclear compartment, but at the physiological protein level the localization could be different. A Cdc25 version was used with a N-terminal triple-HA tag inserted in the *CDC25* locus, which allows a good detection of the protein by western blot [23] at physiological levels of expression. Also when Cdc25 (triple-HA tagged) was not overexpressed (Fig. 2), Cdc25 was enriched in the nuclear extract like Nop1 was, indicating a significant presence of the Cdc25 protein in the nuclei also at physiological levels of expression. Several markers for intracellular proteins were tested in the nuclear extract, since residual internal membranes could still be present as a contamination in the nuclei preparation and a correct evaluation of the influence of these contaminations is necessary. All the markers used for internal membranes were present in traces in the nuclear fraction: in fact the plasma membrane, ER, Golgi, mitochondria or vacuolar membrane markers were almost lost in the nuclei fraction when compared to the total extract, giving similar results both in the OL568-1C strain (Fig. 2) or in the W303-1a strain carrying YEpCDC25eGFP (not shown). Differently from Cdc25, Ras2 was not enriched at all in the nuclear extract, showing a pattern that is comparable to the membrane markers.

3.3. Nuclear localization relies on the central region (aa 353–1100) of Cdc25 protein

Since neither the fusion with eGFP nor the overexpression seemed to affect the localization of Cdc25 protein, several constructs were produced to overexpress the whole N-terminal region (residues 1–1100) or different fragments of this region (Fig. 3A) as eGFP fusions in a multicopy plasmid under a constitutive promoter (*TPI1*), in order to identify the regions of the protein responsible for the nuclear localization. These experiments revealed that either the presence of the aa 353–875 or of the aa 876–1100 regions was sufficient to lead the fusion protein into the nucleus (Fig. 3B), but with some additive effects in the fusion protein spanning aa 353–1100.

The on-line available tool PSORT II [27] was used for prediction of sorting signals and localization sites in Cdc25 sequence. This analysis revealed four putative nuclear localization signals situated at positions 547, 806, 1408 and 1580, giving a prediction of nuclear localization with a 78.3% confidence.

The first two predicted NLSs (situated at positions 547, 806) are situated in the 353–875 region, that shows nuclear localization, but no putative NLS could be identified in the 876–1100 region, which is equally imported into the nucleus. The two NLSs were separately fused with eGFP in the pYX(770–875)eGFP3 and in the pYXeGFP2 (494–685) constructs (Fig. 3A), where eGFP protein was used in more than one copy to obtain proteins big enough not to be efficiently imported into the nucleus without a specific nuclear import signal. Both of these fusion proteins were able to enter the nucleus, but only the pYX(770–875)eGFP3 was observed to accumulate into the nucleus more than in the cytosol, even if none of them accumulated in the nuclear compartment at the same extent as the construct spanning both of them (Fig. 3B, subpanels f and g).

To confirm the role of these regions in the localization of the full-length protein into the nucleus, some deletion constructs were produced, still spanning the catalytical domain and thereby retaining catalytical activity, starting from the YEpCDC25eGFP multicopy plasmid expressing Cdc25(1–1551)eGFP fusion under the *CDC25* promoter control (Fig. 4A). Only the deletion of the entire N-terminal region (1–1146) could abolish the nuclear accumulation of the eGFP fusion protein (Fig. 4B), suggesting that the more C-terminal NLSs identified by PSORT II analysis are actually not functional. The deletion of the single regions, either 354–875 or 876–1100, caused a decrease of the fluorescence ratio of the nucleus compared to the cytoplasm. Western blot analysis revealed that these two constructs were expressed at a level comparable to the Cdc25(1–1551)eGFP, so the deletion of either of the two regions does not affect the production or stability of the protein. Total fluorescence intensity of the whole cell and of the nuclear compartment were measured as described in Materials and methods, and the average proportion of fluorescence in the nucleus was calculated (Table 1): for cells carrying YEpCDC25eGFP, 33.6 ± 0.9% of the fluorescence was nuclear, while for YEp(Δ354–875) eGFP or YEp(Δ876–1100) the nuclear fluorescence dropped to 23.9 ± 1.4% and 22.1 ± 1.5%. This suggests an additive effect of the two regions, 354–875 and 876–1100, in determining either the rate of import or the retention of the fusion proteins in the nucleus.

In order to assess the functionality of the deletion constructs a complementation assay was performed utilizing a *cdc25-5 ts* strain, which has a temperature sensitive growth defect. As shown in Fig. 4A, all the fusions spanning the catalytic domain (which is essential for viability) were able to rescue the growth arrest at restrictive temperature, with minor differences in the efficiency likely due to differences in the expression levels (as could be evaluated from the intensity of the fluorescence observed in cells carrying the different constructs) or to the lower activity of the shortest catalytical domain (1147–1551 aa) [19].

3.4. eGFP fusion proteins spanning either catalytical domain or the central region of Cdc25 are insoluble

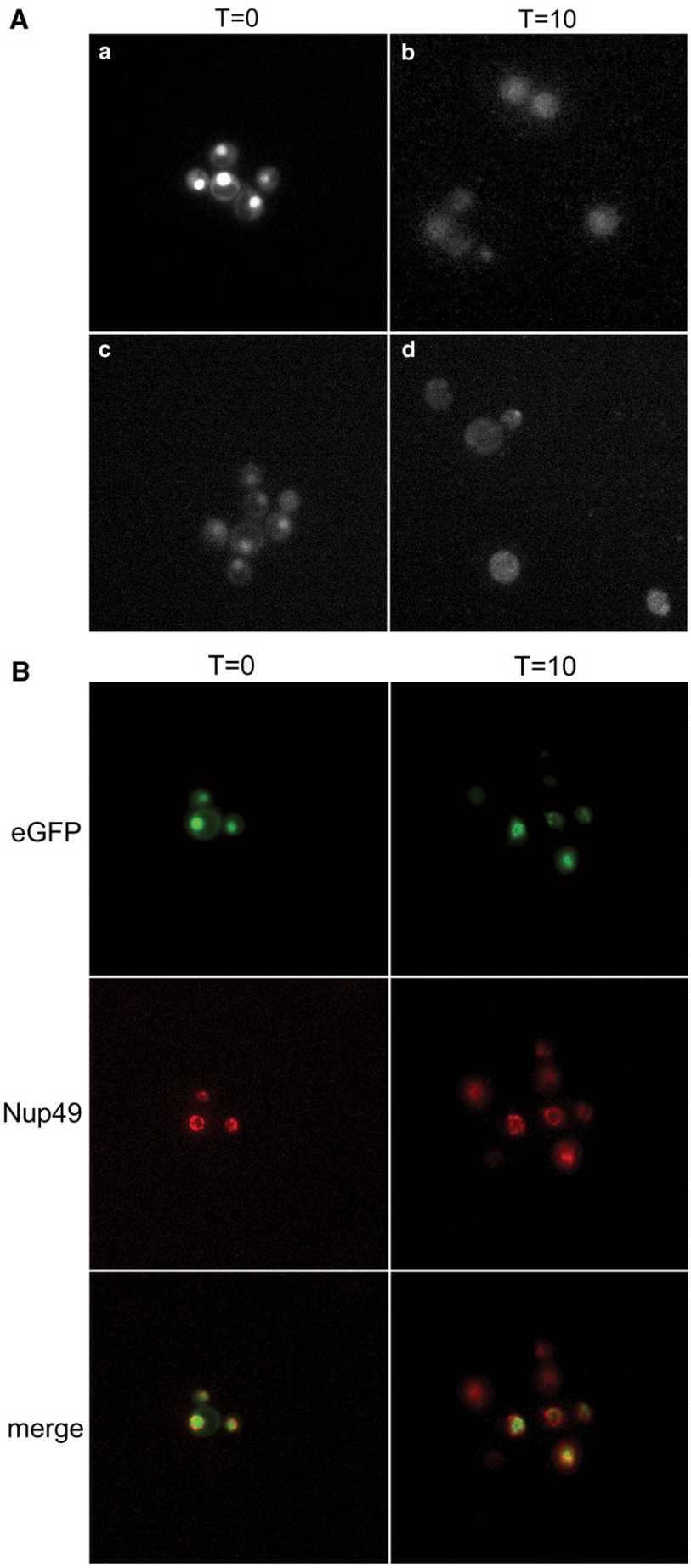
As already mentioned, Cdc25 protein is poorly soluble and after centrifugation always segregates in the pellet fractions [4,8,17,20]. This was often taken as a demonstration that the protein should reside in membranes, notably at the plasma membrane level. More recently, Ras2 protein was shown to accumulate more on internal membranes than on the plasma membrane [34] unless it was overexpressed, and the same could be true for Cdc25 RasGEF. Consistently with literature reported data, in a fractionation assay by differential centrifugation all the fusion proteins spanning the catalytical C-terminal domain resulted highly insoluble, and were associated to the pellet obtained after centrifugation at 5000 ×g (P5) or 16,000 ×g (P16) (data not shown). This indicates that the co-migration in the insoluble fraction in a differential centrifugation assay is not enough to assess the membrane association of these proteins, and that these proteins could associate with some insoluble component inside the nucleus.

On the contrary, the 411 aa N-terminal portion was reported to be at least partially soluble [21], but nothing was known about the central region of the protein. Cells expressing the fusion proteins spanning either the 354–875 aa, the 876–1100 aa, or 354–1100 aa regions were fractionated by centrifugation and resulted completely insoluble. In fact, as it is shown in Fig. 5, they were found in the pellet obtained at 5000 ×g, suggesting that they contribute to the very low solubility of the full length protein. This also suggests that nuclear accumulation of the 354–875 aa, the 876–1100 aa, or 354–1100 aa spanning proteins does not imply that these constructs are soluble: they could associate with the nuclear envelope or with insoluble components of the nuclear matrix.

3.5. Cdc25 accumulates in the non-nucleolar compartment of the nucleus

Nuclear localization was assayed by fluorescence microscopy by colocalization with DNA (DAPI staining) (Fig. 6A). Actually, the eGFP

Fig. 7. Cdc25 can physiologically be exported to the cytoplasm after hyperosmotic shock. The 353–1100 aa region is sufficient to observe the nuclear export induced by hyperosmotic shock. Photographs were obtained with Nikon Eclipse microscope as described in Materials and methods section. (A) Cells transformed with YEpCDC25eGFP (a and b) or pYX(1–1100) eGFP (c and d) were photographed before (a and c) and 10 min after (b and d) being challenged with a hyperosmotic shock. (B) Cells carrying pYX(353–1100)eGFP were photographed before and 10 min after hyperosmotic shock and co-localization is shown with Nup49–tdimer2 fluorescence to visualize the nuclear boundary.


A
T=0
T=10
a
b
c
d
B
T=0
T=10
eGFP
Nup49
merge

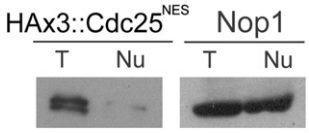


Fig. 8. Cdc25^{NES} protein is excluded from the nuclei. Nuclei were prepared from exponentially growing cells of CDC25NES strain as described in Materials and methods. Total extract (30 μg of proteins) and the corresponding amount of nuclei fraction (1/6th of the proteins loaded for total extract) were loaded on SDS-PAGE and the indicated proteins were visualized by western blot.

fluorescence was not perfectly coincident with DAPI staining pattern, but was nonetheless inside the nuclear compartment, as could be definitively demonstrated by co-localization with the nuclear envelope protein Nup49 (Fig. 6B) [35]. The pattern observed excluded that the Cdc25 fusion protein could be uniquely associated with the nuclear envelope, since the fluorescence was found both inside the nucleus and in its periphery. Furthermore, a construct was obtained by inserting a very strong nuclear export signal (NES), the HIV virus Rev protein NES [26] between Cdc25 portion and GFP tag. In fact, mammalian NES sequences have already been successfully used in yeast [33]. The insertion of Rev NES sequence completely excluded the Cdc25NESeGFP protein from the nucleus (Fig. 6A and B, subpanels c), reinforcing the hypothesis that the protein is localized inside the nucleus. A further more detailed analysis was performed on the more stably expressed fragment, spanning aa 353–1100, by confocal microscopy. In fact, this analysis confirmed that the eGFP fluorescence was adjacent to the DNA, as visualized by propidium iodide staining (Fig. 6C). In yeast, nucleoli often assume a flatten shape, in the peripheral nucleus [32], which could have explained the observed morphology. However, the immunofluorescence against the nucleolar protein Nop1 [36] revealed that the Cdc25(353–1100)eGFP fusion localizes in the non-nucleolar compartment of the nucleus (Fig. 6D).

3.6. Cdc25 nuclear localization can be affected by physiological regulations

It was reported in literature that, when yeast cells are exposed to environmental shocks, many nuclear proteins rapidly relocate to the cytoplasm [37,38]. In order to investigate if the nuclear localization observed for some Cdc25 eGFP fusions was physiologically regulated and could respond to relocalization signals, their behaviour was observed either after exposure to ethanol, hydrogen peroxide or after hyperosmotic shock.

Ethanol and hydrogen peroxide exposure are stress treatments that can inhibit nuclear import [37], but these treatments didn't affect the distribution of any of the fusion proteins (not shown) suggesting a more complex control not only on nuclear import, but also on nuclear retention and export. Conversely, the 1–1551 aa fusion migrated to the nuclear envelope and then was exported from the nucleus within 10 min after the hyperosmotic shock (Fig. 7A, subpanels a and b), relocating to the nucleus within 40 min after the shock (not shown). Also the N-terminal region (1–1100 aa) fusion was regulated in a similar way (Fig. 7A, subpanels c and d), as well as the protein spanning residues 353–875, or 876–1100 (data not shown). A more complex pattern was observed for Cdc25(353–1100)eGFP, probably due to the high level of protein accumulated in the nucleus. This protein was not completely exported from the nucleus, and often stuck into the nuclear envelope, as revealed by comparison with a nuclear envelope protein, Nup49 (Fig. 7B).

No differences in nuclear localization were observed in cells in different phases of the cell cycle, neither starvation for glucose or nitrogen sources nor rapamycin treatment influenced the nuclear localization of Cdc25(1–1551)eGFP fusion. Only PKA activity could partially counteract Cdc25 nuclear import: the overexpression of Tpk1, one of the catalytic subunits of PKA, could reduce the percentage of nuclear fluorescence in the strain expressing the Cdc25eGFP protein at the same extent as the 354–875 aa or 876–1100 aa deletions (Table 1).

These observations indicate that an active transport into and out of the nucleus can regulate Cdc25 protein distribution between nucleus and cytoplasm, suggesting that the protein can shuttle from the nucleus to the cytoplasm and vice versa.

The expression of the Cdc25NESeGFP protein didn't cause any evident phenotype in the cells: growth, percentage of budded cells, cell size, heat shock resistance were similar to the wild-type strain, and no defect in the utilization of galactose could be observed. This could be explained by the presence of the wild-type allele in the strain, that can still operate in the nucleus, although the wild-type protein is also efficiently exported from the nucleus in this strain, probably due to association with the NES containing Cdc25 version (data not shown).

In order to clarify if Cdc25 could have any function in the nuclei, a mutant was created where the Cdc25 protein contained the HIV Rev protein NES sequence. This sequence was able to efficiently exclude the Cdc25^{NES} protein from the nuclei (Fig. 8), but the mutant strain (CDC25^{NES}) was viable and didn't show any growth defect on glucose, nor it was more or less sensitive than the control strain to heat shock, osmotic stress or oxidative stress (data not shown). Interestingly, the CDC25^{NES} strain cannot hardly grow on glycerol (Fig. 9) or ethanol (not shown), suggesting a role for Cdc25 protein in the nucleus either in derepression from glucose or during growth on non-fermentable carbon sources.

4. Discussion

The *S. cerevisiae* RasGEF Cdc25 plays its essential function on membranes, where its main interacting proteins, Ras and the adenylate cyclase, seem to localize. Actually, Ras2 protein was recently found to mainly localize in internal membranes, and not to accumulate in the plasma membrane [34,39], as it was previously suggested [40,41].

Anyway, the nuclear localization of the Cdc25 eGFP fusions is quite surprising, but it is likely to be specific and physiological, since it can respond to relocation signals such as hyperosmotic shock-induced nuclear export. This localization seems to rely on sequences present in the central region of the protein, from aa 353 to aa 1100, which is a largely uncharacterized region of this protein, and no known pattern or profile could be found by online available tools for pattern and

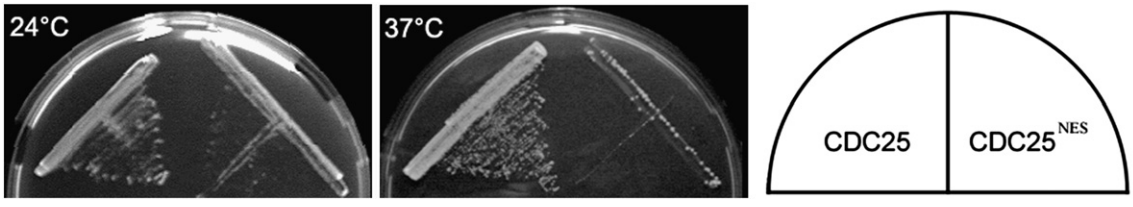


Fig. 9. Cdc25 exclusion from the nucleus impairs growth on non fermentable carbon sources. Cells of OL568-1C strain (CDC25) and CDC25^{NES} strain, growing on YPD plate, were transferred to YP plates supplemented with 3% glycerol and incubated at the indicated temperature.

profile searches at ExPASy (*Ex*pert *P*rotein *A*nalysis *Sy*stem) proteomics server of the Swiss Institute of Bioinformatics (SIB) (MotifScan, InterProScan, ScanProsite and other tools were used). This region is not conserved in RasGEFs of other organisms, except for *S. cerevisiae* Sdc25 and other Cdc25 homologs from other yeasts, like CaCdc25 of *Candida albicans* (accession number S30356), or SkCdc25 of *Saccharomyces kluyveri* (accession number Q02342). An interfering activity of this central region on Cdc25-dependent phenotypes was reported both for the 347–875 aa [18] and the 876–1100 aa [19] regions, but the mechanism of this interference is not known. Either one of the two central regions can be deleted without affecting the rescue of *cdc25-5* temperature sensitive defect; consistently, it was previously reported that a 353–875 residues deletion doesn't inhibit the efficiency of basal or inducible adenylate cyclase activity [14].

The two putative nuclear localization signals situated at positions 547 and 806 (NLSI and NLSII) are able to direct into the nucleus the fragments containing both or either one of them (Fig. 3). The most efficient is the NLSII, a bipartite NLS, also present in Sdc25. Its sequence is KRKKKYPLTVDTLNTMKKKSS: the two final serine residues were previously considered as PKA phosphorylation sites [14], and are highly conserved in other yeasts Cdc25 homologs, as well as the hydrophobic core of the motif and the acidic residue in the middle. Cdc25 was shown to be hyper-phosphorylated in response to glucose, resulting in reduced accessibility to Ras [17]: the serine residues near the NLSII could be targets of PKA regulation on Cdc25 activity or localization.

However, the nuclear localization of Cdc25 is not only due to nuclear import: sensitivity to hyperosmotic shock suggests a more complex regulation on nuclear retention of the protein, since hyperosmotic shock was reported not to affect nuclear import but to cause an immediate dissociation of most proteins from chromatin, rapidly recovered in 5–10 min by activation of the High Osmolarity Glycerol (HOG) pathway [42].

The unexpected nuclear localization of Cdc25 protein could indicate that the protein function can be regulated, apart from the rapid turn-over of the protein, also by relocalization from the cytosol to the nucleus. Nuclear import could subtract Cdc25 from its membrane-attached partners (i.e. Ras1,2 and adenylate cyclase), or have an effect on the degradation rate of the protein. Actually, no difference was observed in PKA activity related phenotypes or in the expression level for the Cdc25eGFP and the Cdc25NESeGFP, which would exclude any effect of down-regulation in Cdc25 activity by being sequestered in the nucleus or more rapidly degraded.

Nuclear localization could also be indicative of a specific nuclear function for Cdc25. In fact, it was previously reported that Cdc25 overexpression was involved in specific transcriptional effects. This could suggest a direct or indirect involvement of Cdc25 in the regulation of transcription, and a requirement of the nuclear localization determinants for the assessment of these functions. It was previously suggested that Cdc25 can be phosphorylated not only by PKA subunits [43], but also by Kss1 and Fus3 MAPK kinases [44]: Cdc25 would be a likely control point for MAPKs cross-regulation on Ras signaling, and this could happen in the nucleus, where the active MAPKs seem to reside [45].

Otherwise, we suggest that Cdc25 could have additional, not essential, Ras-independent functions in the nucleus. In fact, the $CDC25^{NES}$ strain, where Cdc25 is prevented from accumulating in the nucleus, cannot hardly grow on non fermentable carbon sources. Consistently, it was previously suggested that Cdc25 could be involved in peculiar transcriptional effects that are not PKA dependent, such as glucose derepression [46], and that Cdc25 is required for regulation of galactose-induced genes [5,47]; furthermore, besides several interactions of Cdc25 with proteins with a nuclear localization or a nuclear function had independently been reported, such as Clb3 [48], Nfi1 [49], Swc4 [50], Cdc25 was also found in complexes containing nuclear proteins such as Enp1, Glc7 and Fpr3 [51]. Further studies will focus on determining if gene transcription is affected by Cdc25 presence in the nucleus.

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