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The Eliminated Negative Charge at NLS increases the PAK I Binding to Karyopherins

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Abstract

Objective: Pak1 (p21-activated kinase 1) is increasingly linked to cancer. This is surprising since the PAK I proteins, which participate in the actin-cytoskeleton formation, are tightly regulated by the Cdc42 GTPase. However, assaying interactions of Pak1 with β-karyopherins, the nuclear transport factors, suggests that it could cause cancer in the karyopherin-dependent manner. The Pak1-dependent depletion of Kpnβ1 is predicted to strongly increase the requirement for the Kpn β1 expression. In turn, the increased expression of Kpn β1 is predicted to increase nuclear import of the AP-1 transcriptional regulator, cyclin D1 expression, cell proliferation and cancer growth. We propose that increased binding of Pak1 to Kpn β1 initiates vicious circle in which the Pak1-dependent depletion of Kpn β1 is followed by the increased expression of this protein and cell proliferation.

Methods: The mutagenized Ste20 PAK from *S. cerevisiae* was tested for its ability to bind to karyopherins. The mutant-expression phenotypes were examined to establish the β -karyopherin deficiency.

Results: Ste20 forms protein complexes with two β-karyopherins, Kap121 (Kpn β3) and Kap123 (Kpn β1-like karyopherin). The loss-of-phosphorylation mutation (Ser268, Ser269→Ala268, Ala269) increases the binding to Kap123. The deletion of a Cdc42-binding domain (ΔCRIB) increases binding to Kap121. The PAK I binding to karyopherins is separable from its kinase function.

Conclusion: p21-activated kinases could regulate the *in vivo* availability of β-karyopherin(s). Such a regulation involves the sequestering of karyopherins and increasing the requirement for these proteins expression.

Keywords

NLS; p21-activated kinase 1; β-karyopherins

Introduction

Kpn β1 (karyopherin subunit β1) is a nuclear-transport factor that increases cell proliferation and cancer growth [1-4]. The AP-1 transcription factor, which stimulates the expression of cyclin D1, is one of the Kpn β1 cargo proteins. The expression of cyclin D1 activates the CDK4 and CDK6 cyclin-dependent kinases that phosporylate Rb, a retinolastoma protein, which then releases the E2F transcription factor to cause the cell-cycle activation [5]. In addition to AP-1, the Kpn β1 is known to mediate the nuclear import of several other proteins including NFAT that regulates cell motility and metastasis, the NF-kB cytokine and stress-response factor, and NFY.

The overexpressed in mammalian cells Pak1 (p21-activated kinase 1) is also linked to tumor growth [6,7]. Pak1 contains the N-terminal NLS (nuclear localization signal), and the Pak1 overexpression could potentially cause the karyopherin depletion [8]. The depletion increases the requirement for new Kpn β1 expression and the cell proliferation is also increased (Figure 1) [5].

PAK proteins are the serine-threonine kinases which regulate the formation of actin-cytoskeleton, cell motility, and neuronal guidance [6]. Two subfamilies of PAKs were identified: a PAK I subfamily that comprises the mammalian Pak1-Pak3 kinases is responsible for the actin-cytoskeleton formation; while the PAK II subfamily, which consists of Pak4-Pak6 kinases, regulates neuronal outgrowth [6,9]. The PAK I subfamily is regulated by the Cdc42-dependent conformation switch, which causes the kinase activation [6]. The PAK II kinases, on the other hand, are less dependent on a Cdc42 GTPase [9]. The actin-binding myosins are the principal phosphorylation targets of the PAK I proteins, however Pak1 is also known to phosphorylate merlin, the filamin A, and various proteins with a role in signal transduction [6,10].

The re-localizing of Pak1 to the nucleus occurs in the stimulus- and phosphorylation-dependent manner [6,11]. Technically however, the transport of Pak1 (and most other proteins) depends on the α- and β-karyopherin heterodimer. α-karyopherin (Kpn α2, a.k.a. importin β1) is an adaptor protein, which links its cargo to β-karyopherin, while β-karyopherin is responsible for docking the cargo at the nuclear-pore complex [8,12]. The interaction between the cargo protein and β-karyopherin may or may not require α-karyopherin, hence some β -karyopherins are able to recognize the NLS peptides [8,13]. Using a Ste20 PAK from *S. cerevisiae*, we show that this protein could cause the depletion of karyopherins and it could therefore increase the requirement for these proteins expression.

The Ste20p kinase from *S. cerevisiae* is a functional homologue of the PAK I subfamily. A comparative analysis of Ste20 with other PAKs had initially suggested that Ste20 is close to the mammalian Pak2, however based on the overall domain organization and behavior of the truncated Ste20 in solution, this kinase could also be considered the yeast homologue of Pak1 [14,15]. Ste20 contains NLS and predicted C-terminal NES (nuclear export signal), and it could shuttle between nucleus and cytoplasm [16-18]. Under normal growth conditions, the GFP-tagged Ste20 resides in cytoplasm [18,19]. Following oxidative stress, which in yeast cells is linked to inhibited mitochondrial function, Ste20 accumulates in the nucleus: the energy depletion increases nuclear accumulation of the NLS-containing proteins [16,20-22].

Similarly to other PAKs, Ste20 exists as an auto-inhibited kinase whose activation depends on the Cdc42 Rho-type GTPase [6,14]. Together with Cla4 kinase, Ste20 regulates bud emergence, and it also has a role in the activation of kinase-signaling cascade (Ste11-Ste7-Kss1/Fus3) [23]. The Ste 20-mediated signaling cascade regulates yeast mating response and filamentous growth [23-25]. The exact signaling output depends on upstream regulators: the pheromone

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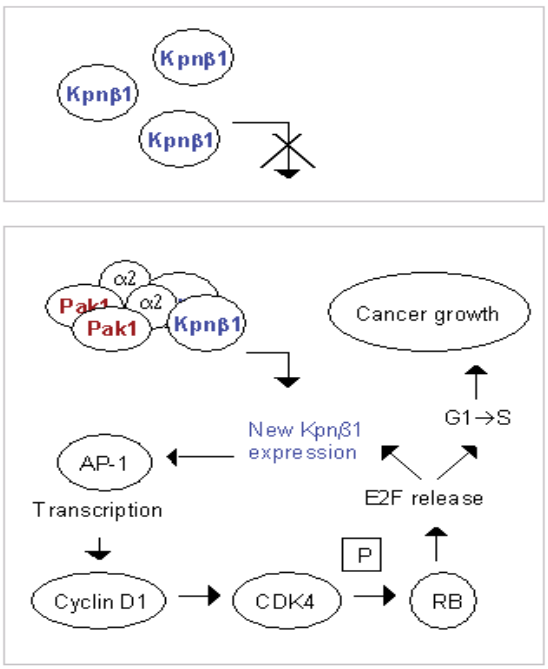

Figure 1: The carcinogenic effect of Pak1 could be linked to the Kpn1β binding [1,3,5].

peptide and Gβγ-subcomplex activate mating response, while Bmh1/Bmh2 and Ras activate filamentous growth [23,26].

In addition to Cdc42, which targets the PAK protein to the sites of polarized growth, the Ste20p localization depends on the Cdc28-Cln2 complex which phosphorylates Ste20 and causes its redistribution to the bud [18-19,27]. As already mentioned, the nuclear localization of Ste20 PAK is associated with its N-terminal lysine-rich peptide (^{272}KK…KKRK^{288}) [16]. Immediately upstream NLS, Ste20 contains Ser268 and Ser269, two amino-acid residues that could undergo phosphorylation [27]. In this study, we show that a double S268,S269→A268,A269 amino-acid substitution, which impairs the phosphorylation, is increasing the Ste20p binding to karyopherins, thereby causing their depletion.

The previously described PAK I mutants were also tested in this study to determine that a ΔCRIB (Ste20$^{\Delta 334\text{-}369}$) mutant, which lacks the Cdc42-binding domain and causes the PAK I conformation switch, is increasing its binding to Kap121/ Pse1 (the homologue of Kpnβ3) [28-31]. The non-mutagenized Ste20 was interacting with Kap123, the Kpnβ1-like nuclear transport factor [29,32,33]. Responsible for the nuclear import of numerous proteins, Kap123 is known to regulate the stability of mitotic microtubules [33]. The S268,S269→A268,A269 amino-acid substitution of Ste20 strongly increased its binding to Kap123, thereby causing the phenotypically detectible depletion of this protein. Our findings suggest that PAKs could regulate the availbility of β-karyopherins in yeast and mammalian cells.

Materials and Methods

Strains and growth conditions

*ste20*Δ cells in the W303-1a (YEL206) background were used to express *STE20* mutants [34]. The mutagenized *STE20* was expressed from the pRS316 (*CEN URA3*) or pRS313 (*CEN HIS3*) plasmids. The Ste20p conformation switch mutant (Ste20$^{\Delta 334\text{-}369}$ or Ste20$^{\Delta CRIB}$) was described previously [14]. The NLS-deleted Ste20$^{\Delta 269\text{-}289}$ and other Ste20 constructs were a gift from Cunle Wu (Biotechnology Research Institute, NRC, Montreal, Canada). The NLS deletion (Δ269-289) was created using the CT TCG AAA AAT CCT TTA AAA AAC TCC TCT//GGT TCA AAT AGT GGT ACA CTA AG and its reverse primer. The Ste20^{A268,A269} was created using the CCT TTA AAA AAC GCC GCT CCA CCT AAA AAG C and its reverse primer, and Ste20^{D268,D269} mutant was created using the CCT TTA AAA AAC GAT GAT CCA CCT AAA AAG C and its reverse primer. The mutations were introduced into the *Bam*HI-*Xho*I *STE20* fragment that was subcloned into pRS313-*STE20*, or pRS316-*GFP-STE20* for yeast expression, and in the pGEX4T-3-*STE20* for the *E. coli* expression [35,36]. The *kap121-41* mutant in DF5 strain (*MAT*a *ura3/ura3 his3/his3 trp1/trp1 leu2/leu2 lys2/lys2 kap121*Δ*/ pkap121-41-URA3*) and *kap123*Δ cells were described previously [33,37].

The karyopherin binding

For immunoprecipitation, the DF5 cells with genome-integrated *KAP60-protA, KAP95-protA, KAP121-protA*, and *Kap123-protA* were used [38]. Yeast were disrupted using a French-pressure Cell® in the Tris-HCl buffer that contained 20 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM DTT, and protein inhibitors. Yeast lysates were cleared by centrifugation and diluted with the same buffer, which however contained 250 mM NaCl and 0.2% Triton X-100. The proteins were extracted and subjected to immunoprecipitation using the anti-Ste20 antibodies [35]. The recombinant GTP-loaded Ran (Gsp1) [37] was added at 0.1 mg/ml.

The *in vitro* binding assay was as described [37]. The bacterially expressed GST-Ste20 was purified on the glutathione-Sepharose in the 20 mM (pH 7.4) HEPES buffer, which contained 110 mM KoAc, 1 mM EDTA and 0.1% Tween 20. The karyopherin-protA enriched yeast cytosols were applied directly to the glutathione-bound GST-Ste20. To obtain cytosols, yeast was processed as above, i.e. as for the immunoprecipitation.

Microscopy

Cells were examined using the Aristoplan microscope (Leitz Wetzlar, Germany). The FV1200 Laser Scanning Confocal Microscope was provided by Carsen Group (Markham, ON, Canada). The oxidative stress was induced in presence of 10 mM hydrogen peroxide or 5 mM sodium azide, as described [20,22]. For immunofluorescence, cells were processed as described [39,40]; the monoclonal anti α-tubulin antibody was used.

Other assays

The Becton Dickinson Flow Cytometer was used to analyze DNA content. Cells were fixed in ethanol and treated with RNAase A and 0.05% propidium iodide. The resistance to benomyl was assayed as described [33].

Results

Ste20 PAK interacts with α- and β-karyopherins

The DF5 yeast cells that express the protA-tagged karyopherins were used to examine the Ste20p interaction with Kap proteins (Figure 2A). Yeast were disrupted with glass beads, and the anti-Ste20 antibodies were used to immunoprecipitate Ste20. The co-immunoprecipiated karyopherins were detected by Western blot,

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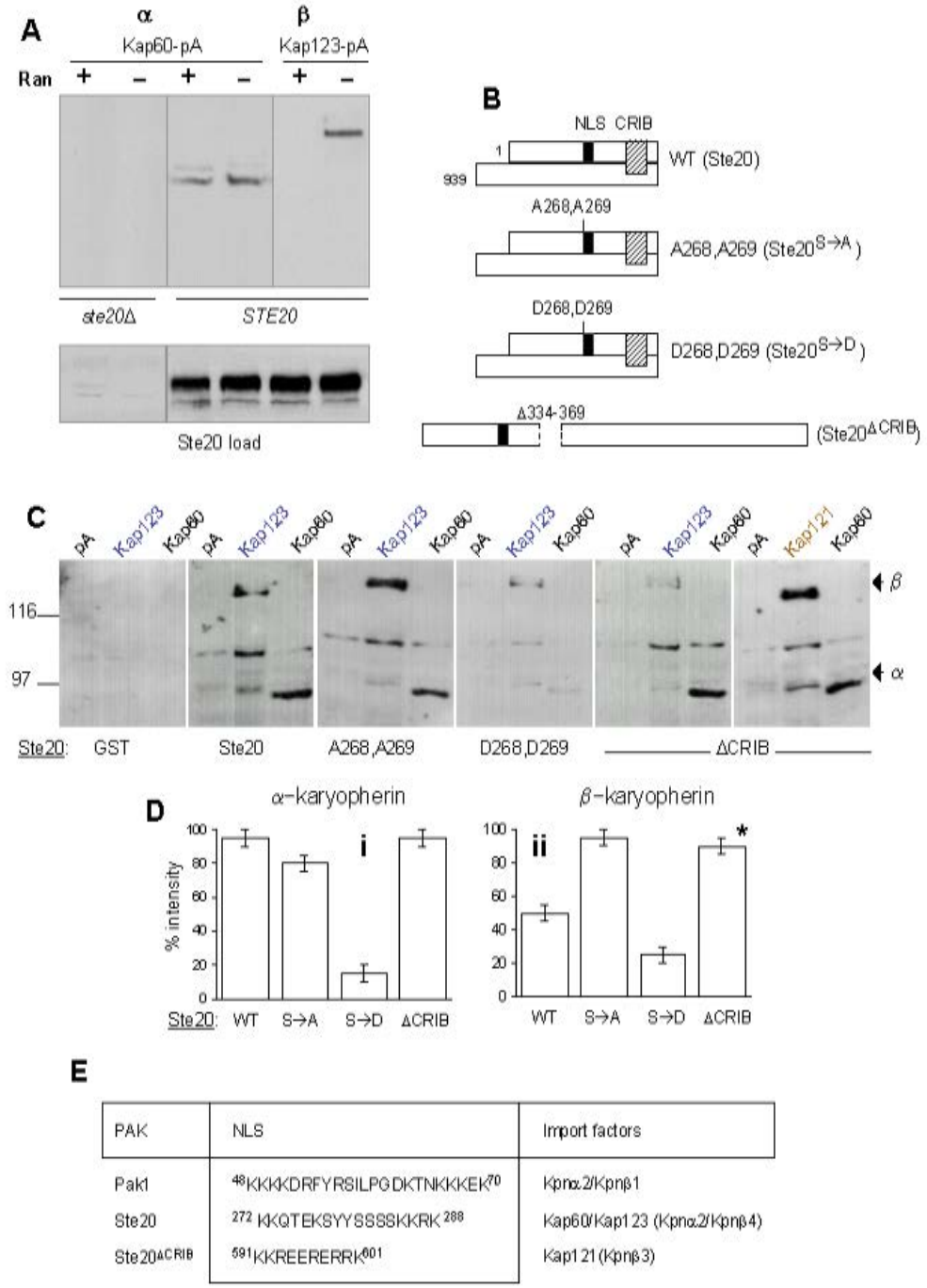

Figure 2: (A) The binding of Ste20 PAK to Kap60-protA (α-karyopherin) and Kap123-protA (β-karyopherin). Ste20 was subjected to immunoprecipiation, and the co-immunoprecipitated proteins were detected by Western blot. The interaction of Ste20 with Kap123 is regulated by Ran. (B) Schematics of the Ste20 PAK mutants used in this study. (C-D) The *in vitro* binding assay and its quantitation. Loss of phosphorylation at NLS (S268, S269→A268,A269) increases the Ste20p binding to Kap123 (Kpnβ1-like karyopherin), while ΔCRIB mutation increases the binding to Kap121-protA (the Kpnβ3 homologue). Error bars indicate the image quantitation error. (E) The ΔCRIB mutation is likely to expose the additional karyopherin-binding epitope.

using the IgG. We determined that Kap60 (the Kpnα2homologue) and Kap123 (the Kpnβ1-like protein) were among the co-immunoprecipitated with Ste20 proteins. The interaction with Kap123 was meaningful since it was sensitive to Ran, a protein that disassociates the karyopherin heterodimer and causes the release of β-karyopherin [12].

The phosphorylation at NLS regulates the Ste20p binding to karyopherins

Immediately upstream NLS (272-288 aa), Ste20 contains the predicted phosphorylation site (Ser268,Ser269), which could be targeted by various yeast kinases including Cdc28, the regulator of

cell-cycle progression [19,27,41]. In order to examine the effect of phosphorylation on the Ste20p binding to Kap123, we introduced A268,A269 and D268,D269 amino-acid substitutions, which imitate the loss or gain of phosphorylation, respectively. We also tested Ste20$^{\Delta CRIB}$, the previously described PAK mutant, which imitates the effect of a Cdc42-dependent conformation switch (Figure 2B) [14].

Using the binding assay for which the recombinant GST-tagged Ste20 proteins were used, we determined that S→A mutation (Ste20^{A268,A269}) increases the Ste20p binding to Kap123, while S→D mutation (Ste20^{D268,D269}) decreases the binding (Figure 2C-D). Ste20$^{\Delta CRIB}$ decreased interaction with Kap123, however it increased interaction with Kap121, yeast homologue of Kpnβ3 [29,33]. We reasoned that a CRIB-domain regulated conformation switch is exposing additional or alternative karyopherin-binding epitope(s), most likely ^{591}KKREERERRK^{601} within the Ste20p kinase domain that increases the ability of this PAK to interact with Kap121 (Figure 2E). Notably, the binding of Kap121 requires other than classical NLS which could contain the negatively charged amino-acid residues and which could therefore bypass the requirement for α-karyopherin [32,37].

The structurally opposite to ΔCRIB Ste20^{H348D} mutation, which locks the kinase in auto-inhibited conformation, did not increase the binding to karyopherins (data not shown) [36]. Not withstanding, overall results of the *in vitro* binding assay (Figure 2 B-C) suggest that auto-inhibited Ste20 conformation is required for the binding to Kap123, while the open conformation is required for binding to Kap121.

Ste20 PAK and the karyopherin-deficient yeast cells

The increased binding of Ste20^{A268,A269} to Kap123 was reflected in its ability to localize to nucleus (Figures 3A-B, Figure 4B, and Supplementry Figure 1). While non-mutagenized Ste20 primarily localizes to nucleus in G_1/G_0 phase, Ste20^{A268,A269} could also localize to the nucleus in the S-phase cells. Ste20$^{\Delta CRIB}$ does not exhibit an apparent nuclear localization, suggesting that it interacts with Kap121 in cytoplasm, but is not very well imported.

The nuclear localization of Ste20 PAK was best visualized in energy-depleted cells. Exposing yeast cells to H_2O_2 or sodium azide is known to inhibit the mitochondrial electron transport and to cause oxidative stress [20,22]. Under energy-depleting conditions, the nuclear export of Ste20 is decreased, while the nuclear retention of this kinase (and many other NLS-containing proteins) is increased [22,42]. The nuclear retention directly depends on the positively charged NLS, which strongly supports the presence of yeast proteins in the DNA-filled environment [42]. The nuclear localization of Ste20 was decreased in the karyopherin-deficient *kap123Δ* and *kap121-41* cells, despite oxidative stress, suggesting that Kap121 and Kap123 are indeed implicated in the Ste20 PAK nuclear transport (Figure 3C).

If we were to summarize our results so far, we would notice that a binding of yeast PAK to karyopherins is regulated by two types of mutations, the phosphorylation-related mutations at NLS and by the CRIB-domain mutation (Figure 4A). The loss-of-phosphorylation mutation (S268,S269→A268,A269) increases the binding of PAK to Kap123, while ΔCRIB increases the binding to Kap121. However, ΔCRIB decreased the binding to Kap123, suggesting that an open Ste20p conformation (Figure 2B) interferes with the interaction. This was not surprising since Kap123 is known to transport compact cargo proteins, such as ribosomal proteins [29]. Overall, the autoinhibited Ste20 was a suitable cargo for Kap123, while Ste20$^{\Delta CRIB}$ was the Kap121 cargo protein.

The Ste20^{D268,D289} mutant, which partially imitates the phosphorylated form of Ste20, failed to interact with either karyopherin (Figure 2C and data not shown) and it was unable to localize to the nucleus. As shown in Figure 4B, the overexpression of Ste20^{D268,289} did not improve its nuclear localization (Figure 4B).

The karyopherin depletion

Kap121 and Kap123 are known to regulate a mitotic progression. Loss of Kap121 activates the mitotic-spindle checkpoint while decreasing the attachment of microtubules to kinetochore, loss of Kap123, on the other hand, decreases stability of mitotic microtubules [30-31,33].

Either Kap121-deficient or *kap123*Δ cells exhibit the G2/M delay and they often retreat to reccurred bud emergence, thereby causing the appearance of mother cells with two or three buds (Figure 5A) [33]. The S→A and ΔCRIB Ste20 mutants, which increase the binding to karyopherins, have similar effect, suggesting that they cause the karyopherins depletion. The reccurred bud emergence was more apparent in the post-diauxic (PD) yeast cultures (Figure 5A, ii) in which the expression of *KAP121* and *KAP123* mRNAs is in decline [43], but not among the logarithmically growing yeast (L-phase). The incidence of binucleation was increased in the Ste20$^{\Delta CRIB}$-expressing cells (Figure 5B) [31].

To ensure that a recurring bud emergence is linked to defective mitosis, we conducted FACS analysis (Figure 5C). We determined that Ste20^{A268,A269} and Ste20$^{\Delta CRIB}$, which were expressed in the hapoloid yeast, strongly increase the number of cells with 2N and 4N DNA content (Figure 5C). Delayed at mitosis, yeast cells were unable to complete cell division and to enter, in a timely manner, a pheromone-inducible growth arrest (Figure 5C, right). Similar defects were reported previously for the karyopherin-deficient *kap121* and *kap123*Δ cells [30,33].

Using immunofluorescence with the anti-α-tubulin antibodies, we determined that expression of Ste20^{A268,A269} negatively influences the mitotic-spindle formation, while increasing yeast sensitivity to benomyl, the microtubule-destabilizing compound (Figure 6A-B). The additional evidence therefore was obtained that Ste20^{A268,A269} is depleting Kap123. The Ste20$^{\Delta CRIB}$-expressing cells were not very sensitive to benomyl (Figure 6B), suggesting that mitotic microtubules are not affected in these cells. Based on the shown in Figure 5 A and B results, we hypothesize that Ste20$^{\Delta CRIB}$ decreases, in the Kap121-involving manner, the attachment of microtubules to kinetochore, but not their stability [31,33]. However, either Kap123-or Kap121-deficiency could cause the G2/M delay and it could increase the percentage of haploid cells with 2N/4N DNA content.

The deletion of NLS modifies the signaling specificity of PAK

In addition to its role in the regulated protein localization, the Ste20p NLS appears to define this PAK signaling specificity (Supplementary Figure 2). Lysine-rich NLS of Ste20 is a so-called red-loop sequence which increases protein disorder upstream CRIB domain [43] (Supplementary Figure 2A). The deletion of NLS increases the ability of Ste20 to dimerize (data not shown), and it modifies this kinase phosphoproteome.

The PAK I proteins form two types of dimers: the anti-parallel dimer increases the inhibitory effect of CRIB domain, while the parallel dimer increases kinase activity or modifies this activity so

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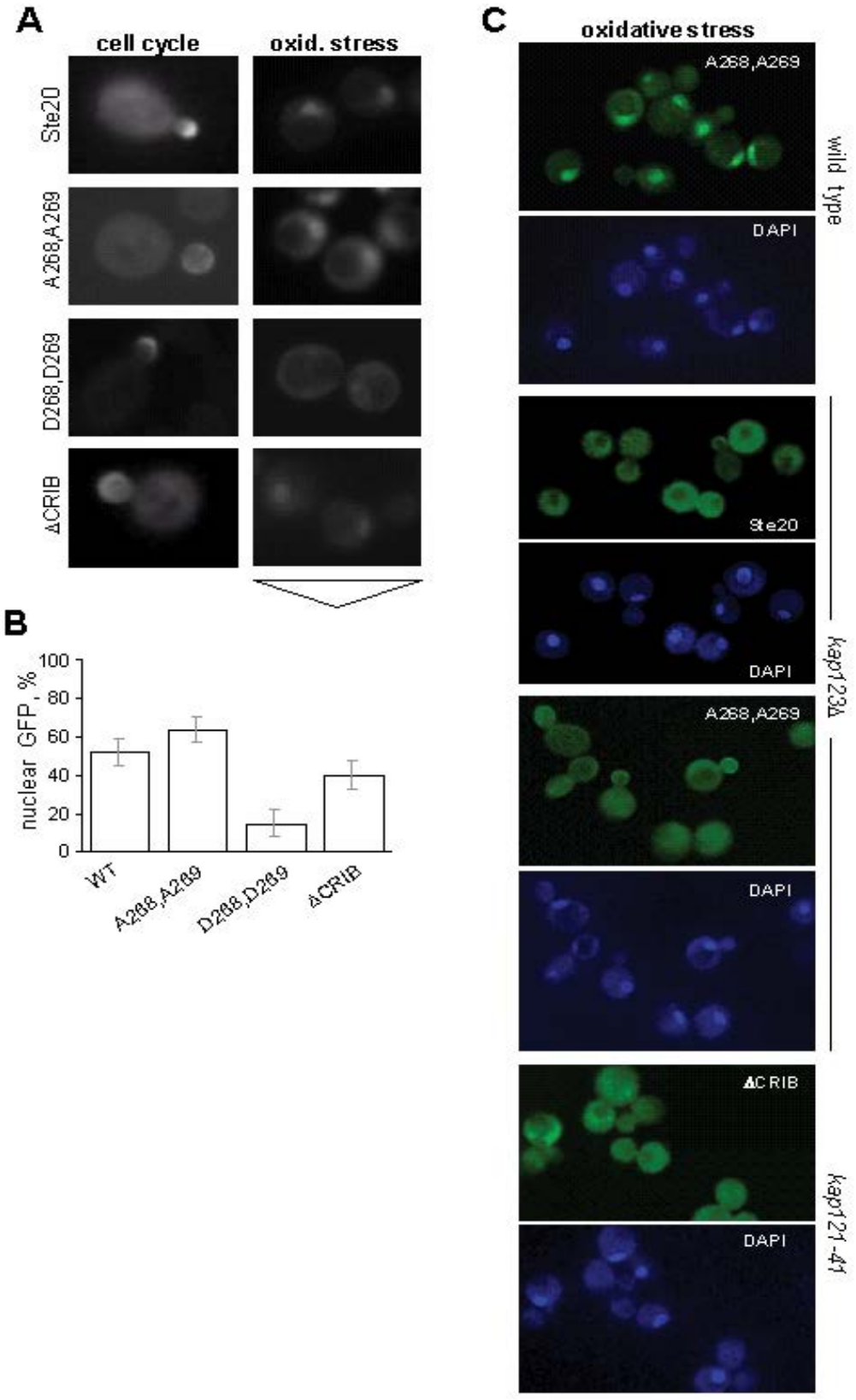

Figure 3: (A-B) The nuclear localization of Ste20 PAK mutants and its quantitation. (C) Nuclear localization of Ste20 PAK is decreased in the *kap121-41* and *kap123*Δ cells. Yeast were grown at 32ºC (the semi-permissive temperature for *kap 121-41* mutant).

A

Ste20 PAK	Type of mutation	β-Kap binding	Nuclear localization
WT	none	moderate	moderate
A268,269	Loss of phosphorylation	increased	moderate to increased
D268,D269	Increased phosphorylation	decreased	impaired
ΔCRIB	Conformation switch	increased*	decreased

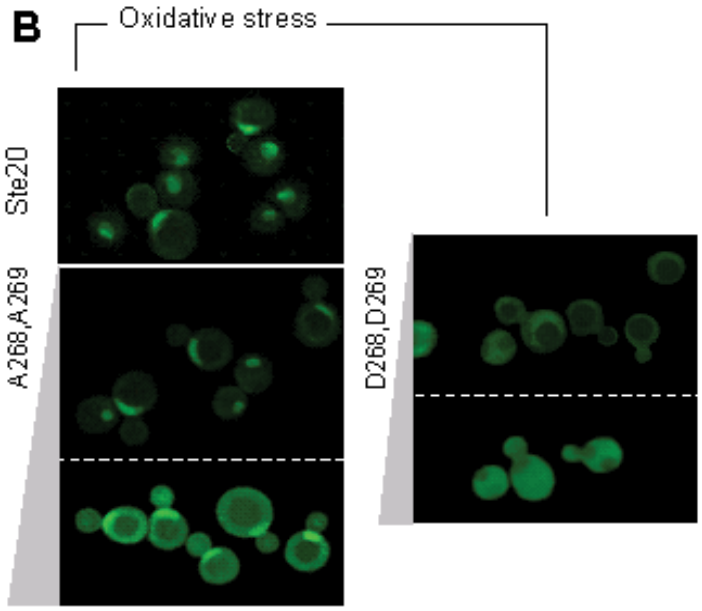

Figure 4: (A) The summary of examined mutant phenotypes. (B) The nuclear localization of Ste20^{D268,D269} is not increased following its overexpression.

that additional proteins could become phosphorylated [15,44,45]. We determined that NLS-deleted Ste20 increases the phosphorylation of the ribosome-assembly and translation-initiation factors (Supplementary Figure 2), suggesting that this PAK mutant probably acquires the substrate specificity of other kinase(s), such as Sch9 for example : yeast homologue of the S6K and the signaling effector of TORC1 signaling [46,47]. The phosphorylation of Ste11, the downstream kinase target of Ste20 PAK was also increased in presence of Ste20$^{\Delta NLS}$ (data not shown) [24]. Our findings suggest that Ste20p NLS not only regulates nuclear localization, but is also responsible for the signaling specificity of this kinase.

Discussion

The Ste20 PAK is known for its role in the polarized yeast growth, mating signaling pathway, and the oxidative stress response [10,14,16,20]. The oxidative stress involves nuclear localization of Ste20p kinase. In this study, we sought to assay the karyopherin-binding specificity of Ste20 in order to determine whether or not this PAK I protein causes the depletion of karyopherins: in a context of mammalian cell, the Pak1-dependent depletion of Kpnβ1 is thought to increase the requirement for this protein expression and cell proliferation (Figure 1)[5].

Yeast cells express three PAK I kinases: Ste20, Cla4, and Skm1. The role of Skm1 was insufficiently studied in budding yeast, however this kinase was linked to cell division in fission yeast (*S. pombe*) [48]. Cla4 has a prominent role in the bud formation and it contains NLS, however: (i) this PAK remains bound to the actin-cytoskeleton througout the entire cell cycle, and (ii) it functionally resembles both the PAK I and PAK II kinases, while Ste20 only resembles PAK I [9,16,49-51].

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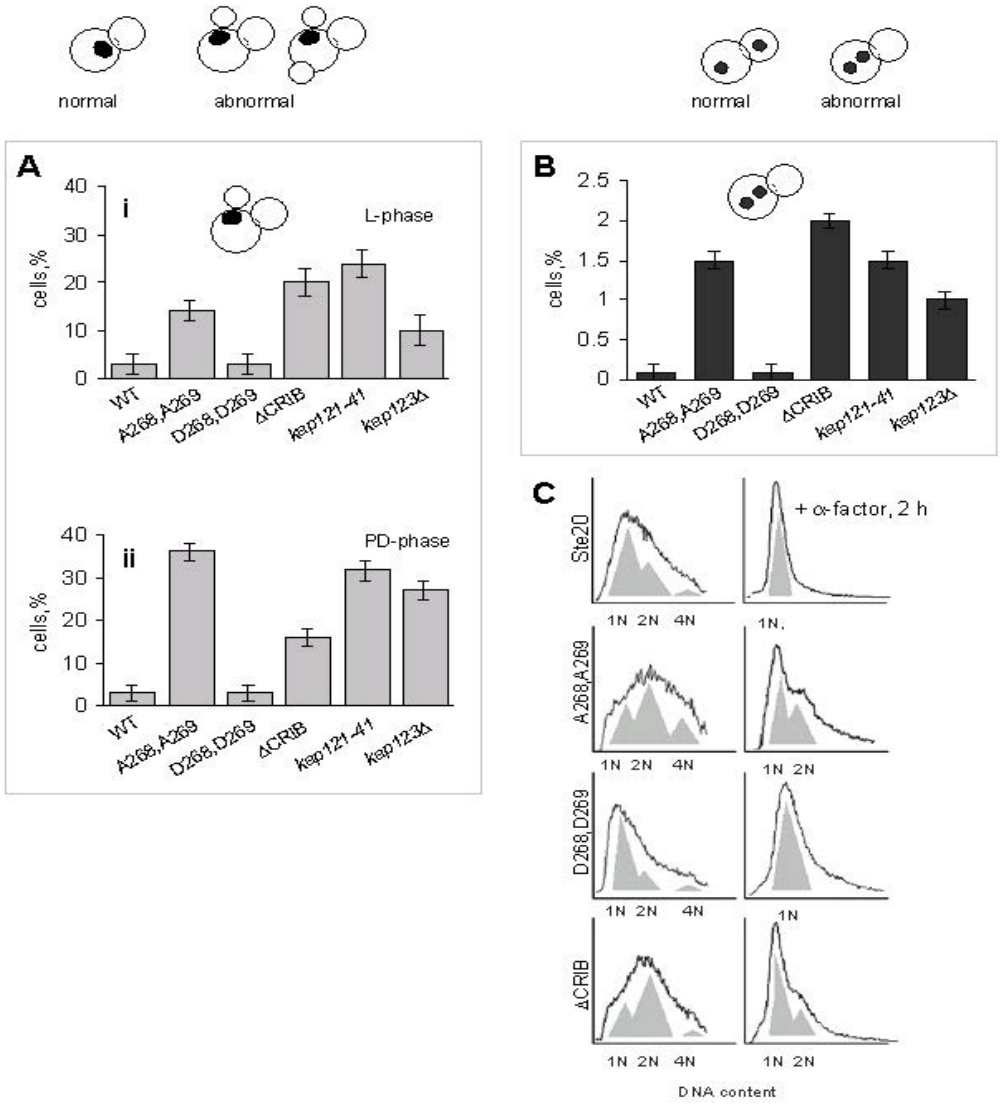

Figure 5: PAK-dependent karyopherin depletion and the mitotic deficiency. (A) Unable to proceed with mitosis, Ste20^{A268,A269}- and Ste20$^{\Delta CRIB}$-expressing cells often contain 2 or more buds. Previously, such a morphogenic defect was demonstrated for the mitosis- and microtubule- deficient *kap121* and *kap123*□ cells [33]. (B) Ste20$^{\Delta CRIB}$ increases incidence of binucleate cells. (C) The results of FACS analysis suggest that mitotic progression is impaired in presence of the karyopherin-sequestering Ste20^{A268,A269} and Ste20$^{\Delta CRIB}$ PAK mutants.

In this study, we assayed the interaction of Ste20 PAK with karyopherins and determined that it strongly interacts with Kap123 (the Kpnβ1-like karyopherin) (Figure 2, A and C)[29,32,33]. In addition, the Ste20$^{\Delta CRIB}$ mutant, which undergoes a conformation switch (Figure 2B), interacted with Kap121/Pse1 [28,29]. The interaction of Ste20 with Kap95 (yeast Kpn1β) was quite weak (data not shown), however the binding to Kap123 was indicative of the overall ability to interact with the Kpnβ1-like proteins [8,29,32]. The Ste20-Kap123 complex formation was likely to involve the Kap60 adaptor protein, and it was sensitive to Ran(Gsp1), a protein that disassociates the karyopherin-protein complexes in the nucleus (Figure 2, A and C) [8,12].

In contrast to Kap123, which primarily imports ribosomal proteins, Kap121 is responsible for the nuclear import of specific transcription factors, incuding Yap1 and Ste12 [30,31,37]. Yap1 is involved in the nutrient-related oxidative stress, while Ste12 is linked to the MAPK-signaling response [37].

Kap121 is linked to mitotic progression [30-31]. Its regulates nuclear recruitment of the mitotic spindle checkpoint proteins (Mad1 and Mad2) and is responsible for the mitotic kinetochore bi-orientation [30,31,33]. The CRIB-domain deletion of Ste20, a mutation that is frequently used to study the effect of PAK I conformation switch (Figure 2B) [6,14] increases the Ste20p binding to Kap121 (Figures 2C) [14,36]. This mutation is predicted to expose the additional karyopherin-binding epitope (Figure 2E), which triggers the binding. The auto-inhibited Ste20^{H348D} mutant and wild-type Ste20 poorly interact with Kap121 (Figures 2C and data not shown), however Ste20 PAK goes back and forth between the auto-inhibited and open conformation [6], suggesting that its interaction with Kap121 could be increased when the kinase undergoes a Cdc42-dependent conformation switch.

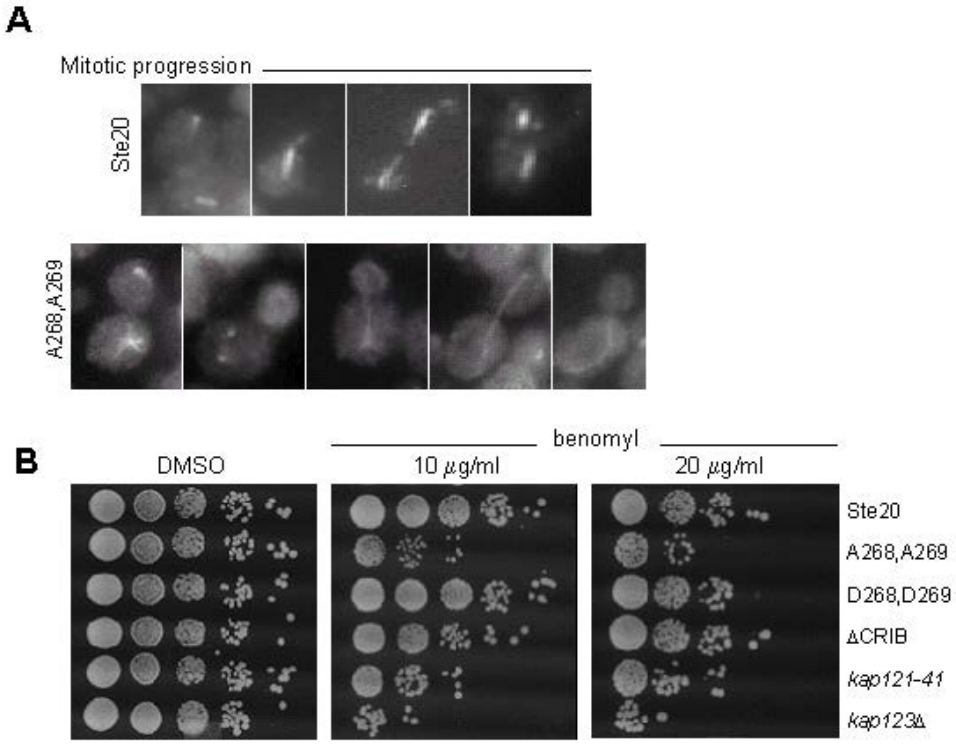

Figure 6: (A) The immunofluorescence using the anti α-tubulin antibody. (B) The increased sensitivity to benomyl is indicative of the decreased stability of mitotic microtubules.

The role of Kap123p in mitotic progression is restricted to regulating the stability of mitotic microtubules [33]. In addition to tubulins, the thickness and stability of mitotic spindle is regulated by the Cdc28 and several other proteins [52,53]. Complexed with the late cyclins, Cdc28 ensures the phosphorylation of γ-tubulin, a spindle-pole body protein, which regulates the thickness of a spindle [52,53]. The effect of Kap123, on the other hand, is associated with its imported Caj1, the member of Hsp40 family that regulates the resistance to benomyl, the microtubule-destabilizing drug [33].

Our findings suggest that either in the Kap121- or Kap123-dependent manner, Ste20 could interfere with mitotic progression. Indeed, the expression of *STE20* gene is known to decrease yeast proliferation, while *ste20*Δ knockout, which lacks the gene, exhibits a faster growth rate [54,55]. The *ste20*Δ knockout also exhibits higher incidence of the chromosome loss, which was previously linked to the Ste20p role in MEN (mitotic exit network): the MEN defects pause mitotic progression and slow cell division [55]. The genetic evidence however strongly suggested that a Ste20p role in mitosis is linked to kinetochore, a place of the microtubule attachment at chromosome, and not to MEN [56]. Our study confirms that genetic evidence and it also suggests that a Ste20p link to kinetochore involves the karyopherins.

The S268,S269→D268,D269 amino-acid sustitution decreases the Ste20p binding to karyopherins (Figures 2C). This was not surprising since S→D is a phosphorylation-mimicking mutation, which decreases nuclear localization of Ste20p [19,57]. The expression of $Ste20^{D268,D269}$ decreased the coordination between the G1-S switch related events [58]: the bud emergence was way ahead of the DNA replication; consequently, $Ste20^{D268,D269}$-expressing cells experienced a replication-related S-phase delay, which however was not related to karyopherins.

The mammalian Pak1 is similar to $Ste20^{D268,D269}$ mutant, which contains the negatively charged amino-acid residues at NLS. The mammalian Pak1 has the ^{46}EEKKKK…KKK^{68} signal which is predicted to weakly interact with karyopherins. There is a possibility however that a negative charge at NLS is somehow eliminated in cancer cells, to increase the interaction of Pak1 with Kpnβ1. One could not exclude a post-translational modification, however cancer is known to correlate with the increased incidence of a codon replacement, which could occur either due to replication or transcription error. The transcription error could be triggered by the NLS-coding sequence itself: the tandem of lysine residues (the AAA, AAG codons) has the appearance of poly-A like sequence, which could pause transcription and plant an error. The likelihood of an error would be increased further in response to the nucleotide disequilibrium which is not uncommon for cancer cell. The random mutagenesis experiments suggest that the reduced levels of one nucleotide dramatically increase the likelihood of amino-acid substitutions [37]. The decrease could occur naturally, in response to various metabolic changes that selectively decrease or increse the levels of specific nucleotides, or in response to chemical treatment which selectively decreases the availability of dCTP or uridine. Most likely, a combination of the nucleotide disequilibrium and paused at the NLS transcription are causing the codon substitution in cancer cells. Future studies will verify whether or not breast cancer cells are more likely to express the Pak 1 mRNA with mutation at NLS.

Indeed, one could speculate that a Pak1 overexpression, which was reported in cancer cells [7], is an important factor that increases

the binding to Kpnβ1 and causes its depletion, however (i) the overexpression of Ste20 (Figure 4B) does not cause the karyopherin deficiency, and (ii) a specific mutation rather than increased protein expression is required to substantially increase the binding of PAK to β1-karyopherin.

In this study, we also report the unusual role of Ste20p NLS which, in addition to nuclear localization, appears to regulate the PAK I signaling specificity. The deletion of Ste20p NLS increases the phosphorylation of Ste11, the signaling target of Ste20p [24,45,59] (data not shown). Apart from Ste11, $Ste20^{\Delta NLS}$ increases the phosphorylation of several ribosome-assembly and translation-initiation factors, suggesting that a signaling specificity of this kinase is modified to resemble the AGC kinases, such as Sch9 [46,47]. The proteins that were phosphorylated in presence of $Ste20^{\Delta NLS}$ included the components of SSU (small subunit processome) and the chromatin remodeling Rsc3-Rsc30 complex [60,61]. The phosphorylation of Bms1 and Utp7 components of the SSU was also visibly increased (Supplementary Figure 2A). Using the radioactive labeling (Supplementary Figure 2C-E), we established that rRNA maturation was delayed in $Ste20^{\Delta NLS}$-expressing cells, while the protein synthesis was increased, suggesting the increased activation of the TORC1 signaling pathway.

In addition to N-terminal NLS, the Ste20p kinase contains a predicted NES, which overalps with the C-terminal Gβ-binding domain [17,62]. The C-terminal GFP tagging decreases the accessibility of NES, thereby decreasing the Ste20p nuclear export while increasing the nuclear localization [18]. The C-terminal Ste20p truncation, which eliminates NES, also causes the accumulation of yeast PAK I in the nucleus (data not shown). If the Ste20p NES is neither obstracted nor deleted, then nuclear retention of this protein is likely to involve oxidative stress, which pauses yeast growth in G1 (strong oxidative stress) or G2/M (mild oxidative stress) and which increases nuclear accumulation of most NLS-containing proteins [16,20-22,63].

Ste20 has a role in the oxidative-stress response. Primarily, this role is linked to the phospho-deacetylation of histone H2B [20,64-66]. In order to rule out that a PAK I-dependent histone modification has direct effect on the proliferative cell growth, we conducted the DNA microarray analysis (Supplementary Figure 3). We expressed the C-terminal *STE20* fragment ($Ste20^{497-939}$) [14] behind the cNLS and determined that it strongly modifies the expression of the H_2O_2- and acid-inducible genes. This is consistent with a previously expressed idea that nuclear localization of Ste20 PAK is linked to apoptosis, which in yeast involves changes in redox homeostasis, but not to cell proliferation [20].

Overall, our study suggests that increased binding of PAK I to karyopherins could have devastating consequences for yeast and mammalian cells. The exact consequences however would be quite different: the karyopherin deficiency in yeast impairs the mitotic progression, while the Kpnβ1 deficiency in mammalian cells is predicted to increase the requirement for this protein expression and cell proliferation [5].

References

1. van der Watt PJ, Maske CP, Hendricks DT, Parker MI, Denny L, et al. (2009) The Karyopherin proteins, Crm and Karyopherin beta, are overexpressed in cervical cancer and are critical for cancer cell survival and proliferation. Int J Cancer 124: 1829-1840.
2. Angus L, van der Watt PJ, Leaner VD (2014) Inhibition of the nuclear transporter, Kpnß1, results in prolonged mitotic arrest and activation of the intrinsic apoptotic pathway in cervical cancer cells. Carcinogenesis 35: 1121-1131.
3. Kuusisto HV, Jans DA (2015) Hyper-dependence of breast cancer cell types on the nuclear transporter importin ß1. Biochim Biophys Acta. 1853: 1870-1878.
4. Kim YH, Ha S, Kim J, Ham SW (2016) Identification of KPNB1 as a Cellular Target of Aminothiazole Derivatives with Anticancer Activity. Chem Med Chem 11: 1406-1409.
5. van der Watt PJ, Ngarande E, Leaner VD (2011) Overexpression of Kpnβ1 and Kpnα2 Importin Proteins in Cancer Derives from Deregulated E2F Activity. PLoS One 6: e27723.
6. Bokoch GM (2003) Biology of the p21-activated kinases. Annu Rev Biochem 72: 743-781.
7. Kumar R, Li DQ (2016) PAKs in Human Cancer Progression: From Inception to Cancer Therapeutic to Future Oncobiology. Adv Cancer Res 130: 137-209.
8. Marfori M, Mynott A, Ellis JJ, Mehdi AM, Saunders NF, et al. (2011) Molecular basis for specificity of nuclear import and prediction of nuclear localization. Biochim Biophys Acta 1813: 1562-1577.
9. Wells CM, Jones GE (2010) The emerging importance of group II PAKs. Biochem J 425: 465-473.
10. Wu C, Lee SF, Furmaniak-Kazmierczak E, Côté GP, Thomas DY, et al. (1996) Activation of myosin-I by members of the Ste20p protein kinase family. J Biol Chem 271: 31787-31790.
11. Oladimeji P, Diakonova M (2016) PAK1 translocates into nucleus in response to prolactin but not to estrogen. Biochem Biophys Res Commun 473: 206-211.
12. Görlich D, Kutay U (1999) Transport between the cell nucleus and the cytoplasm. Annu Rev Cell Dev Biol 15: 607-660.
13. Wozniak RW, Rout MP, Aitchison JD (1998) Karyopherins and kissing cousins. Trends Cell Biol 8: 184-188.
14. Leberer E, Wu C, Leeuw T, Fourest-Lieuvin A, Segall JE, et al. (1997) Functional characterization of the Cdc42p binding domain of yeast Ste20p protein kinase. EMBO J 16: 83-97.
15. Lei M, Lu W, Meng W, Parrini MC, Eck MJ, et al. (2000) Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. Cell 102: 387-397.
16. Lin M, Unden H, Jacquier N, Schneiter R, Just U, et al. (2009) The Cdc42 effectors Ste20, Cla4, and Skm1 down-regulate the expression of genes involved in sterol uptake by a mitogen-activated protein kinase-independent pathway. Mol Biol Cell 20:4826-4837.
17. Kutay U, Güttinger S (2005) Leucine-rich nuclear-export signals: born to be weak. Trends Cell Biol 15: 121-124.
18. Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, et al. (2003) Global analysis of protein localization in budding yeast. Nature 425: 686-691.
19. Wu C, Leeuw T, Leberer E, Thomas DY, Whiteway M (1998) Cell cycle-and Cln2p-Cdc28p-dependent phosphorylation of the yeast Ste20p protein kinase. J Biol Chem 273: 28107-28115.
20. Ahn SH, Cheung WL, Hsu JY, Diaz RL, Smith MM, et al. (2005) Sterile 20 kinase phosphorylates histone H2B at serine 10 during hydrogen peroxide-induced apoptosis in S. cerevisiae. Cell 120: 25-36.
21. Severin FF, Hyman AA (2002) Pheromone induces programmed cell death in S. cerevisiae. Curr Biol 12: R233-R235.
22. Shulga N, Roberts P, Gu Z, Spitz L, Tabb MM, et al. (1996) In vivo nuclear transport kinetics in Saccharomyces cerevisiae: a role for heat shock protein 70 during targeting and translocation. J Cell Biol 135: 329-339.
23. Dohlman HG, Thorner JW (2001) Regulation of G protein-initiated signal transduction in yeast: paradigms and principles. Annu Rev Biochem 70: 703-754.
24. Drogen F, O'Rourke SM, Stucke VM, Jaquenoud M, Neiman AM, et al. (2000) Phosphorylation of the MEKK Ste11p by the PAK-like kinase Ste20p is required for MAP kinase signaling in vivo. Curr Biol 10: 630-639.
25. Flatauer LJ, Zadeh SF, Bardwell L (2005) Mitogen-activated protein kinases with distinct requirements for Ste5 scaffolding influence signaling specificity in Saccharomyces cerevisiae. Mol Cell Biol 25: 1793-1803.

26. Mösch HU, Roberts RL, Fink GR (1996) Ras2 signals via the Cdc42/Ste20/mitogen-activated protein kinase module to induce filamentous growth in Saccharomyces cerevisiae. Proc Natl Acad Sci USA 93: 5352-5356.

27. Oehlen LJ, Cross FR (1998) Potential regulation of Ste20 function by the Cln1-Cdc28 and Cln2-Cdc28 cyclin-dependent protein kinases. J Biol Chem 273: 25089-25097.

28. Chow TY, Ash JJ, Dignard D, Thomas DY (1992) Screening and identification of a gene, PSE-1, that affects protein secretion in Saccharomyces cerevisiae. J Cell Sci 101 : 709-719.

29. Rout MP, Blobel G, Aitchison JD (1997) A distinct nuclear import pathway used by ribosomal proteins. Cell 89: 715-725.

30. Iouk T, Kerscher O, Scott RJ, Basrai MA, Wozniak RW (2002) The yeast nuclear pore complex functionally interacts with components of the spindle assembly checkpoint. J Cell Biol 159: 807-819.

31. Cairo LV, Wozniak RW (2016) The Nuclear Transport Factor Kap121 Is Required for Stability of the Dam1 Complex and Mitotic Kinetochore Bi-orientation. Cell Rep 14: 2440-2450.

32. Wozniak RW, Rout MP, Aitchison JD (1998) Karyopherins and kissing cousins. Trends Cell Biol 8: 184-188.

33. Ptak C, Anderson AM, Scott RJ, Van de Vosse D, Rogers RS, et al. (2009) A role for the karyopherin Kap123p in microtubule stability. Traffic 10: 1619-1634.

34. Leberer E, Wu C, Leeuw T, Fourest-Lieuvin A, Segall JE, et al. (1997) Functional characterization of the Cdc42p binding domain of yeast Ste20p protein kinase. EMBO J 16: 83-97.

35. Wu C, Whiteway M, Thomas DY, Leberer E (1995) Molecular characterization of Ste20p, a potential mitogen-activated protein or extracellular signal-regulated kinase kinase (MEK) kinase kinase from Saccharomyces cerevisiae. J Biol Chem 270: 15984-15992.

36. Ash J, Wu C, Larocque R, Jamal M, Stevens W, et al. (2003) Genetic analysis of the interface between Cdc42p and the CRIB domain of Ste20p in Saccharomyces cerevisiae. Genetics 163: 19-20.

37. Leslie DM, Grill B, Rout MP, Wozniak RW, Aitchison JD (2002) Kap121p-mediated nuclear import is required for mating and cellular differentiation in yeast. Mol Cell Biol 22: 2544-2555.

38. Rout MP, Aitchison JD, Suprapto A, Hjertaas K, Zhao Y, et al. (2000) The yeast nuclear pore complex: composition, architecture, and transport mechanism. J Cell Biol 148: 635-651.

39. Oberholzer U, Iouk TL, Thomas DY, Whiteway M (2004) Functional characterization of myosin I tail regions in Candida albicans. Eukaryot Cell 3: 1272-1286.

40. Iouk TL, Aitchison JD, Maguire S, Wozniak RW (2001) Rrb1p, a yeast nuclear WD-repeat protein involved in the regulation of ribosome biosynthesis. Mol Cell Biol 21: 1260-1271.

41. Oda Y, Huang K, Cross FR, Cowburn D, Chait BT (1999) Accurate quantitation of protein expression and site-specific phosphorylation. Proc Natl Acad Sci U S A 96: 6591-6596.

42. Meinema AC, Laba JK, Hapsari RA, Otten R, Mulder FA, et al. (2011) Long unfolded linkers facilitate membrane protein import through the nuclear pore complex. Science 333: 90-93.

43. Linding R, Jensen LJ, Diella F, Bork P, Gibson TJ, et al. (2003) Protein disorder prediction: implications for structural proteomics. Structure 11: 1453-1459.

44. Buchwald G, Hostinova E, Rudolph MG, Kraemer A, Sickmann A, et al. (2001) Conformational switch and role of phosphorylation in PAK activation. Mol Cell Biol 21: 5179-5189.

45. Pirruccello M, Sondermann H, Pelton JG, Pellicena P, Hoelz A, et al. (2006) A dimeric kinase assembly underlying autophosphorylation in the p21 activated kinases. J Mol Biol 361: 312-326.

46. Pultz D, Bennetzen MV, Rødkær SV, Zimmermann C, Enserink JM, et al. (2012) Global mapping of protein phosphorylation events identifies Ste20, Sch9 and the cell-cycle regulatorykinases Cdc28/Pho85 as mediators of fatty acid starvation responses in Saccharomyces cerevisiae. Mol Biosyst 8: 796-803.

47. Urban J, Soulard A, Huber A, Lippman S, Mukhopadhyay D, et al. (2007) Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. Mol Cell 26: 663-674.

48. Merla A, Johnson D (2001) The Schizosaccharomyces pombe Cdc42p GTPase signals through Pak2p and the Mkh1p-Pek1p-Spm1p MAP kinase pathway. Curr Genet 39: 205-209.

49. Cvrcková F, De Virgilio C, Manser E, Pringle JR, Nasmyth K (1995) Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. Genes Dev 9: 1817-1830.

50. Mitchell DA, Sprague GF (2001) The phosphotyrosyl phosphatase activator, Ncs1p (Rrd1p), functions with Cla4p to regulate the G(2)/M transition in Saccharomyces cerevisiae. Mol Cell Biol 21:488-500.

51. Cohen BA, Pilpel Y, Mitra RD, Church GM (2002) Discrimination between paralogs using microarray analysis: application to the Yap1p and Yap2p transcriptional networks. Mol Biol Cell 13: 1608-1614.

52. Nazarova E, O'Toole E, Kaitna S, Francois P, Winey M, et al. (2013) Distinct roles for antiparallel microtubule pairing and overlap during early spindle assembly. Mol Biol Cell 24: 3238-3250.

53. Winey M, O'Toole ET (2001) The spindle cycle in budding yeast. Nat Cell Biol 3: E23-27.

54. DeRisi JL, Iyer VR, Brown PO (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 278:680-686.

55. Chiroli E, Fraschini R, Beretta A, Tonelli M, Lucchini G, et al. (2003) Budding yeast PAK kinases regulate mitotic exit by two different mechanisms. J Cell Biol 160: 857-874.

56. Tong AH, Lesage G, Bader GD, Ding H, Xu H, et al. (2004) Global mapping of the yeast genetic interaction network. Science 303: 808-813.

57. Gruhler A, Olsen JV, Mohammed S, Mortensen P, Faergeman NJ, et al. (2005) Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. Mol Cell Proteomics 4: 310-327.

58. Skotheim JM, Di Talia S, Siggia ED, Cross FR (2008) Positive feedback of G1 cyclins ensures coherent cell cycle entry. Nature 454: 291-296.

59. Bhattacharjya S, Gingras R, Xu P (2006) An NMR-based identification of a peptide fragment from the beta-subunit of a G-protein showing specific interactions with the GBB domain of the Ste20 kinase in budding yeast. Biochem Biophys Res Commun 347: 1145-1150.

60. Dragon F, Gallagher JE, Compagnone-Post PA, Mitchell BM, Porwancher KA, et al. (2002) A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. Nature 417: 967-970.

61. Cairns BR, Lorch Y, Li Y, Zhang M, Lacomis L, et al. (1996) RSC, an essential, abundant chromatin-remodeling complex. Cell 87: 1249-1260.

62. Leeuw T, Wu C, Schrag JD, Whiteway M, Thomas DY, et al. (1998) Interaction of a G-protein beta-subunit with a conserved sequence in Ste20/PAK family protein kinases. Nature 391: 191-195.

63. Flattery-O'Brien JA, Dawes IW (1998) Hydrogen peroxide RAD9-dependent cell cycle arrest in G2 in Saccharomyces cerevisiae whereas menadione causes G1 arrest independent of RAD9 function. J Biol Chem 273: 8564-8571.

64. Ahn SH, Diaz RL, Grunstein M, Allis CD (2006) Histone H2B deacetylation at lysine 11 is required for yeast apoptosis induced by phosphorylation of H2B at serine 10. Mol Cell 24: 211-220.

65. Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, et al. (2001) Remodeling of yeast genome expression in response to environmental changes. Mol Biol Cell 12: 323-337.

66. Oberholzer U, Nantel A, Berman J, Whiteway M (2006) Transcript profiles of Candida albicans cortical actin patch mutants reflect their cellular defects: contribution of the Hog1 and Mkc1p signaling pathways. Eukaryot Cell 8: 1252-1265.

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