Fertility and Polarized Cell Growth Depends on eIF5A for Translation of Polyproline-Rich Formins in *Saccharomyces cerevisiae*

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ABSTRACT eIF5A is an essential and evolutionary conserved translation elongation factor, which has recently been proposed to be required for the translation of proteins with consecutive prolines. The binding of eIF5A to ribosomes occurs upon its activation by hypusination, a modification that requires spermidine, an essential factor for mammalian fertility that also promotes yeast mating. We show that in response to pheromone, hypusinated eIF5A is required for shmoo formation, localization of polarisome components, induction of cell fusion proteins, and actin assembly in yeast. We also show that eIF5A is required for the translation of Bni1, a proline-rich formin involved in polarized growth during shmoo formation. Our data indicate that translation of the polyproline motifs in Bni1 is eIF5A dependent and this translation dependency is lost upon deletion of the polyprolines. Moreover, an exogenous increase in Bni1 protein levels partially restores the defect in shmoo formation seen in eIF5A mutants. Overall, our results identify eIF5A as a novel and essential regulator of yeast mating through formin translation. Since eIF5A and polyproline formins are conserved across species, our results also suggest that eIF5A-dependent translation of formins could regulate polarized growth in such processes as fertility and cancer in higher eukaryotes.

THE essential eIF5A protein is highly conserved from archaea to mammals, and has a distant ortholog, EF-P, in eubacteria (Park *et al.* 2010). Interestingly, eIF5A is the only known protein to undergo a unique and essential post-translational modification to form the hypusine residue, and this modification is necessary for its interaction with the 80S ribosome (Jao and Chen 2006; Zanelli *et al.* 2006). eIF5A was initially described to be involved in the formation of the first peptide bond in translation initiation (Benne and Hershey 1978). Later reports showed that eIF5A acts in translation elongation as its depletion resulted in the accumulation of polysomes in ribosome run-off experiments, yielding polysome profiles similar to those of elongation factor mutants.

Additionally, depletion of eIF5A increased the transit time of ribosomes along mRNAs (Gregio *et al.* 2009; Saini *et al.* 2009; Li *et al.* 2010).

Recently, it has been shown that bacterial EF-P is essential for translation of proteins with consecutive proline residues (Doerfel *et al.* 2013; Ude *et al.* 2013), and during the course of our studies, this function was also proven for eIF5A in *Saccharomyces cerevisiae* (Gutierrez *et al.* 2013). *In vitro* experiments showed that eIF5A relieves the stall of ribosomes during translation of at least three consecutive prolines, and depletion of eIF5A in yeast cells strongly reduced the translation of polyproline (polyPro) proteins (Gutierrez *et al.* 2013). However, the connection between the molecular function, physiological roles, and possible targets of eIF5A is still missing.

Two or more isoforms of eIF5A have been identified in all eukaryotes (Park *et al.* 2010). In humans, two hypusinated isoforms exist; eIF5A-1 is constitutively expressed and is abundant in proliferating cells and some human cancer tissues. In contrast, the expression of eIF5A-2 is normally very low and tissue specific and is mainly found in testis, brain, and certain cancer cell lines. The connection between eIF5A and cancer development is further supported by studies

indicating that overexpression of eIF5A-2 is associated with metastasis in several cancer types (Caraglia *et al.* 2013). The knockout of eIF5A in mice causes early embryonic lethality, indicating a role in mammalian development (Nishimura *et al.* 2012). In *C. elegans*, mutation of eIF5A produces slow larval growth, structural abnormalities, and sterile adults (Hanazawa *et al.* 2004). In *Saccharomyces cerevisiae*, eIF5A is encoded by two homologous genes, *TIF51A* and *TIF51B*. *TIF51A* is essential and mainly expressed under aerobic conditions, whereas the nonessential *TIF51B* is only expressed under anaerobic conditions (Schnier *et al.* 1991; Wohl *et al.* 1993). The synthesis of the hypusine residue of eIF5A takes place in two steps. The first step involves the enzyme deoxyhypusine synthase, encoded by the yeast essential gene *DYS1*, transferring an aminobutyl moiety from the polyamine spermidine to a specific lysine group in the N terminus of eIF5A. In a second step, the enzyme deoxyhypusine hydroxylase, encoded in yeast by the nonessential gene *LIA1*, yields the active hypusinated eIF5A protein through the addition of a hydroxyl group. It is remarkable that both enzymes, which act on just one protein, are evolutionary conserved (Park *et al.* 2010).

The physiological role of eIF5A has begun to be visualized from several functional studies. Depletion of eIF5A in yeast and inhibition of eIF5A hypusination in higher eukaryotes both cause cell cycle arrest at G1 phase (Hanauske-Abel *et al.* 1994; Kang and Hershey 1994). Additionally, eIF5A has been identified as a factor involved in the control of mRNA decay, although it is still unclear whether this function is direct or indirect (Zuk and Jacobson 1998; Valentini *et al.* 2002; Schrader *et al.* 2006). In yeast temperature-sensitive mutants, the overexpression of factors involved in proper actin organization can suppress the temperature-sensitive phenotype of eIF5A mutants (Valentini *et al.* 2002; Zanelli and Valentini 2005). It was also documented that eIF5A temperature-sensitive mutants showed changes in actin dynamics at restrictive temperature (Chatterjee *et al.* 2006). A functional association between eIF5A and cell wall integrity has been established from the studies showing that growth defects of eIF5A and deoxyhypusine synthase (*DYS1*) temperature-sensitive mutants are partially suppressed in the presence of the osmotic stabilizer sorbitol and that *DYS1* mutant is sensitive to cell-wall-perturbing compounds (Valentini *et al.* 2002; Galvao *et al.* 2013). Additionally, the overexpression of several components of the PKC pathway also restores growth of eIF5A mutants (Valentini *et al.* 2002).

Polyamines (putrescine, spermidine, and spermine) are essential for life and their mechanism of action in cells has been thoroughly investigated, although their exact mode of action is far from being resolved. In *S. cerevisiae*, the metabolic synthesis of spermidine requires the proteins encoded by the genes *SPE1*, *-2* and *-3*. Under spermidine limiting conditions, yeast *spe2* mutants show morphological abnormalities and most of intracellular spermidine is used for the hypusine modification of eIF5A, suggesting that activation of eIF5A is probably the most important function of spermidine in yeast

cells (Chattopadhyay *et al.* 2008). In mammalian cells, spermidine regulates translation at initiation and elongation steps by unknown mechanisms that are partially dependent on eIF5A. These data suggest that the role of polyamines in translation can account for their essentiality in cellular proliferation (Landau *et al.* 2010).

Spermidine is also indispensable for fertilization efficiency in eukaryotes, from humans to yeast (Lefevre *et al.* 2011). During sexual reproduction of *S. cerevisiae*, haploid cells grow a mating projection, the shmoo, toward a pheromone gradient produced by the partner cell. Shmoo formation requires spermidine (Bauer *et al.* 2013) and allows the fusion of yeast cells of different sexual type to form a diploid zygote. Yeast pheromone response involves the activation of a conserved mitogen-activated protein kinase (MAPK) module through its recruitment by the Ste5 scaffold protein to the pheromone receptor-associated G-protein. The activated MAPK Fus3 modulates the expression of mating-specific genes, promotes cell-cycle arrest, and controls polarized morphogenesis (reviewed in Slaughter *et al.* 2009; Merlini *et al.* 2013). One of the targets of Fus3 is the formin Bni1 (Matheos *et al.* 2004). Like other eukaryotic formins, Bni1 functions in actin cable polymerization (Evangelista *et al.* 1997; Evangelista *et al.* 2002; Pruyne *et al.* 2002; Sagot *et al.* 2002a,b) and contains several conserved protein domains, which allow its proper localization at sites of polarized growth and its interaction with proteins of the polarisome, such as Spa2 (Fujiwara *et al.* 1998; Liu *et al.* 2012). Upon pheromone detection, phosphorylation of Bni1 by Fus3 promotes its localization to the shmoo tip, and thus resulting in the polarized assembly of actin cables (Matheos *et al.* 2004). Actin plays an essential and complex role in shmoo formation since Bni1-induced actin cables are required for stable recruitment of the Ste5 scaffold at the shmoo, yielding polarized recruitment and full activation of MAPK Fus3 (Qi and Elion 2005). Spermidine is not only crucial for yeast mating but also essential for effective fertilization in *Caenorhabditis elegans*, suggesting that its role in fusion of haploid gametes is conserved in higher eukaryotes (Bauer *et al.* 2013).

We investigated the connection between spermidine-hypusinated eIF5A and polarized growth during shmoo formation in *S. cerevisiae*. Our results show a mechanistic connection between eIF5A and translation of Bni1 formin to allow organization of actin cables to form the shmoo. Eukaryotic formins, like Bni1, contain polyPro segments in their sequences. Our data suggest that eIF5A has an essential role in fertility and polarized cell growth in eukaryotes through the translation of polyPro formins.

Materials and Methods

Yeast strains and growth conditions

The strains and plasmids used in this study are listed in Supporting Information, Table S1 and Table S2, respectively, and primers are listed in Table S3. All strains were generated in the *S. cerevisiae* haploid strain BY4741 (*MAT***a** *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) and grown in liquid YPD medium

(1% yeast extract, 2% peptone, 2% glucose). A PCR-based genomic tagging technique was employed to tag genomic full-length *BNI1* or *BNI1* harboring the deletion of amino acids 1240–1954 at the C terminus (*BNI1ΔCt*) with 6HA. The plasmid PA171 was used as a template for PCR reactions using primers BNI1-1 and BNI1-2 for HA tagging of full-length *BNI1*, and BNI1-2 and BNI1-5 for C-terminus-truncated *BNI1* (Table S3). The resulting cassettes were transformed in *tif51A-1* and wild-type strains and transformants were selected in synthetic complete medium lacking histidine. To generate the strains containing a genomic 6HA tag of a *BNI1* mutant harboring the deletion of polyPro stretches (amino acids 1239–1307) (*BNI1ΔPro*), the C terminus *BNI1-HA* sequence was amplified from genomic DNA of the strain PAY723 using primers BNI1–2 and BNI1–6. The use of the primer BNI1–6 resulted in the deletion of nucleotides 3715–3921 in *BNI1*, which encode for the polyPro stretches of the Bni1 protein. The resulting PCR product was transformed in *tif51A-1* and wild-type cells, and transformants were selected in synthetic complete medium lacking histidine.

For experiments with null mutants, strains were grown at 30° to the mid-log phase. For experiments with temperature-sensitive mutants, strains were maintained at the permissive temperature of 25° and transferred to the nonpermissive temperature of 37° during mid-log phase. Otherwise indicated, all pheromone treatments were done with 10 μg/ml of α-factor (Sigma) for 2 hr.

To overexpress Bni1 protein, a multicopy plasmid (PA291) containing a *GAL* promoter fused to *BNI1-4HA* (*pGAL-BNI-HA*) was used (kindly provided by David Pellman) (Kono *et al.* 2012) (Table S2). Wild type, *tif51A-1*, *tif51A-3*, and *bni1Δ* were transformed with PA291, and transformants were selected in synthetic complete media lacking uracil. To induce Bni1 overexpression, cells were grown in liquid synthetic complete media lacking uracil and containing 2% galactose as the only carbon source (Sgal −ura).

Shmooing efficiency and Spa2 localization

Strains were grown in experimental temperatures and treated with α-factor for 2 hr. Cells were visualized by microscopy and ∼300 cells were analyzed per sample from at least two independent experiments. To specifically visualize the sublocalization of Spa2, an overexpression plasmid containing *SPA2-GFP* fusion was used (Arkowitz and Lowe 1997). Localization of Spa2-GFP was monitored by visual inspection in at least 100 cells from two independent experiments.

F-actin staining

For the visualization of F-actin, cells were fixed with 4% formaldehyde for 1 hr at experimental temperatures and then collected by centrifugation. Pellets were incubated overnight with 9% Alexa Fluor 488-phalloidin (Molecular Probes) and resuspended in PBS to be visualized by fluorescence microscopy.

Microscopy

All microscopy were carried out at room temperature using the Axioskop 2 fluorescence microscope (Zeiss), and images
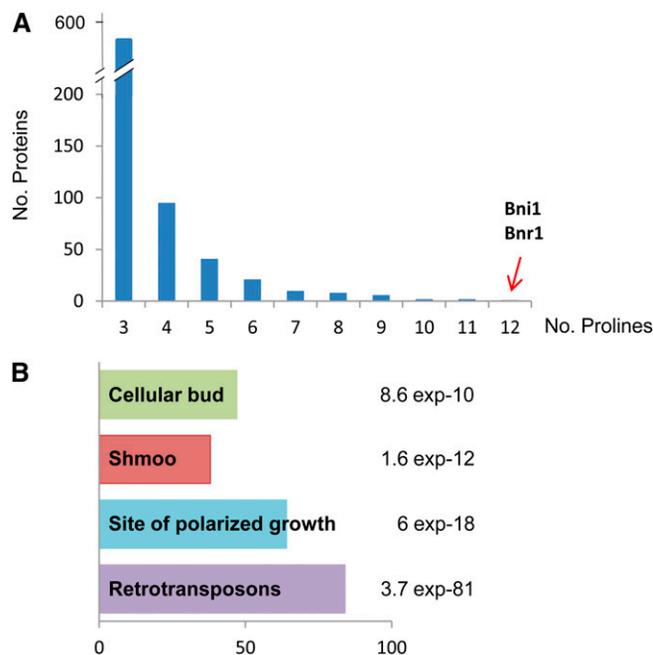
Figure 1 Number and functional categories of *S. cerevisiae* proteins containing three or more consecutive prolines. (A) Number of yeast proteins containing polyPro stretches with the indicated consecutive prolines. Formins Bni1 and Bnr1 are the only yeast proteins with polyproline stretches with 12 or more prolines. (B) GO functional categories significantly overrepresented in the 549 yeast genes containing a polyPro stretch with at least three consecutive prolines. Bars represent the number of genes found in each category and the *P*-value is indicated for each category.

were taken using an AxioCam MRm (Zeiss). For fluorescent microscopy, the power unit AttoArc HBO 100W (Zeiss) was used to visualize the indicated fluorophores.

Western blot and quantitative RT-PCR

Western blot analyses were performed as previously described (Miguel *et al.* 2013). Antibodies used were anti-HA 12C5A (Roche), anti-Hxk2 (a kind gift from F. Rández-Gil, Instituto de Agroquímica y Tecnología de los Alimentos, Valencia), and anti-eIF5A antibody (kindly provided by S. R. Valentini, Säo Paulo State University, Säo Paulo, Brazil) (Valentini *et al.* 2002). Band intensities of immunoblots were detected and quantified with the luminescent image analyzer ImageQuant LAS 4000 mini (GE Healthcare). Reverse transcription and quantitative PCR (RT-qPCR) analyses, performed as described in Garre *et al.* (2013), were used to quantify *BNI1-HA*, *BNI1-ΔCt-HA*, and *BNI1ΔPro-HA* mRNA amount (primers BNI1–3 and BNI1–4, Table S3), and *HXK2* mRNA amount (primers HXK2-1 and HXK2-2, Table S3) was determined as a control. Transcript amount was normalized against endogenous *ACT1* expression using established primers ACT1-1 and ACT1-2 (Table S3). Translation efficiencies of *BNI1-HA*, *BNI1ΔCt-HA*, *BNI1ΔPro-HA*, and *HXK2* were estimated as the protein/mRNA ratio, using the Western and RT-qPCR data (normalized against *ACT1* expression) obtained from cell samples of the same experiment. *BNI1-HA*, *BNI1ΔCt-HA*, and *BNI1ΔPro-HA* translation efficiencies are expressed relative to *HXK2*
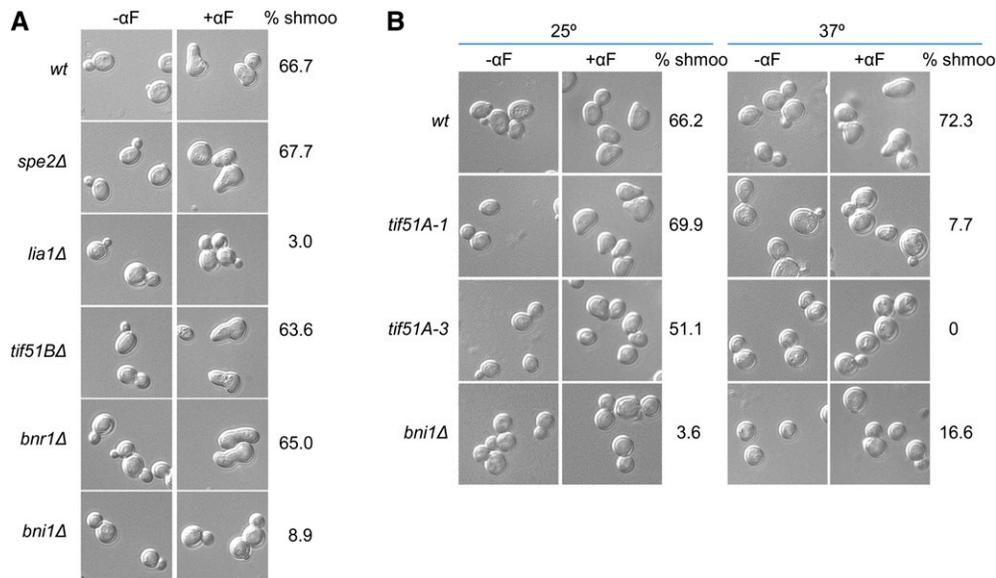
Figure 2 Yeast shmoo formation requires hypusinated eIF5A. (A and B) Representative DIC images of wild type and mutants with or without treatment of 10 μg/ml α-factor for 2 hr. Null mutants were maintained in 30° (A), and temperature-sensitive mutants were maintained at 25° or transferred to 37° for 4 hr (B). Quantification of percentage of cells containing shmoos in samples treated with α-factor are indicated. Approximately 300 cells were manually counted for each sample from at least two independent experiments.

translation efficiency and represented as a fraction against 25° for each strain.

β-Galactosidase assay

To investigate the induction of cell fusion genes in response to pheromone, the membrane protein *FUS1* was expressed in a fusion plasmid containing the reporter gene *LacZ* (Table S2). As a positive control, osmotic stress-induced gene *STL1* coupled with *LacZ* was used (Table S2). Biological triplicates were grown at 25° to the mid-log phase and transferred to the nonpermissive temperature of 37° for 4 hr and then incubated with 10 μg/ml of α-factor for 1 or 2 hr. For *STL1* induction, control cells were stressed with 0.6 M KCl for 45 min. β-Galactosidase activity was determined from three biological triplicates as previously described (Garre *et al.* 2012).

Results

Pheromone-induced shmoo formation is inhibited in eIF5A mutants

Recently, it was discovered that the bacterial eIF5A-ortholog factor EF-P is essential for the translation of proteins with consecutive prolines (polyPro proteins) (Doerfel *et al.* 2013; Ude *et al.* 2013). Furthermore, *S. cerevisiae* mutant in the spermidine biosynthesis *SPE2* gene is unable to mate in low-spermidine media (Bauer *et al.* 2013), and given the role of spermidine in eIF5A activation (Park *et al.* 2010), we proposed that hypusinated eIF5A may be involved in fertility through the translation of polyPro proteins with roles in fertilization. To test this hypothesis, we first investigated the number and functional roles of the polyPro proteins in *S. cerevisiae*. As shown in Figure 1A, 549 yeast proteins were found to contain at least one motif of three consecutive proline residues and the number of proteins is much reduced for those with four or more consecutive prolines. A search in the Gene Ontology database (GO) revealed four significant over-

represented categories, including "site of polarized growth" and "mating projection (shmoo)" (Figure 1B). Only two yeast proteins, Bnr1 and Bni1, contain 10 or more consecutive prolines. These two proteins are evolutionary conserved formins, regulating actin cytoskeleton during budding (Imamura *et al.* 1997; Pruyne *et al.* 2004), with Bni1 also involved in shmoo formation during mating (Evangelista *et al.* 1997). From these data, we investigated whether eIF5A is involved in shmoo formation through the regulation of Bni1.

With this aim, we first evaluated the ability to form shmoo projections under pheromone treatment of mutants in genes *SPE2* (biosynthesis of spermidine), *LIA1* (deoxyhypusine hydroxylase of eIF5A), mutants in the formins *BNR1* and *BNI1*, and mutant in the nonessential eIF5A gene, *TIF51B*. The *lia1Δ* mutant was unable to form shmoos in the presence of α-factor, whereas, as expected in a rich media where spermidine is available, *spe2Δ* showed a percentage of shmoo formation similar to the wild type (Figure 2A). Since the only known target of Lia1 is eIF5A, these results suggest that hypusination of eIF5A is necessary for shmoo formation and that perhaps the vital requirement of spermidine relies on eIF5A activation by hypusination. Furthermore, *tif51BΔ* was able to shmoo, and, as it has been described, the formin Bni1, but not Bnr1, is essential for shmooing (Figure 2A).

Next, since eIF5A is an essential protein in yeast, we used eIF5A temperature-sensitive strains previously described (Valentini *et al.* 2002; Li *et al.* 2011) to evaluate the formation of mating projections. Mutants *tif51A-1* and *tif51A-3* contain a single (Pro83 to Ser) and double (Cys39 to Tyr, Gly118 to Asp) mutation, respectively, in eIF5A protein. Both mutants showed a drop in viability after 8 hr of incubation at 37°, with a more severe growth defect in *tif51A-3* (Figure S1, A and B). Accordingly, eIF5A protein was mostly depleted after 4 hr of incubation at 37° in both mutants (Figure S1, C and D). Treatment with 10 μg/ml α-factor induced shmoo formation in eIF5A mutants and wild type at permissive
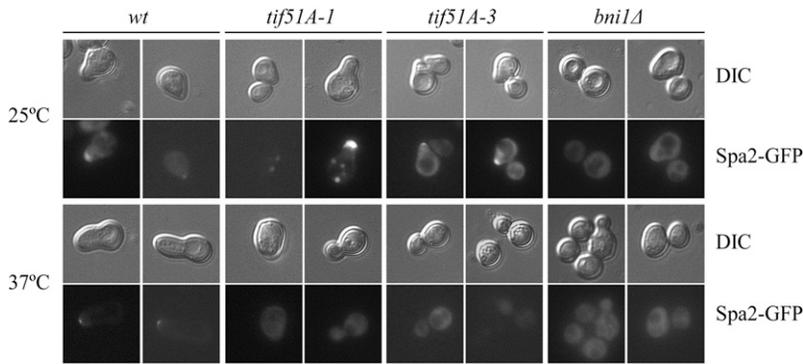
Figure 3 Localization of the polarisome protein Spa2 at the shmoo tip in response to pheromone requires eIF5A. Representative GFP and their corresponding DIC images of wild type, *bni1Δ*, *tif51A-1*, and *tif51A-3* mutants over-expressing Spa2-GFP. Cells were maintained at 25° or transferred to 37° for 4 hr. Cells were examined after treatment with 10 μg/ml α-factor for 2 hr.

temperature (25°) (Figure 2B). However, at restrictive temperature (37°), *tif51A-1* yielded very low shmooing (7.7%) and *tif51A-3* was unable to shmoo, and most cells in both strains appear ellipsoidal. As previously described, *bni1* mutant showed a low percentage of shmoo formation at both temperatures (Figure 2B). As positive controls in shmoo formation at 37°, we also used two other temperature-sensitive mutants in essential genes (*top2-1* mutation in topoisomerase II and *xpo1-1* mutation in exportine Crm1/Xpo1) and observed that, in both, the percentage of shmoo formation was similar at permissive and restrictive temperatures (Figure S2). Taken together, these results indicate that hypusinated eIF5A is required for successful shmoo formation.

eIF5A is required to shmoo localize Spa2, induce FUS1 gene, and assemble actin cables during pheromone stimulation

We further investigated the basis for the defect in shmoo formation in eIF5A mutants by visualizing the localization of Spa2, a component of the polarisome that localizes at the shmoo tip during mating through its interaction with Bni1 (Fujiwara *et al.* 1998; Liu *et al.* 2012). Upon pheromone treatment at 25°, wild-type and eIF5A mutant cells showed Spa2-GFP localization at the shmoo tip, meanwhile Spa2 was delocalized in *bni1Δ* (Figure 3). Inactivation of eIF5A by incubation at 37° inhibited polarized localization of Spa2 in *tif51A-1* and *tif51A-3* mutants (Figure 3). Furthermore, to determine the full activation of the MAP kinase Fus3 with α-factor, we examined the expression of the *FUS1* gene, which encodes a pheromone-induced transmembrane protein involved in cell fusion (Trueheart and Fink 1989). A *FUS1-lacZ* fusion was induced under pheromone treatment in wild type and to a lower level in *tif51A* mutants at permissive temperature. However, no *FUS1-lacZ* induction was observed in eIF5A mutants at restrictive temperature (Figure 4A). In *bni1Δ*, *FUS1* gene was not induced at 37°, and surprisingly, *FUS1* induction was observed at 25° (Figure 4A). In contrast to the observed defects in *FUS1* induction, *tif51A* mutants were able to induce an osmotic stress-responsive gene (*STL1*) fused to *LacZ* during osmotic stress treatment, indicating that, at experimental conditions, *tif51A* mutants maintain the ability to induce signaling pathways in response to external stimuli (Figure 4B).

It has been shown that depletion of eIF5A causes changes in the actin cytoskeleton by unknown mechanisms (Chatterjee *et al.* 2006; Zanelli and Valentini 2007). We therefore investigated the requirements of eIF5A for pheromone-induced polarization of actin cables. Phalloidine staining of actin before and after treatment with pheromone was visualized by fluorescent microscopy (Figure 5). At 25°, in the absence of α-factor, eIF5A mutants showed a normal pattern of actin with patches and cables (Figure 5). Addition of α-factor at permissive temperature induced the formation of actin cables from the shmoo in *tif51A-1* and wild-type cells, and actin was visualized mainly as patches in *tif51A-3*. However, at 37° with α-factor, both eIF5A mutants were unable to form actin cables, but contained delocalized actin patches. Consistent with previous reports (Matheos *et al.* 2004), actin was observed as intense delocalized patches in all conditions in *bni1* mutant. Therefore, eIF5A is necessary for the formation of actin cables upon pheromone stimulation.

Taken together, these results indicate that eIF5A controls different outcomes of pheromone signaling, including induction of pheromone-responsive genes and localization of components of the polarisome at the shmoo. Given the essential role of actin in the induction of cell polarization during mating (Slaughter *et al.* 2009), these downstream effects could be explained by a direct role for eIF5A in actin polymerization.

eIF5A is required for the translation of the polyproline formin Bni1

Cells depleted in eIF5A show similar defects in polarization during mating as the *bni1* mutant. The formin Bni1 is a large protein containing three segments of 10, 12, and 5 consecutive proline residues in the formin homology domain 1 (FH1), the domain required for assembly of actin filaments (Figure 6A) (Sagot *et al.* 2002b). Since it has been shown that the bacterial ortholog of eIF5A, EF-P, enhances the synthesis of proteins containing polyPro stretches (Doerfel *et al.* 2013; Ude *et al.* 2013), we hypothesized that yeast eIF5A may be involved in the translation of the polyPro motifs of Bni1 and that low levels of Bni1 in eIF5A mutants is the basis for their inability to shmoo. To test this, we obtained two genomic HA-tagged versions of *BNI1* in wild type and *tif51A-1*; a full-length *BNI1* with HA after the coding region (*BNI1-HA*) and a truncated *BNI1* with HA fused before the first polyPro stretch
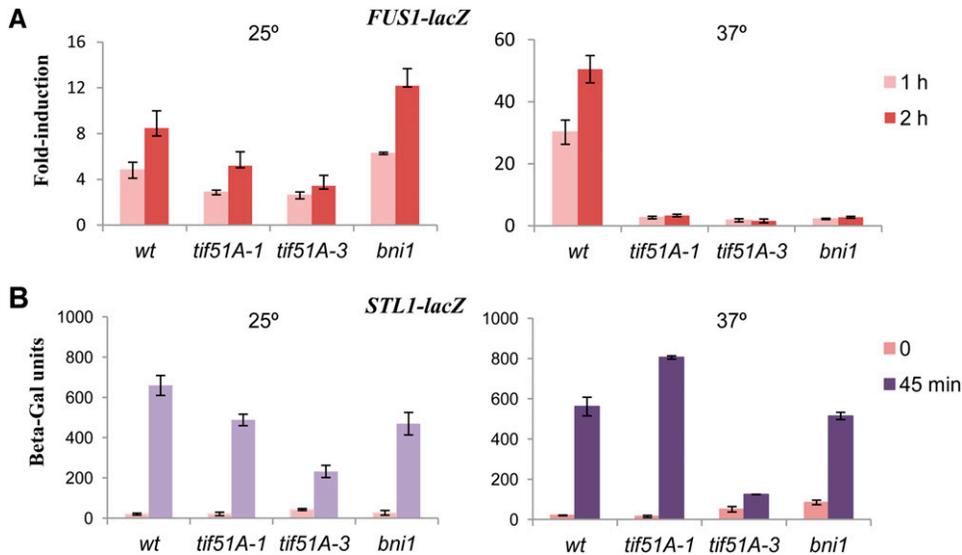
Figure 4 Expression of *FUS1-lacZ* in response to pheromone requires eIF5A. (A and B) Expression of *FUS1-LacZ* (A) or *STL1-LacZ* fusion (B) in wild type and mutants. Cells were grown at 25° or 4 hr at 37° and then treated with 10 μg/ml α-factor for either 1 or 2 hr (A) or stressed with 0.6 M KCl for 45 min (B). Error bars represent the standard error of the mean of three or more biological replicates.

(*BNI1ΔCt-HA*) (Figure 6A). The shift of wild-type cells from 25° to 37° slightly reduced the protein levels of Bni1-HA and Bni1ΔCt-HA. However, Bni1-HA level was importantly reduced in *tif51A-1* cells after 2 hr of incubation at 37° and was almost undetectable after 6 hr. Conversely, we only observed a partial reduction in Bni1ΔCt-HA protein levels in *tif51A-1* after the shift to 37°, which was sustained up to 6 hr (Figure 6B). We analyzed *BNI1-HA* and *BNI1ΔCt-HA* mRNA expression in the same cell cultures by RT-qPCR and calculated the protein/mRNA ratios as an indication of translation efficiency, which was compared to the translation efficiency of a control gene, *HXK2*, encoding hexokinase 2, a protein with no polyPro motifs. After temperature shift, *tif51A-1*

showed an important decrease in the translation efficiency of full-length *BNI1*, reaching almost zero after 6 hr. In comparison, the translation efficiency of C-terminus truncated *BNI1* lacking the polyPro motifs was sustained from 2 to 6 hr upon eIF5A depletion (Figure 6C). These results suggested that eIF5A controls the expression of the Bni1 formin and is required for the translation of the polyPro motifs. To further demonstrate that the polyPro motifs are responsible for the *BNI1* translation dependency on eIF5A, we obtained an additional *bni1* mutant, in which only a short genomic region of the FH1 domain containing the polyPro stretches was deleted, maintaining intact the rest of the *BNI1* C terminus (*BNI1ΔPro-HA*) (Figure 6A). In *tif51A-1*, the translation efficiency of Bni1ΔPro also reduced
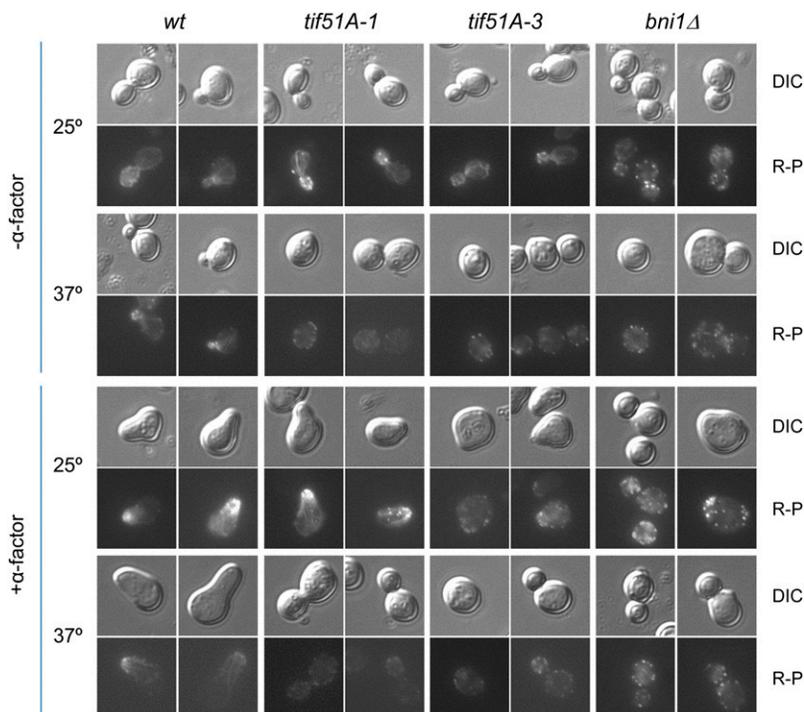
Figure 5 Polymerization and localization of actin cables require eIF5A during shmoo formation. Representative fluorescence and their corresponding DIC images of wild type, *tif51A-1*, *tif51A-3*, and *bni1Δ* showing F-actin staining by phalloidin at 25° or after incubation for 4 hr at 37°, in the presence or absence of treatment with 10 μg/ml α-factor for 2 hr.
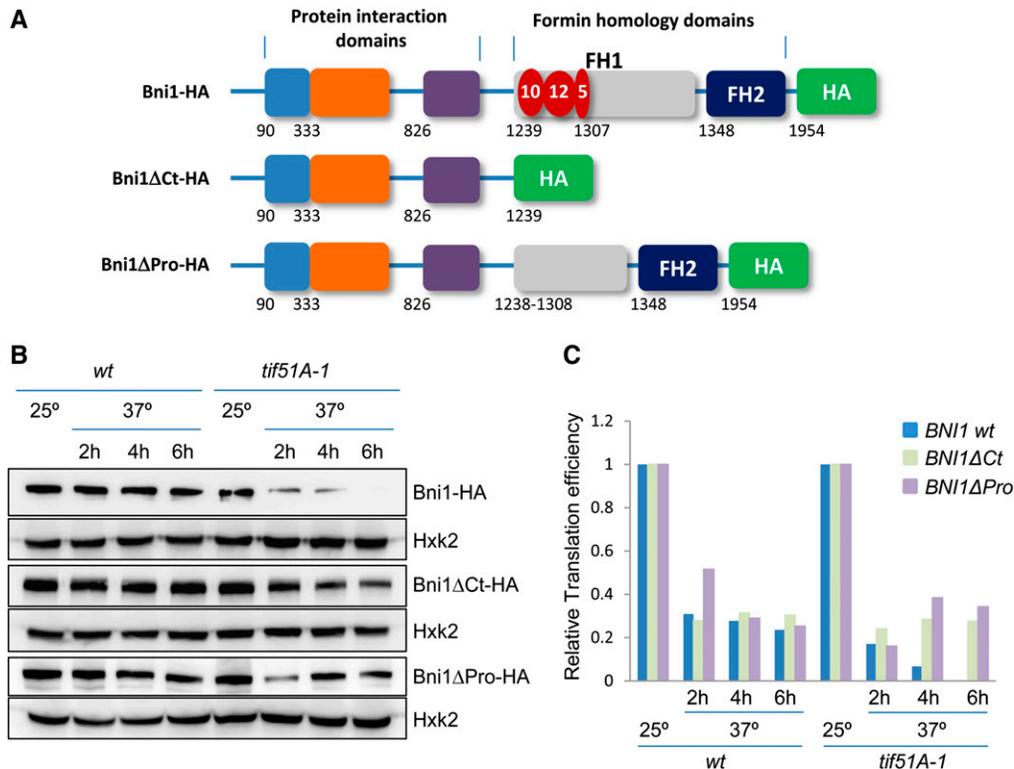
Figure 6 Translation of the proline stretches of the formin *BNI1* requires eIF5A. (A) Schematic diagrams showing C-terminal HA genomic tagging of full-length (*BNI1-HA*), C-terminal truncated (Δ1240–1954, *BNI1ΔCt-HA*), and proline-deleted (Δ1239–1307, *BNI1ΔPro-HA*) Bni1. Formin homology (FH) and protein interaction domains are indicated, as well as the polyPro stretches with the number of consecutive prolines represented in red. The numbers below the boxes indicate the first amino acid of each domain or stretch. (B) Immunoblots with antibodies against HA and hexokinase 2 protein (Hxk2) to show expression of full-length Bni1, C-terminal truncated Bni1, proline-deleted Bni1 and Hxk2, in wild type and *tif51A-1* at 25° or 37° at the indicated times. (C) Translation efficiency of *BNI1-HA*, *BNI1ΔCt-HA*, and *BNI1ΔPro-HA* relative to translation efficiency of *HXK2* in wild type and *tif51A-1*. Protein/mRNA ratios for Bni1, Bni1ΔCt-HA, Bni1ΔPro-HA, and Hxk2 were calculated by Western (B) and quantitative PCR from the same samples. Translation efficiencies of *BNI1-HA*, *BNI1ΔCt-HA*, and *BNI1ΔPro-HA* are calculated relative to translation efficiency of *HXK2* and represented as a fraction against 25° for each strain.

at 37° but was sustained at 4 and 6 hr of incubation at restrictive temperature, with similar efficiencies observed in the wild-type strain (Figure 6, B and C). This result, showing eIF5A independency of this *BNI1* version with the polyPro stretches deleted, strongly supports the conclusion that eIF5A is involved in the translation of the proline-rich motifs of Bni1.

It is noteworthy to mention that the above results could have an alternative interpretation, where the differences in protein amount seen between Bni1 wild type and Bni1 mutants are due to different protein degradation rates, and it could be that eIF5A is involved in the stabilization of only the wild-type Bni1 protein. Although we cannot exclude this possibility, the fact that the specific elimination of the Bni1 polyPro stretches renders the protein eIF5A independent, deems the interpretation that the eIF5A elongation factor is involved in Bni1 translation rather than in the regulation of protein stability to be more reasonable. Bni1 protein stability has been studied in a previous work in which it was demonstrated that local cell wall/membrane damage results in the PKC-regulated proteosomal degradation of Bni1 (Kono *et al.* 2012). Moreover, it was described that the Bni1 N-terminal region (amino acids 1–642), which does not contain the polyPro motifs, is responsible for this regulated degradation that can be provoked by heat shock at 39° (Kono *et al.* 2012). Perhaps, our observation that Bni1 protein levels slightly drop in all strains during the shift from 25° to 37° (Figure 6B), could be explained by a regulated Bni1 degradation during a mild heat shock.

Overexpression of Bni1 protein partially restores the shmoo formation defect of eIF5A mutants

Finally, we explored the possibility of suppression of the eIF5A mutant shmoo defect by increasing the cellular levels of Bni1. To do so, we used a multicopy plasmid containing a *BNI1-HA* fusion under the control of the strong *GAL* promoter (Kono *et al.* 2012). Given the unessential nature of Bni1 to cell viability, the lack of this protein cannot explain the essentiality of eIF5A, and therefore, as expected, the growth defect at restrictive temperature of *tif51A-1* and *tif51A-3* mutant cells was not recovered when *BNI1* was overexpressed in media containing galactose (Figure 7A). Upon galactose overexpression conditions, we observed a high increase in Bni1 protein levels in wild type and in *tif51A-1* at permissive temperature with respect to the genomic endogenous Bni1 levels; however, Bni1 protein level increased only slightly in *tif51A-1* at restrictive temperature (Figure 7B). This result further confirms that eIF5A is necessary for the production of Bni1 protein, even when overexpressing the *BNI1* gene. Nevertheless, a slight increase in Bni1 protein was obtained in eIF5A mutants at restrictive temperature; therefore, we studied whether this increase could improve the shmoo formation of eIF5A mutants. As expected, a complete complementation of the shmoo formation defect in *bni1* mutant was observed by inducing *pGAL-BNI1*. We also observed, at restrictive temperature, higher percentages of shmoo formation in *tif51A-1* (19.5%) and *tif51A-3* (6.9%) (Figure 7C) with respect to what was observed in these strains without *BNI1* overexpression (7.7 and 0%, respectively;
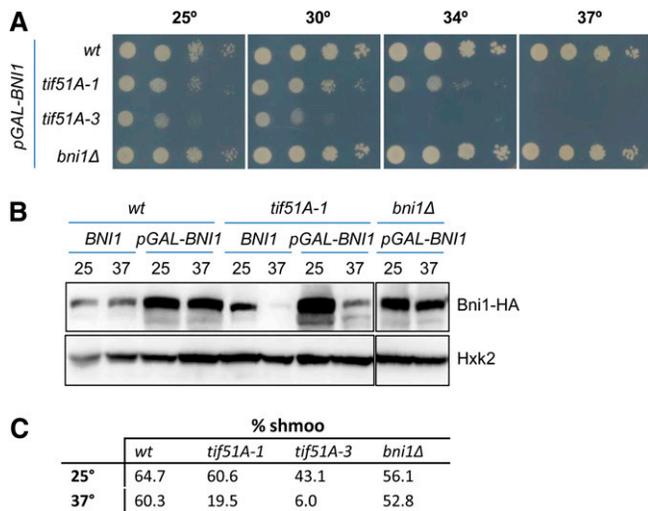
Figure 7 Overexpression of the formin *BNI1* partially restores the shmoo formation defect of eIF5A mutants. (A) Overexpression of *BNI1* does not restore the growth defect of eIF5A thermosensitive mutants at restrictive temperature. Growth of the indicated yeast strains containing a plasmid expressing *BNI1* under the *GAL* promoter (*pGAL-BNI1*) was tested in Sgal −ura plates incubated at the indicated temperatures. (B) Immunoblots with antibodies against HA and hexokinase 2 protein (Hxk2) to show the expression of genomic Bni1 (*BNI1*) or the overexpression of Bni1 (*pGAL-BNI1*) at 25° or 37° for 4 hr. (C) Shmoo formation is partially restored in eIF5A mutants. The indicated strains containing a *pGAL-BNI1* plasmid were grown at 25° in Sgal−ura media and then were maintained at 25° or transferred to 37° for 4 hr. Quantification of percentage of cells forming shmoos in samples treated with 10 μg/ml α-factor are indicated. Approximately 300 cells were manually counted for each sample from at least two independent experiments.

Figure 2B) This partial suppression of shmoo defect seen in eIF5A mutants by a slight increase of Bni1 protein levels further supports that the shmooing defect in these mutants is due to the low levels in Bni1 formin. Additionally, since increasing Bni1 protein levels does not produce a total restoration of shmoo formation ability in eIF5A mutants, it suggests that more Bni1 protein would be necessary for a full complementation or that other proteins involved in shmoo formation might be under the translational control of eIF5A.

Discussion

The budding yeast is a model for studying the establishment of cellular asymmetry, in particular during the formation of a mating projection (shmoo) toward a pheromone gradient. It is remarkable that many of the genes that control cell polarity are conserved between yeast and more complex eukaryotes. Polarized growth requires the asymmetric organization of the cytoskeleton and the formation of actin cables nucleated by formins (Slaughter *et al.* 2009). *S. cerevisiae* formin Bni1 contains long stretches of consecutive prolines located in the formin homology domain 1, which has been conserved among eukaryotic formins, and is required for the formation of actin cables to form a shmoo in response to pheromone (Pruyne *et al.* 2002; Sagot *et al.* 2002b). In our study, we discovered that the translation factor eIF5A and the enzyme

Lia1 required for its activation through hypusination are both essential for shmoo formation. Moreover, eIF5A mutants showed defects in all downstream events (polarized localization of the polarisome component Spa2, induction of the fusion gene *FUS1*, and actin polarization) of the pheromone response. Since Bni1 is activated by pheromone-responsive MAP kinase Fus3 and, concurrently, Bni1 is required for full activation of Fus3 by mediating its polarized localization to the shmoo (Matheos *et al.* 2004; Qi and Elion 2005), our data suggest that the defect in shmoo formation in eIF5A mutants is a direct consequence of dramatically low levels of Bni1 protein upon eIF5A depletion. Our results using three genomically tagged *BNI1* versions, a full-length Bni1 whose translation is sensitive to eIF5A and two Bni1 proteins containing deletions of the polyPro stretches, by the deletion of the C terminus (Bni1ΔCt) or by a specific deletion of the three polyPro motifs (Bni1ΔPro), for which translation is eIF5A independent, strongly suggest that eIF5A is required for translation of these proline-rich sequences. Overall, our results connect, for the first time, eIF5A molecular function with a physiological role and a specific target.

It is worth mentioning that the defects in shmooing (Figure 2) and actin cable formation (Figure 5) are more severe in eIF5A mutants than in *bni1* mutant. Additionally, the restoration of shmoo formation is only partial when increasing Bni1 protein levels in eIF5A mutants (Figure 7). These results can be explained by the putative role of eIF5A in the translation of other polyPro proteins involved in shmoo formation and actin organization. Indeed, the GO functional category of "Mating Projection" was significantly represented in the group of proteins with three or more consecutive prolines (Figure 1). Specifically, 38 proteins of the 119 in this GO functional category are polyPro proteins, and five of them contain at least six consecutive prolines. In respect to the observed actin cable formation and cytoskeleton organization defects in eIF5A mutants, five yeast proteins with these functions contain poly-Pro motifs with at least seven consecutive prolines, with one of them being the only other yeast formin, Bnr1. All of these polyPro proteins are putative targets of eIF5A, which should be further investigated.

The role of eIF5A in yeast mating could be extended to fertility in higher eukaryotes. Spermidine is essential for successful reproduction in eukaryotes (Bauer *et al.* 2013) and is also the substrate for eIF5A hypusination, a modification that is required for eIF5A binding to the ribosomes (Park *et al.* 2010). Given that reproduction and cell fusion during eukaryotic reproduction requires polarized growth, our results suggest that translation of polyPro formins, conserved across species from yeast to humans, could regulate this process.

Yeast and mammalian essential eIF5A proteins are 65% identical. Moreover, human eIF5A expressed in yeast cells becomes hypusinated and can functionally substitute yeast eIF5A in strains carrying eIF5A mutation (Schnier *et al.* 1991; Magdolen *et al.* 1994). Additionally, eukaryotic formins are large proteins with conserved C-terminal FH domains, which contain multiple proline-rich motifs that recruit actin

monomers and nucleate actin filaments (Breitsprecher and Goode 2013). From our results and the existence of high sequence and functional homologies in eIF5A and formins, it can be conceptualized that other important roles of human eIF5A could be a consequence of formin-controlled translation. In humans, 15 different formins exist and are involved in actin organization during cell adhesion, cytokinesis, cell polarity or morphogenesis, and have essential roles in cell proliferation and migration in the context of human disease (Deward et al. 2010; Breitsprecher and Goode 2013; Favaro et al. 2013). As eIF5A has been reported as a novel oncogenic protein in many types of human tumors and its overexpression has been reported to be associated with metastasis in multiple cancer types (Caraglia et al. 2013; Wang et al. 2013), and, additionally, formins participate in cancer cell migration and invasion (Lizarraga et al. 2009; Kitzing et al. 2010; Zhu et al. 2011; Favaro et al. 2013), it would also be of great interest to explore the connection between eIF5A and formin translation in cancer and metastasis.

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GENETICS

Fertility and Polarized Cell Growth Depends on eIF5A for Translation of Polyproline-Rich Formins in *Saccharomyces cerevisiae*

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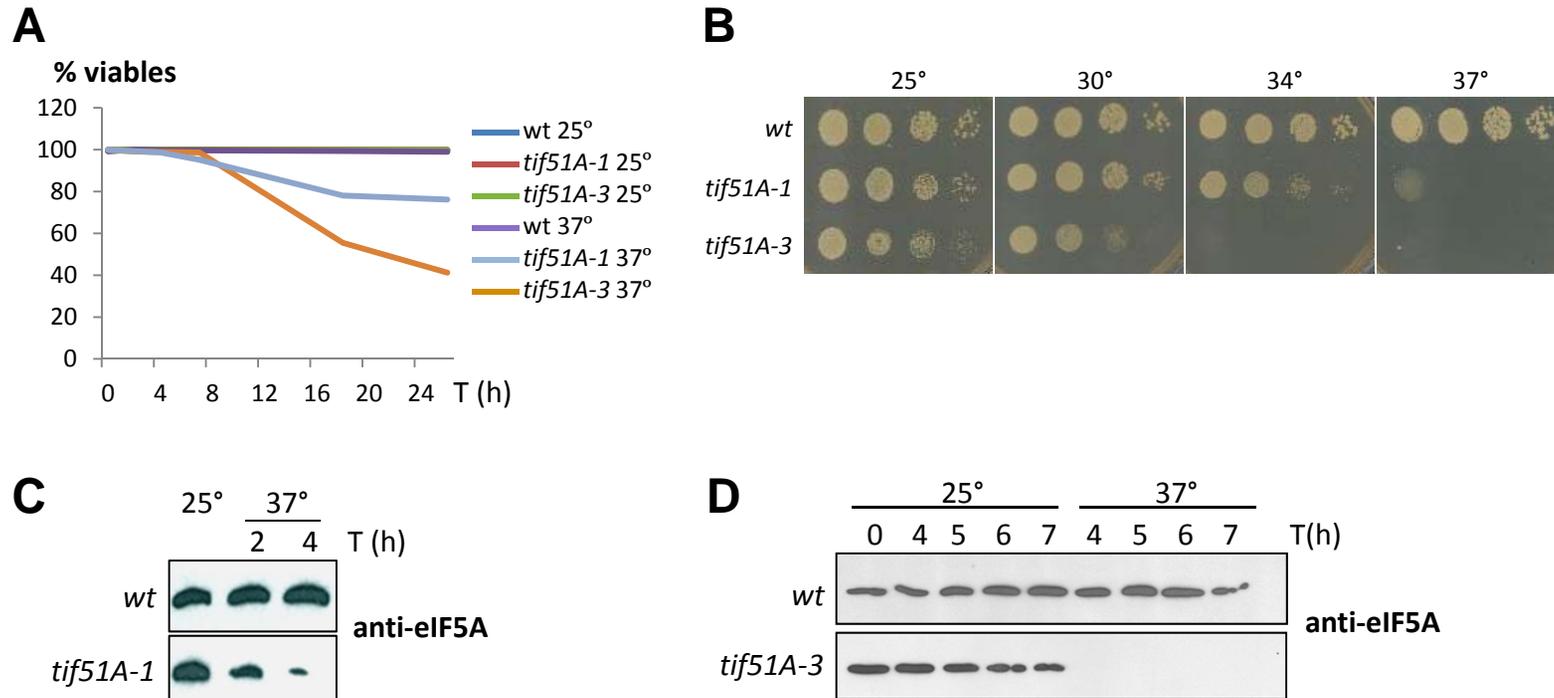
Figure S1 Depletion of the Tif51A protein results in lethality. (A) Percentage of viable cells in wild-type, *tif51A-1* and *tif51A-3* grown in synthetic media at 25° or after incubation at non-permissive (37°) at the times indicated. 3000 cells were counted for each sample. Cell viability was measured at the indicated times using the Muse Count & Viability Assay Kit and the Muse™ Cell Analyser (Millipore, Billerica, MA, USA) according to manufactures' instructions. (B) 10-fold serial dilutions of wild-type, *tif51A-1* and *tif51A-3* were plated onto YPD medium and incubated at the indicated temperatures. (C, D) Immunoblots showing eIF5A protein depletion in *tif51A-1* (C) and *tif51A-3* (D) after incubation at 37° for the indicated times. eIF5A protein expression was also visualized in wild-type under the same conditions.

A *top2-1*

25⁰ 37⁰

% shmoo 63.0 62.5

B *xpo1-1*

25⁰ 37⁰

% shmoo 40.5 38.5
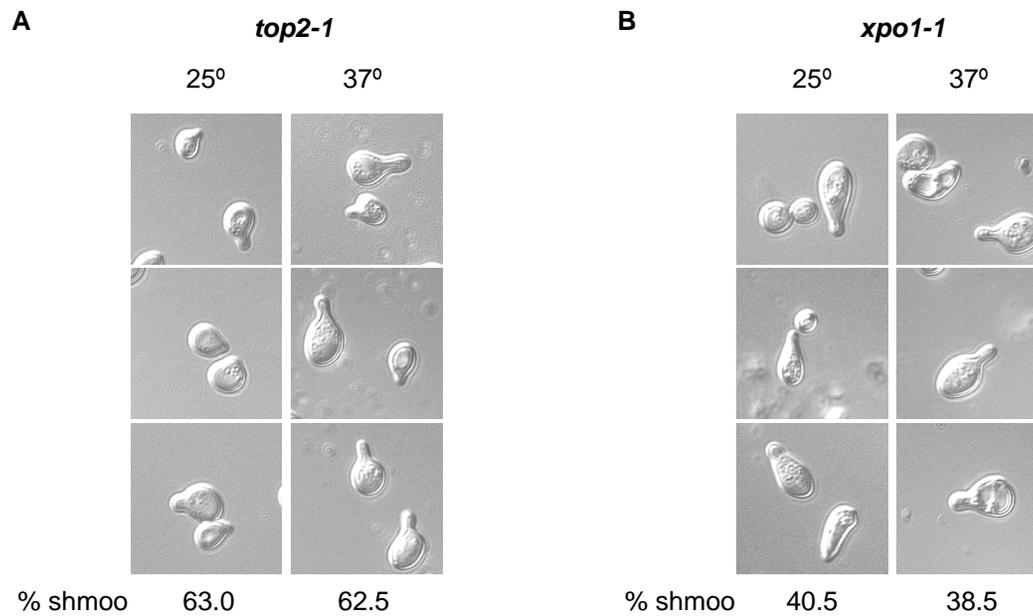
Figure S2 Yeast shmoo formation in temperature-sensitive mutants. (A, B) Representative DIC images of mutants containing temperature sensitive alleles in the essential proteins topoisomerase II (*top2-1*) (A) and karyopherine Crm1/Xpo1 (*xpo1-1*) (B). Mutants were grown at 25° until exponential phase and were maintained at 25° or transferred to 37° for 4h. Cells were then treated with 10 µg/ml α-factor for 2h. Percentage of cells containing shmoos in samples treated with α-factor are indicated. Approximately 300 cells were manually counted for each sample from at least two independent experiments.

Table S1   Yeast strains used in this study.

Strain	Genotype	Source
BY4741	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
lia1Δ	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 lia1Δ::KanR*	Euroscarf
spe2Δ	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 spe2Δ::KanR*	Euroscarf
tif51BΔ	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 tif51BΔ::KanR*	Euroscarf
bnr1Δ	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 bnr1Δ::KanR*	Euroscarf
bni1Δ	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 bni1Δ::KanR*	Euroscarf
tif51A-1	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 tif51A-1::KanMX*	(Lı *et al.* 2011)
tif51A-3	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 tif51A-3::KanMX*	(Lı *et al.* 2011)
PAY723	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 BNI1-6HA::HIS3*	This study
PAY725	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 tif51A-1::KanMX BNI1-6HA::HIS3*	This study
PAY727	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 bni1Δ1240-1954-6HA::HIS3*	This study
PAY729	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 tif51A-1::KanMX bni1Δ1240-1954-6HA::HIS3*	This study
PAY739	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 bni1Δ1239-1307-6HA::HIS3*	This study
PAY741	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 tif51A-1::KanMX bni1Δ1239-1307-6HA::HIS3*	This study
top2-1	MATa *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 top2-1*	(Brill and Sternglanz 1988)
xpo1-1	MATa *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 xpo1::LEU2 pKW457 (xpo1-1 in pRS313)*	(Stade *et al.* 1997)

Table S2   List of plasmids used in this study.

Plasmid	Description	Source
PA171	GA2256 *HA6-HIS3*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PA283	pRS416 *SPA2-GFP, URA3*, 2µ	Dr. R. Arkowitz, CNRS, Nice, France
PA288	GA1815 *FUS1-LacZ, URA3*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PA159	Yep368 *STL1-LacZ, LEU2,* 2µ	Dr. E. de Nadal, University Pompeu Fabra, Barcelona, Spain
PA291	PB1028 *GAL-BNI1-4HA, URA3*, 2µ	Dr. D. Pellman (Dana-Farber Cancer Institute and Harvard Medical School, Boston, USA)

Table S3 List of primers used in this study.

Primer	Sequence	Use
BNI1-1	5´-AGATCCATAGGTGAGGCTAGCACAGGTAACAGGCTAAG TTTCAAATCCGGTTCTGCTGCTAG-3´	*BNI1* full length genomic tagging
BNI1-2	5´-GTTTGTTTTGGTATTACTGTTGTCATAATTTTTTGGTTTAA TATTCCTCGAGGCCAGAAGAC-3´	*BNI1* full length, C terminus truncated, and polyPro deletion genomic tagging
BNI1-3	5´-ACATGTGGAAAACGGAAAGC-3´	qPCR
BNI1-4	5´-AGATCTTCTGCGCCATCTGT-3´	qPCR
BNI1-5	5´-GCAGAAGATCTCTCTACTCAATCATCTGTACTCTCCTCAC AGCCGTCCGGTTCTGCTGCTAG-3´	*BNI1* C-truncated genomic tagging
BNI1-6	5´-GGCGCAGAAGATCTCTCTACTCAATCATCTGTACTCTCC TCACAGCTACCATCTGTATTATCT-3´	*BNI1* polyPro deletion genomic tagging
ACT1-1	5´-TCGTTCCAATTTACGCTGGTT-3´	qPCR
ACT1-2	5´-CGGCCAAATCGATTCTCAA-3´	qPCR
HXK2-1	5´-CAATTCCATTGGGTTTCACC-3´	qPCR
HXK2-2	5´-GCAACATTGGAACAACATCG-3´	qPCR

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