**Schizosaccharomyces pombe** Cells Lacking the Ran-binding Protein Hba1 Show a Multidrug Resistance Phenotype Due to Constitutive Nuclear Accumulation of Pap1*

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Esther A. Castillo‡§, Ana P. Vivancos‡¶, Nic Jones, José Aytés, and Elena Hidalgo‡**

From the 3Cell Signaling Unit, Departament de Ciencies Experimental i de la Salut, Universitat Pompeu Fabra, C/Dr. Aigüader 80, 08003 Barcelona, Spain and 4Paterson Institute for Cancer Research, Christie Hospital National Health Science Trust, Wilmslow Rd., M20 4BX Manchester, United Kingdom

In *Schizosaccharomyces pombe*, the transcription factor Pap1, and the mitogen-activated protein kinase Sty1 are excluded from the nucleus in a Crm1-dependent manner under non-stressed conditions. Upon oxidant treatment, both Sty1 and Pap1 concentrate into the nucleus, due to an enhanced import or an impaired export. Hba1, a protein that when overexpressed confers brefeldin A resistance, contains a Ran binding domain. The purpose of this project was to understand at the molecular level the role of Hba1 in the *S. pombe* oxidative stress response. Fluorescent and confocal microscopy studies demonstrate that Hba1 is located at the nucleoplasm and not at the nuclear envelope. We also demonstrate that either multiple copies or deletion of the *hba1* gene induces nuclear accumulation of Pap1 and Sty1. We propose that Hba1 assists Crm1 to export some nuclear export signal-containing proteins. Pap1 nuclear accumulation is sufficient for constitutive activation of its specific antioxidant response. On the contrary, constitutive nuclear localization of Sty1 in the Δhba1 strain does not trigger the Sty1-specific, Atf1-dependent antioxidant response in the absence of stress. We conclude that the increased multidrug resistance of strains lacking or overexpressing Hba1 is due to the accumulation of Pap1 in the nucleus under non-stressed conditions.

In eukaryotic cells, compartmentation calls for the existence of specific transport of macromolecules among compartments. Import and export out of the nucleus are processes equally important; all nuclear proteins have to be imported from its cytosolic synthesis compartment, and many proteins shuttle continuously between the nucleus and the cytoplasm. Furthermore, RNAs synthesized in the nucleus have to be exported to the cytoplasm by means of the formation of ribonuclear complexes. This active transport between nucleus and cytoplasm requires the formation of transport complexes that, once assembled, are sent to one direction across the nuclear pore complex. The different conditions between both compartments will facilitate the complex to disassemble once it has crossed the nuclear pore complex. The Ran GTPase is a protein present at both sides of the nuclear envelope, its GTP/GDP forms being preferential at each one of them. Ran is, therefore, essential to generate the asymmetry between nucleus and cytoplasm after GTP hydrolysis. The receptor protein Crm1 binds cooperatively with the nuclear form of Ran, Ran-GTP, and with export substrates. This tripartite complex will be exported and disassembled once it reaches the cytoplasm. Crm1 binds its substrates by recognizing some leucine-rich nuclear export signals (NES)1 for reviews, see Refs. 1–3).

Regulation of nuclear transport is an essential process utilized by eukaryotic cells to activate transcription factors or other regulatory proteins (for reviews, see Refs. 4 and 5). In *Schizosaccharomyces pombe* that is the case for the transcription factor Pap1 and for the MAP kinase Sty1 (6, 7). During normal exponential growth both proteins are mainly located at the cytoplasm, but they rapidly transfer to the nucleus upon stress conditions, where they trigger specific cellular responses (6, 8–10). This relocalization is transient, and the proteins rapidly shuttle back to its inactive cytoplasmic localization. For both proteins it has been described that their nuclear export is Crm1-dependent (6, 11).

Sty1, also known as Spc1 and Phh1, is a MAP kinase required for the cell to survive different stress conditions such as osmotic stress, heat shock, nutrient starvation, or multidrug/oxidative stress (6, 12–14). Some of the stress responses, including osmotic and oxidative stress, rely on the activation by Sty1 of the transcription factor Atf1 (15), a constitutively nuclear protein that upon Sty1 activation turns on the expression of anti-osmotic (*i.e.* *gpd1*) and antioxidant (*i.e.* *ctt1*) genes among others (14). Phosphorylation of Sty1 by the MAP kinase kinase Wis1 upon stress triggers Sty1 transport to the nucleus, where it transiently associates with and phosphorylates Atf1 (10), which in turn activates transcription of the stress response genes. According to previous reports (11), stress-induced nuclear accumulation of Sty1 is dependent on an active nuclear import process; partial loss of function of Crm1 does not lead to a constitutively nuclear Sty1, but to an enhanced retention of Sty1 in the nucleus upon stress.

The *pap1* gene has been consistently cloned as a multicopy plasmid conferring resistance to different types of drugs in *S. pombe*, such as brefeldin A (16), caffeine (17), or staurosporin (18). When overexpressed or stress-activated, Pap1 triggers transcription of a set of genes whose products constitute a

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To whom correspondence should be addressed. Tel.: 34-93-542-2891; Fax: 34-93-542-2802; E-mail: elena.hidalgo@upf.edu.

1 The abbreviations used are: NES, nuclear export signal; MAP, mitogen-activated protein; GFP, green fluorescent protein; GST, glutathione S-transferase.
powerful antioxidant cellular response; strains lacking pap1 are hypersensitive to oxidants such as tert-butylhydroperoxide or diamide, heavy metals, and multidrugs. Interestingly enough, similar phenotypes have been described for a Δsty1 strain (6). Upon stress, Pap1 suffers post-translational modifications, which may probably hinder its carboxy-terminal NES and impede Crm1 binding and nuclear export (8). Thus, a strain with the crm1-809 allele, exhibiting a partial nuclear export defect at permissive temperature (19), shows constitutive nuclear localization for Pap1. Mutation rendering a defective Crm1 on overexpression of some of the gene products regulated by Pap1, give rise to strains with increased resistance to drugs and oxidants (17, 19–22).

hba1 is one of the few genes whose overexpression was associated with increased multidrug resistance but had not been connected to Pap1 (23). It was described as an essential gene, although it has more recently been demonstrated that the hba1 knockout is viable (20). Hba1 is a nuclear protein (23) that contains a Ran binding domain (24). Benko et al. (20) described the isolation of a strain resistant to caffeine and brefeldin A harboring a mutation at the hba1 locus, although it was claimed that this phenotype might be due to hyperactivity of the gene product rather than to its inactivation (20). The closest homologue to Hba1 in the gene product rather than to its inactivation (20) described that this phenotype might be due to hyperactivity of Hba1 and Yrb2, RanBP3 (28), in Crm1-mediated export of NES-containing substrates has been recently characterized. RanBP3 has been described to stabilize the interaction between Crm1 and its substrates and influence substrate choice in vitro and in vivo (29, 30). It has been shown not only to enhance the affinity of Crm1 for Ran-GTP and cargo but also for the Ran guanine nucleotide exchange factor RCC1 (31).

In this study we show that overexpression of Hba1 enhances caffeine resistance of S. pombe in a Pap1-dependent manner. We demonstrate that the same effect is accomplished by deletion of the hba1 gene; that is, the lack of function of Hba1 confers caffeine resistance to strains harboring the mutation. Furthermore, either deletion of hba1 or overexpression of its gene product trigger nuclear accumulation of Pap1, and in those strains the transcription of Pap1-dependent genes is constitutively increased. Another reported substrate of Crm1, the MAP kinase Sty1, also shows constitutive nuclear localization under those conditions, although its specific antioxidant response is not switched on in this case. Implications of these results regarding the role of Hba1 in nuclear export and the activation mechanisms of Pap1 and Sty1 are discussed.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—We used in our experiments the wild-type strains HM123 (h leu1) and PN513 (h leu1 ura4; P. Nurse laboratory stock), the Δpap1 strain EHH108 (h leu1 his2 ura4 pap1Δ::ura4−8 (8)), the crm1 mutant TP113.8B (h leu1 ura4 crm1-809) (19), and the Δsty1 strain NT224 (h leu1 ura4 sty1-1). We used strain EHH14 (EHH108 with an integrated copy of the wild-type GFP:pap1 chimera gene under the control of the nmt1 (no message in thimidine) promoter (8)) to visualize the localization of a GFP. Pap1 fusion as well as strain EHH14.C501A.C523A.C532A, encoding a constitutively nuclear GFP. Pap1 mutant activation (8). Dr. Benko kindly provided strains caf1-21 (ura4-D18 h−) and caf1−Δ::ura4−8 (ura4-D18 ade6-704 leu1-32 h−) harboring mutations at the hba1 locus (20). Strain caf1−Δ::ura4−8 was transformed with linearized plasmid pRS54.8X, which contains the nmt-driven GFP:pap1 gene (see above); yielding strain was named EA33. To construct an S. pombe strain expressing a GFP-tagged version of Hba1, we designed specific primers to PCR-amplify a fragment containing the carboxyl-terminal hba1-coding sequence fused in-frame to a GFP-coding sequence followed by the heterologous selectable marker kanMX6 (33, 34). A non-coding hba1 coding sequence using as template plasmid pFA6a-GFP:6S57T::kanMX6 (32). Strain HM123 was transformed with the linear fragment, and recombinants were selected on their ability to grow on G418/Gentamicin (Invitrogen), as described before (32). The resulting strain was named EHH2 (HM123 with hba1::GFP linked to kanMX6). The same strategy was used to generate strain EHH5 (HM123 with sty1::GFP linked to kanMX6). EA16 (TP113.8B with sty1::GFP linked to kanMX6), and EA15 (wild-type PN513 with sty1::GFP linked to kanMX6). We then crossed EA15 with the caf1::ura4−4 strain and selected for ura+, kanamycin-resistant clones, yielding strain EA7 (h leu1 ura4 caf1−4::ura4::GFP::GFP linked to kanMX6). To construct an S. pombe strain with the pap1 and hba1 loci deleted, we first transformed the wild-type strain with the linear fragment containing pap1::kanMX6, obtained by PCR amplification using pap1-specific primers and plasmid pFA6a-kanMX6 as a template (32). We then crossed the resulting strain, AV4 (h leu1 ura4 pap1::kanMX6 with the hba1 strain caf1−4::ura4 and selected for ura+, kanamycin-resistant clones, yielding strain AV6 (h leu1 ura4 caf1−4::ura4 ade6::pap1::kanMX6). We constructed a double Δhba1 Δsty1 strain transforming the caf1−4::ura4−4 strain with a linear fragment containing sty1::kanMX6, obtained by PCR amplification using sty1-specific primers and plasmid pFA6a-kanMX6 as a template, as described above. We named this strain EA32. Cells were grown in rich medium (YES5) or in synthetic minimal medium (EMM2) as described (32, 33, 34).

Plasmids—We used plasmid pREP41-GFP:pap1 (6) to trigger the expression of a GFP.Pap1 fusion protein in S. pombe cells. We PCR-amplified the hba1-coding sequence from an S. pombe cDNA library using primers specific for the gene and containing BamH1 and Smal restriction sites. After digestion of pREP41-GFP:pap1 with BamH1 and Smal, we subcloned the digested PCR fragment containing the hba1 gene, yielding plasmid pG40/1. The same strategy was followed to clone the crm1 open reading frame in pREP41-GFP, yielding plasmid pG30/1.

Caffeine Sensitivity Assay—S. pombe strains were grown in liquid minimal (MM) or rich (YES5) media to an absorbance at 600 nm of 0.5. Cells were then diluted in the same media, and the indicated number of cells for each dilution was spotted on plates with YEPD or YES agar and grown with 30 m M caffeine. The spots were allowed to dry, and the plates were incubated at 30 °C for 2–4 days.

RNA Analysis—Cells grown in minimal media to a final absorbance at 600 nm of 0.5 were left either untreated or were treated for the indicated times with H2O2 at the indicated concentrations or with 4 m M diethylsulfate, also known to trigger Pap1 activation (8). 25–50 ml of yeast cultures were then centrifuged at 1500 rpm for 3 min and washed with H2O, and cell pellets were immediately frozen in dry ice. Each sample was then resuspended in 0.4 ml of AE buffer (50 m M sodium acetate, pH 5.3, 10 m M EDTA, pH 8.0). Sodium dodecyl sulfate was then added to a final concentration of 1%, and proteins and DNA were extracted by adding 0.3 ml of acidic phenol and incubation at 65 °C for 4 min. Samples were cooled down in dry ice, 0.3 ml of chloroform was added, and the aqueous phase was separated by centrifugation at 10,000 × g for 2 min at 4 °C. After chloroform extraction, RNA was precipitated with ethanol. RNA concentration was determined by absorbance at 260 nm, and equal amounts (10 μg) were loaded in form aldehyde-agarose gels (38) containing ethidium bromide to confirm equal loading by visualizing ribosomal RNA. RNA was then transferred to GeneScreen Plus nylon membranes (PerkinElmer Life Sciences). Hybridization and washes were performed as recommended by the manufacturer. The blots were hybridized with the Pap1-dependent apt1 probe containing the complete open reading frame of the p25-encoding region of the Sty1−, Aft1−, and Aft2−dependent gpx1 probe, coding a 419-bp fragment of the glutathione peroxidase-encoding gene. Probes were labeled with [32P]dCTP using a random primer system from Roche Applied Science.

Western Blot Analysis—To purify a GST.Hba1 fusion protein, E. coli strain F8910 cells (37) transformed with the pGEX-derivatives plasmid pGEX.Hba1 were incubated in LB broth with 100 m M of ampicillin and incubated at 37 °C for ~16 h with vigorous shaking. The overnight culture was diluted 25-fold into 100 ml of fresh medium and incubated at 37 °C until the culture reached an optical density of 1 at 600 nm. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 0.5 m M, and shaking was continued at 25 °C for 16 h. Cells were then harvested, and the cell pellets were resuspended in 4 ml of STET extraction buffer (50 m M Tris-HCl, pH 8.0, 150 m M sodium chloride, 1 m M EDTA pH 8.0, 1% Triton X-100) and broken by sonication. Debris and unbroken cells were removed by centrifugation. GST-Hba1 was bound to glutathione S-
**RESULTS**

**Overexpression of Hba1 Triggers Activation of Pap1**—The fungal metabolite brefeldin A, an inhibitor of protein secretion, was used by Rose and co-workers (16) in a genetic screening to identify proteins capable of conferring resistance to this drug. Mutations at the *pap1* or *crm1* loci leading to constitutive activation of the Pap1-dependent antioxidant response yielded strains resistant to brefeldin A. Interestingly, overexpression of another protein, Hba1, also led to the same resistant phenotype (23). We decided to analyze whether Hba1 could have a role in protein nuclear transport and, consequently, might exert its role in brefeldin A resistance through regulation of Pap1 subcellular localization, as it had been previously described for Crm1. A wild-type strain containing an integrated GFP-*pap1* allele was transformed with a plasmid containing the *hba1* gene under the control of the thiamine-repressible, intermediate strength promoter *nmt* 41x (35). The localization of the GFP-Pap1 fusion protein, as determined by fluorescence microscopy under basal or non-stressed conditions, was cytosolic (Fig. 1A). However, when Hba1 was overexpressed, the localization of the fusion protein was nuclear even in the absence of stress. Once established that Pap1 was constitutively nuclear upon Hba1 overexpression, we analyzed whether the change of cellular localization was sufficient to trigger Pap1-dependent activation. As shown in Fig. 1B, the level of *p25* transcript, as determined by Northern blot analysis, was constitutively high in a wild-type strain overexpressing Hba1 (HM123-p20.41x). As a control of regulation by nuclear export, the same strain overexpressing Crm1 (HM123-p30.41x) showed undetectable levels of *p25* expression in the absence of oxidative stress when compared with the basal level of wild-type HM123.

Pap1 overexpression or activation has been frequently associated to multidrug resistance. We determined that Hba1 overexpression could also lead not only to brefeldin A resistance, as described by Rose and co-workers (23), but also to caffeine resistance (Fig. 1C). Such phenotype was abolished when Hba1 was deleted. This suggests that Hba1 may have a role in multidrug resistance through regulation of Pap1. A localization of the GFP-Pap1 fusion protein in a strain overexpressing Hba1 is shown in Fig. 1B. The cellular distribution of GFP-Pap1 protein under non-stressed conditions was determined by fluorescence microscopy in both strains. B, Northern blot analysis of Pap1-dependent *p25* expression. Total RNA from wild-type strain HM123, HM123 transformed with plasmid p20.41x, or with plasmid p30.41x containing the *crm1* gene under the control of the *nmt* promoter was obtained. Cultures had been left untreated (−) or were treated for 15 min with 0.5 mM H2O2, previous to RNA extraction (+). C, caffeine resistance analysis of strains overexpressing Hba1 and carrying or not carrying a deletion on the *pap1* gene. Strains HM123 (WT), HM123 transformed with p20.41x, and EHH108 (Δ*pap1*) transformed with p20.41x, were grown in minimal media (MM) to a final *A*₅₀₀ of 0.5 and spotted onto minimal media plates with or without 30 mM caffeine. Plates were incubated at 30 °C for 3–4 days.

**Fig. 1. Overexpression of Hba1 triggers Pap1 activation.** A, localization of GFP.Pap1 protein in a strain overexpressing Hba1. Strain EHH14 (Δ*pap1* with an integrated version of the GFP.*pap1* gene under the control of the *nmt* promoter) was transformed or not with a plasmid containing the *hba1* gene under the control of the *nmt* promoter (p20.41x or p*pha1*). The cellular distribution of GFP.Pap1 protein under non-stressed conditions was determined by fluorescence microscopy in both strains. B, Northern blot analysis of Pap1-dependent *p25* expression. Total RNA from wild-type strain HM123, HM123 transformed with plasmid p20.41x, or with plasmid p30.41x containing the *crm1* gene under the control of the *nmt* promoter was obtained. Cultures had been left untreated (−) or were treated for 15 min with 0.5 mM H₂O₂, previous to RNA extraction (+). C, caffeine resistance analysis of strains overexpressing Hba1 and carrying or not carrying a deletion on the *pap1* gene. Strains HM123 (WT), HM123 transformed with p20.41x, and EHH108 (Δ*pap1*) transformed with p20.41x, were grown in minimal media (MM) to a final *A*₅₀₀ of 0.5 and spotted onto minimal media plates with or without 30 mM caffeine. Plates were incubated at 30 °C for 3–4 days.
was overexpressed in a Δpap1 strain (Fig. 1C), indicating that the multidrug resistant phenotype conferred by Hba1 overexpression is dependent on Pap1. Whereas loss of function of the nuclear exporter Crm1 leads to a nuclear and constitutively active Pap1 (6, 17), overexpression of Hba1 can also trigger Pap1 activation, which led us to believe that Hba1 could have some role in Pap1 nuclear import.

**Lack of Function of Hba1 Also Induces Pap1 Activation**—A strain carrying a mutation at the hba1 locus was isolated in a screening for caffeine resistant S. pombe cells (20). The authors claimed that the caffeine resistance conferred by the mutated allele, named caf1-21, had to be consequence of hyperactivity rather than inactivation of the gene product (20). We PCR-amplified and sequenced the mutated allele, detecting a GC to TA nonsense mutation at base pair 265, leading to an incomplete protein of only 88 residues (full-length Hba1 is 399 amino acids long). We further confirmed the absence of a full-length Hba1 protein in the caf1-21 strain by Western blot analysis of total protein extracts, using antibodies raised against GST.Hba1 (Fig. 2A). Hba1 could be detected in extracts prepared from a wild-type strain as a single band of ~60 kDa with denaturing electrophoresis (lane 1, Fig. 2A). Overexpression of the protein in a strain carrying plasmid p20.41x led to a >10-fold increase in Hba1 concentration (lane 4, Fig. 2A). Extracts from strains carrying the caf1-21 allele or deleted for hba1 showed no detectable full-length Hba1 (lanes 3 and 2, respectively, Fig. 2A).

Once confirmed that the caffeine-resistant caf1-21 strain was defective for Hba1, we tested whether Pap1 cellular localization was altered in this strain. A GFP.Pap1 fusion protein was constitutively nuclear in a caf1-21 background (data not shown), as it was in a Δhba1 strain (Fig. 2B). Northern blot analysis confirmed that either multiple copies or deletion of hba1 triggered Pap1-dependent transcription of the p25/ap1 gene even under non-stressed conditions and that such an effect was totally dependent upon the presence of Pap1 (Fig. 2C). Thus, the activation of the Pap1-dependent antioxidant/multidrug response of a strain deleted for hba1 led to a caffeine-resistant phenotype, as shown in Fig. 2D, whereas a double hba1/pap1 knockout strain showed a sensitivity to the drug similar to a wild-type or a Δpap1 strain.

**Hba1 Is Required for the Nuclear Export of Both Pap1 and Sty1**—Originally, Hba1 was described as a protein containing a Ran-binding protein domain, and therefore, it could be involved in either nuclear import or export (23, 24). Hba1 has homology to the S. cerevisiae protein Yrb2 and to the human RanBP3, especially at the Ran binding domain (Fig. 3A). When overexpressed all three orthologs were localized to the nucleus (23, 25, 30). We decided to confirm Hba1 nuclear localization expressing an Hba1.GFP fusion protein at wild-type level. As shown in Fig. 3B, the fusion protein conferred nuclear staining by fluorescence microscopy. We also performed confocal microscopy studies of the Hba1.GFP strain, which further demonstrated that the fusion protein was located at the nuclear matrix and not at the nuclear membrane (data not shown). That seems to indicate that Hba1 participates in the protein export pathway rather than in protein nuclear import.

Pap1 is not the only oxidative stress sensor which activity is regulated at the level of cellular localization. Sty1 also concentrates at the nucleus upon H$_2$O$_2$ stress (11). We decided to
study how Hba1 function could affect the localization of NES-containing cargo Sty1 (Fig. 4A). Inhibition of nuclear export with the Crm1 binding drug leptomycin B or expression of the GFP fusions in a crm1 mutant background (19) resulted in nuclear localization of GFP.Pap1 (Fig. 4B) as it had been described before (6, 9, 39). A Sty1.GFP fusion, expressed at wild-type levels, also accumulated in the nucleus upon Crm1 inhibition (both by leptomycin B treatment or in the crm1 mutant background), even though the same crm1 mutation had been reported to trigger an enhanced retention of Sty1 in the nucleus after stress withdrawal but not the basal nuclear localization of Sty1 (11).

Both GFP.Pap1 and Sty1.GFP fusion proteins had nuclear localization under non-stressed conditions when Hba1 function was impaired either by deletion or overexpression of the hba1 gene (Fig. 4B). Therefore, Hba1 seems to participate, together with Crm1, in both Pap1 and Sty1 export. The protein levels of GFP.Pap1 and Sty1.GFP were not modified in the different strain backgrounds, as determined by Western blot analysis (data not shown).

Nuclear Accumulation of Pap1, but Not of Sty1, Triggers an Antioxidant Response as Well as Growth Defects and Caffeine-resistant Phenotypes—Recent studies found that S. pombe mounts two separate responses upon H$_2$O$_2$ stress, an adaptive response to low levels of H$_2$O$_2$, which protects the cell from subsequent exposures to higher concentrations of H$_2$O$_2$, and an acute or survival response, which allows the cell to survive a sudden and potentially lethal exposure to H$_2$O$_2$ (13, 40). Pap1 is required for the response to low level H$_2$O$_2$ stress, whereas Sty1/Atf1 regulate the response to high levels of the oxidant. Combination of Pap1-dependent and Sty1/Atf1-dependent responses would protect S. pombe against a wide range of oxidants and drugs at different concentrations (40).

Drug and antioxidant resistance of strains with impaired Hba1 function could be due to nuclear accumulation of either Pap1, Sty1, or both. Therefore, it was essential to verify whether the Sty1-dependent transscriptional response was also constitutively active in those strains and could, therefore, exert some protective role against caffeine. Sty1 is phosphorylated and transferred to the nucleus by a variety of environmental stimuli including oxidative, osmotic, and physical stresses (41–43). First, we analyzed whether the change of Sty1 cellular sublocalization upon export defects was concomitant with a phosphorylation of the MAP kinase. A wild-type and a Δhba1 strain were subjected to stress by hydrogen peroxide (Fig. 5A) and sodium arsenite (data not shown), and phosphorylation of Sty1 was determined by Western blot analysis using an antiphospho-p38 MAPK antibody. No significant differences at the basal or induced levels were detected between both strains.

Transcription of the gene gpx1, encoding glutathione peroxidase, upon oxidative stress has recently been reported to depend exclusively on Sty1 and Atf1 activation but not on Pap1 (40). As shown in Fig. 5B, expression of gpx1 was unaffected in a Δhba1 strain compared with a wild-type strain, both at the basal and oxidative stress-induced levels. Unlike Pap1, nuclear localization of Sty1 upon Hba1 loss-of-function is not concomitant with a Sty1 gain-of-function, since it is not phosphorylated in the absence of stress, and its specific antioxidant response is not constitutively triggered. We confirmed this fact analyzing the multidrug resistant phenotype of a double knockout strain; cells lacking both Hba1 and Sty1 retained the caffeine resistance phenotype of a Δhba1 strain (Fig. 5C).

According to the previous data, the phenotypes observed in strains lacking a functional Hba1 (growth defects (44) and increased caffeine tolerance; see Figs. 1 and 2) could be exclusively due to the constitutive nuclear location of Pap1. To test this hypothesis we analyzed the phenotype of a strain, EHH14.C501A,C523A,C532A, harboring an integrated GFP.pap1 allele encoding a fusion protein with three cysteine to alanine substitutions close to the NES of Pap1, which renders a constitutively nuclear and transcriptionally active GFP.Pap1 (8). As shown in Fig. 6A, such a strain is as resistant to the presence of 30 mM caffeine in plates as the hba1 knockout when compared with a wild-type strain. Furthermore, the same growth defects were observed in the Δhba1 strain and in the EHH14.C501A,C523A,C532A strain, as reflected by their reduced colony size (data not shown) and their impaired growth curves, which presented an elongated duplication time (3.08 ± 0.02 h for the wild-type strain (HM123), 4.82 ± 0.38 h for Δhba1 strain (cafl1::ura4)), and 5.10 ± 0.65 h for EHH14.C501A,C523A,C532A strain, all grown in minimal media at 30 °C) and a reduced maximum of absorbance at 600 nm when compared with a wild-type strain (Fig. 6B).

DISCUSSION

Antioxidant defenses and multidrug resistance often use the same signal transduction pathways for cell signaling both in prokaryotic and eukaryotic cells. Thus, the identification of molecular components of antioxidant pathways has often been accomplished through genetic screenings of mutant clones resistant to different drugs. In particular, mutations at different loci whose gene products up-regulate the activity of the transcriptional factor Pap1 have been previously associated with increased resistance to several drugs. It had been reported that overexpression of Hba1, a RanBP-like protein, could confer brefeldin A resistance (23) and that certain mutations at the hba1 locus could also increase caffeine tolerance (20). In this manuscript we demonstrate that Hba1 participates in the Crm1-
dependent nuclear export of some NES-containing cargoes and that loss of function of Hba1 either by mutation or overexpression triggers basal nuclear accumulation of the antioxidant activating proteins Pap1 and Sty1. As a consequence of such nuclear accumulation, the Pap1-dependent transcriptional response, but not the Sty1-dependent response, is constitutively active under non-stressed conditions. The same constitutive gain-of-function is accomplished when *S. pombe* cells express Pap1 mutant proteins with impaired nuclear export. In both cases, the Pap1-dependent transcriptional response is constitutively activated, and that constitutes the molecular basis for an increased tolerance to caffeine, but it also causes defects in cell cycle progression in the absence of stress.

Many of the gene products isolated through genetic screenings to multidrug resistance exert their protective role due to its connection to the Pap1-dependent transcriptional response. Thus, overexpression of the multidrug ABC-type transporters Hba2 and Pmd1 confer pleiotropic multidrug resistance (21, 22); interestingly, they both are transcriptionally up-regulated by Pap1 in response to oxidative stress (6). So far the loss-of-function of three gene products can lead to Pap1 nuclear accumulation, the Pap1-dependent transcriptional response triggers basal nuclear accumulation of the antioxidant function of three gene products can lead to Pap1 nuclear accumulation, the Pap1-dependent transcriptional response triggers basal nuclear accumulation of the antioxidant function.

![Diagram A](image1.png)

**Diagram A.** A scheme of GFP.Pap1 and Sty1.GFP fusion proteins; the NES within the cysteine-rich domain (CRD) of Pap1 is indicated.

![Diagram B](image2.png)

**Diagram B.** Localization of GFP.Pap1 and Sty1.GFP fusion proteins in wild-type (WT) cells, cells carrying the *crm1-809* allele (*crm1*), cells treated with 100 μM leptomycin B for 50 min (LMB), Δhba1 cells, and wild-type cells transformed with a plasmid containing hba1 under the control of the nmt promoter (phba1). The cellular distribution of the fusion proteins was determined by fluorescence microscopy. Strain names from left to right and top to bottom panels are as follows: EHH14 (Δpap1 with integrated GFP.pap1 allele), TP113.6B (*crm1-809*) with pRep41-GFP.pap1, EHH14 after leptomycin B treatment, EA33 (caf1::ura4Δ Δhba1) with integrated GFP.pap1 allele), EHH14 with p20.41x (phba1), EHH5 (wild-type strain with integrated sty1.GFP), EA16 (TP113.6B with integrated sty1.GFP), EHH5 after leptomycin B treatment, EA11 (caf1::ura4Δ with integrated sty1.GFP), and EHH5 with p20.41x. The percentage of cells showing nuclear accumulation of GFP.Pap1 is indicated.

![Diagram C](image3.png)

**Diagram C.** Nuclear accumulation of Sty1 in a Δhba1 strain does not trigger the Sty1-dependent antioxidant response. A. Western blot analysis of phosphorylated Sty1 in wild-type and Δhba1 strains. Total protein extracts of strains HM123 (WT) and caf1::ura4Δ (Δhba1) were obtained before and after 10 or 30 min of 1 mM H2O2 stress. 10 μg of each extract were loaded on denaturing gels and immunoblotted with anti-phosphorylated p38 polyclonal antibody. B. Northern blot analysis of the Sty1-ATF1-dependent gpx1 transcription in wild-type and Δhba1 strains. Total RNA from the same strains as in A was obtained before and after 15 and 60 min of 1 mM H2O2 stress. C. Caffeine resistance analysis of strains carrying deletions on the hba1 and/or sty1 genes. Strains HM123 (WT), caf1::ura4Δ (Δhba1), NT224 (Δsty1), and EA32 (Δhba1 Δsty1) were grown in minimal media (MM) to a final absorbance at 600 nm of 0.5 and spotted onto minimal media plates containing or not containing 30 mM caffeine. Plates were incubated at 30 °C for 3–4 days.

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2 A. P. Vivancos, E. A. Castillo, J. Ayte, and E. Hidalgo, unpublished data.
expression are concomitant, indicating that the post-transcrip-
tional modifications suffered by the protein upon signal activa-
tion may alter its conformation and affect its interaction with
the export complexes, but those changes are not required for its
positive modulation of the transcription machinery.

We have shown that constitutive activation of Pap1 not only
confers multiple drug resistance, but it also impairs growth.
The level of reactive oxygen species and the intracellular redox
state are tightly modulated by enzymatic and non-enzymatic
activities (45). At least for E. coli it has been described
that the steady-state concentrations of H$_2$O$_2$ are kept within a
2-fold range among different strains by modulating the levels of
catalase (46). Furthermore, those levels are very similar to the
concentration of intracellular H$_2$O$_2$, which could impair cell
antioxidant activities and to a decrease in the steady-state
may be required as a mitogenic signal. Constitutive activation
of the cells.

The results shown here have important implications regard-
ing Sty1 regulation of gene expression. Sty1 activity has been
described to rely on its level of phosphorylation and is, there-
fore, regulated by its immediate upstream kinase, Wis1, and
two phosphatases, Pyp1 and Pyp2. Sty1 phosphorylation and
Sty1 nuclear translocation have always been described to be
linked. We describe here that, as for Pap1, inhibition of the
export machinery (either of Crm1 or of Hba1) can lead to
constitutive nuclear accumulation of the MAP kinase, contrary
to what had been previously described (11). Nevertheless, that
change of cellular sub-localization under uninduced conditions
does not dramatically modify either the basal level of phos-
phorylation of Sty1 nor its transcriptional activity. Furthermore,
the constitutively nuclear Sty1 kinase seems to respond to
stress following similar kinetics to those described for cytosolic
Sty1, even though its main regulators may be physically sep-
parated in different cell compartments. It has been described
that Wis1 and the tyrosine phosphatases Pyp1/2 are always
kept at the cytosol (11); therefore, the constitutive nuclear
accumulation of Sty1 upon export inhibition could lead to tran-
sient isolation from its regulatory proteins. Nevertheless, re-
cent work indicates that Wis1 could also accumulate at the
nucleus upon certain types of stress (48). Further work will be
required to unravel how the stress-induced kinetics of Sty1
activation in a wild-type versus a Δhba1 strains can be so
similar.

The role of Hba1 on Crm1-dependent export of NES-contain-
ing cargoes is not clear and will need further elucidation. If
export efficiency in vivo correlates with cargo exporter affinity
in vitro then Crm1 affinity for NES-containing substrates is
not sufficient to guarantee a rapid and efficient export in vivo;
under those circumstances, Hba1 and other family members
could act as cofactors of such multiple export interactions and
stabilize the otherwise weak tripartite complex formed by
Crm1-RanGTP export cargo. Regarding this role of Hba1 as an
export cofactor, it is not surprising that both deletion of hba1 or
overexpression of its gene product can lead to the same export
defect phenotype, since the same ability to inhibit export com-
plex formation at higher concentrations have been previously
described for the S. cerevisiae homologue Yrb2 (26, 27) and
for the human homologue RanBP3 in vivo (29). An excess of any of these cofactor family members could be
sequestering Ran-GTP or even the putative exported cargoes,
removing them, and inhibiting their export, since Crm1 con-
centration would be limiting. It is also worth noting that some
NES-containing proteins, such as the NES of protein kinase
inhibitor, do not seem to require Hba1 function to be exported,
at least in S. pombe cells (3). It remains unclear which substrates
do require the participation of these export cofactors. Hba1
might be working as a scaffold to promote the efficient assem-
bly of export complexes, probably degreasing the $K_D$ of Crm1 for
some but not all NES-containing substrates. Another interesting
feature of Hba1 is that it is a phosphoprotein (23), although it
has not been described as a stress- or drug-dependent phos-
phorylation in vivo. In fact, Sty1 MAP kinase cannot phospho-
rylate Hba1 in vitro, ruling out the possibility that the in vivo
observed phosphorylation of Hba1 is Sty1-dependent.

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3. E. A. Castillo, J. Auté, and E. Hidalgo, unpublished data.
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