The translation termination factor eRF1 (Sup45p) of Saccharomyces cerevisiae is required for pseudohyphal growth and invasion

Alexandra Petrova†, Denis Kiktev‡, Olga Askinazi§, Svetlana Chabelskaya¶, Svetlana Moskalenko, Olga Zemlyanko and Galina Zhouravleva∗

Department of Genetics and Biotechnology, St Petersburg State University and St Petersburg Branch Vavilov Institute of General Genetics, Russian Academy of Science, Universitetskaya emb. 7/9, 199034, St Petersburg, Russia

*Corresponding author: Department of Genetics and Biotechnology, St Petersburg State University, Universitetskaya emb. 7/9, 199034 St Petersburg, Russia. Tel: +79119384080; E-mail zhouravleva@rambler.ru
†Present address: Department of Radiation Oncology, Emory School of Medicine, Emory University, Atlanta, GA 30322, USA
‡Present address: Laboratory of amyloid biology, St Petersburg State University, Universitetskaya emb. 7/9, 199034 St Petersburg, Russia
§Present address: Department of Biology, University of Virginia, P.O. Box 400328, Charlottesville, VA 22904-4328, USA
¶Present address: Inserm U835, Upres EA2311, Université Rennes 1, 2, avenue du prof. Léon Bernard, 35043 Rennes Cedex, France

One sentence summary: Mutations in the SUP45, but not in the SUP35, gene abolish diploid pseudohyphal growth and suggest that they are defective in the cAMP-dependent cascade inducing pseudohyphal growth.

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ABSTRACT
Mutations in the essential genes SUP45 and SUP35, encoding yeast translation termination factors eRF1 and eRF3, respectively, lead to a wide range of phenotypes and affect various cell processes. In this work, we show that nonsense and missense mutations in the SUP45, but not the SUP35, gene abolish diploid pseudohyphal and haploid invasive growth. Missense mutations that change phosphorylation sites of Sup45 protein do not affect the ability of yeast strains to form pseudohyphae. Deletion of the C-terminal part of eRF1 did not lead to impairment of filamentation. We show a correlation between the filamentation defect and the budding pattern in sup45 strains. Inhibition of translation with specific antibiotics causes a significant reduction in pseudohyphal growth in the wild-type strain, suggesting a strong correlation between translation and the ability for filamentous growth. Partial restoration of pseudohyphal growth by addition of exogenous cAMP assumes that sup45 mutants are defective in the cAMP-dependent pathway that control filament formation.

Keywords: translation termination; nonsense mutations; eRF1; eRF3; pseudohyphal growth; budding; hygromycin B; cycloheximide; paromomycin; cAMP

INTRODUCTION
In eukaryotes, two protein factors, eRF1 and eRF3, are essential for the translation termination (Frolova et al. 1994; Zhouravleva et al. 1995). Factor eRF1 recognizes all three stop codons (UAA, UAG and UGA) and releases a newly synthesized peptide (Frolova et al. 1994). Factor eRF3 stimulates termination activity of eRF1 in the GTP-dependent manner (Frolova et al. 1996). Both genes, SUP45 and SUP35, encoding eRF1 and eRF3, respectively, are essential in yeast Saccharomyces cerevisiae (Himmelfarb, Maicas and Friesen 1985; Wilson and Culbertson 1988). Despite this, we have previously isolated non-lethal nonsense mutations in SUP45 and SUP35 genes (designated sup45-n and sup35-n, respectively), which reduced the amount of eRF1 or eRF3 (Moskalenko
et al. 2003; Chabelskaya et al. 2004). Strains carrying these nonsense mutations were shown to have increased levels of endogenous tRNAs, that may be responsible for the viability of cells with impaired translation termination (Zhouravleva et al. 2006).

Most of the mutations in SUP45 and SUP35 genes selected as omnipotent suppressors are characterized by pleiotropic manifestations (Inge-Vechtomov, Zhouravleva and Philippe 2003). In most cases, it is not known whether these effects are a direct result of a translation termination defect, or whether they are mediated by participation of eRF1 and/or eRF3 proteins in cellular processes not directly related to translation termination. Data obtained in yeast prove that translation termination factors interact with components of different cellular systems, including the NMD complex (Czaplinski et al. 1998), poly(A)-binding protein (Hoshino et al. 1999; Cosson et al. 2002; Uchida et al. 2002), a system of mRNA export from the nucleus (Gross et al. 2007; Bolger et al. 2008), as well as actin (Bailleul et al. 1999) and tubulin (Valouev et al. 2004) cytoskeletons. Furthermore, several eRF3-linked phenotypes (e.g. sensitivity to caffeine, inability to utilize non-fermentable carbon sources) were recently shown to be not linked to translation termination (Valouev, Kushnirov and Ter-Avanesyan 2002; Merritt et al. 2010).

Mutations sup45 and sup35 are associated with the loss of chromosomes (Borchsenius, Tchourikova and Inge-Vechtomov 2000) and cytokinesis defects (Valouev et al. 2004). A decrease in the amount of eRF1 or eRF3c (a C-terminal fragment of eRF3) in the cells influences cell morphology and/or cell cycle progression (Valouev, Kushnirov and Ter-Avanesyan 2002). It is assumed that eRF1 is involved in regulation of the cell cycle in Arabidopsis thaliana (Petsch, Mylne and Botella 2005). A decrease in eRF3c in yeast leads to actin depolymerization (Valouev, Kushnirov and Ter-Avanesyan 2002). In mammalian cells, eRF3 depletion induces cell cycle arrest at the G1 phase through inhibition of the pathway, controlled by mTOR protein (Chauvin, Sali and Jean-Jean 2007). Nonsense mutations in the SUP45 gene lead to impairment in the pseudohyphal growth of strains from Peterhof Genetic Collection of St Petersburg State University (PGC) (Zhouravleva and Petrova 2010).

Yeast is a dimorphic fungus able to grow in two morphologically different forms. The 'yeast' form is characterized by ovoid cells, which separate shortly after mitosis. In the filamentous form, cells become elongated, stay attached to each other and form branched chains. The term 'pseudohyphal (PH) growth' is used to distinguish this filamentous form from true hyphal growth typical of Candida albicans, a pathogenic yeast species. Signal cascades that regulate morphological shift in these species are highly conserved (Whiteway, Dignard and Thomas 1992; Liu, Kohler and Fink 1994; Kohler and Fink 1996; Leberer et al. 1996). Only diploid yeast strains possess the ability to form pseudohyphae in response to environmental changes, though haploid strains develop a related phenotype, characterized by the invasion of a growing colony into the agar (Roberts and Fink 1994). PH growth of diploids and invasive growth of haploids are triggered by largely overlapping regulatory cascades (see Gagiano, Bauer and Pretorius 2002 for review). FLO11, a target gene of these cascades, encodes GPI-anchored cell surface glycoprotein and plays a crucial role in filamentous growth development in both diploid and haploid strains (Guo et al. 2000).

In this paper, using strains of different origin, we show that sup45 mutations that reduce the efficiency of translation termination result in disruption of the PH growth of diploid strains, while mutations that disrupt eRF1 phosphorylation or improve the efficiency of translation termination do not have this effect. Missense and nonsense sup45 mutations lead to a change in the budding pattern in both diploid and haploid strains, and abolish the invasive growth of haploid strains. However, sup45 mutations do not impair PH growth, proving that this effect is specific for defects of eRF1.

MATERIALS AND METHODS

Strains

Escherichia coli strain used was XL1-Blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1 [F', proAB, lacIq, ΔlacZΔM15, Tn10(tet)] (Sambrook, Fritsch and Maniatis 1989).

All yeast strains (Table 1) used in this study (except 1a-D1628 and D1631) are congenic to the Σ1278b genetic background (Liu, Styles and Fink 1993).

Table 1. Yeast strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or description</th>
<th>Background</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a-D1628</td>
<td>MATα ade1-14 his3 leu2 ura3 trp1 lys2 sup45::HIS3 [SUP45]</td>
<td>GT81</td>
<td>Moskalenko et al. (2003)</td>
</tr>
<tr>
<td>1b-D1628</td>
<td>MATα ade1-14 his3 leu2 ura3 trp1 lys2 sup45::HIS3 [SUP45]</td>
<td>GT81</td>
<td>Moskalenko unpublished</td>
</tr>
<tr>
<td>D1631</td>
<td>MATα/MATα diploid of 1a-D1628 × 1b-D1628</td>
<td>GT81</td>
<td>This study</td>
</tr>
<tr>
<td>16a-D1608</td>
<td>MATα ade1-14 his7-1 lys2-87 met13-A1 thr4-B15 trp1 ura3-52 leu2-3,112 SUP35::TRP1</td>
<td>GT81</td>
<td>Chabelskaya et al. (2004)</td>
</tr>
<tr>
<td>10560-23c</td>
<td>MATα ura3-52 his3::HIS leu2::HISG</td>
<td>Σ1278b</td>
<td>From H-U. Mosch</td>
</tr>
<tr>
<td>10560-4a</td>
<td>MATα ura3-52 trp1::H his2::H his3::H his3::H his3::H</td>
<td>Σ1278b</td>
<td>This study</td>
</tr>
<tr>
<td>D1639</td>
<td>MATα/MATα ura3-52 ura3-52 leu2::H his3::H his2::H his3::H</td>
<td>Σ1278b</td>
<td>This study</td>
</tr>
<tr>
<td>D1640</td>
<td>MATα/MATα ura3-52 ura3-52 leu2::H his3::H trp1::H his3::H SUP35::SUP35::HIS3</td>
<td>Σ1278b</td>
<td>This study</td>
</tr>
<tr>
<td>8a-D1640</td>
<td>MATα ura3-52 his3::H leu2::H trp1::H his3::H sup35::HIS3 [SUP35]</td>
<td>Σ1278b</td>
<td>This study</td>
</tr>
<tr>
<td>D1641</td>
<td>MATα/MATα ura3-52 ura3-52 leu2::H his3::H his3::H trp1::H his3::H SUP35::SUP35::HIS3</td>
<td>Σ1278b</td>
<td>This study</td>
</tr>
<tr>
<td>9a-D1641</td>
<td>MATα ura3-52 his3::H leu2::H his3::H SUP35::HIS3 [SUP45]</td>
<td>Σ1278b</td>
<td>This study</td>
</tr>
<tr>
<td>D1642</td>
<td>MATα/MATα ura3-52 ura3-52 his3::H his3::H his3::H leu2::H his3::H trp1::H his3::H SUP35::SUP35::HIS3</td>
<td>Σ1278b</td>
<td>This study</td>
</tr>
<tr>
<td>D1643</td>
<td>MATα/MATα ura3-52 ura3-52 his3::H his3::H his3::H leu2::H his3::H trp1::H his3::H SUP35::SUP35::HIS3 [SUP45]</td>
<td>Σ1278b</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 2. Mutations in SUP45 and SUP35 genes, studied in this work.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Amino acid change</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>In SUP45 gene:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sup45-101</td>
<td>E266 → stop</td>
<td>Moskalenko et al. (2003)</td>
</tr>
<tr>
<td>sup45-102</td>
<td>Y 53 → stop</td>
<td></td>
</tr>
<tr>
<td>sup45-104</td>
<td>L283 → stop</td>
<td></td>
</tr>
<tr>
<td>sup45-105</td>
<td>E385 → stop</td>
<td></td>
</tr>
<tr>
<td>sup45-107</td>
<td>L317 → stop</td>
<td></td>
</tr>
<tr>
<td>sup45-103</td>
<td>L215</td>
<td></td>
</tr>
<tr>
<td>sup45-113</td>
<td>M48I</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>E360V</td>
<td>Hatin et al. (2009)</td>
</tr>
<tr>
<td>A8</td>
<td>E104K</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>Q76R</td>
<td></td>
</tr>
<tr>
<td>C6</td>
<td>Q76K</td>
<td></td>
</tr>
<tr>
<td>C11</td>
<td>N58K</td>
<td></td>
</tr>
<tr>
<td>S421D/S432D</td>
<td>S421D, S432D</td>
<td>Kallmeyer et al. (2006)</td>
</tr>
<tr>
<td>S421A/S432A</td>
<td>S421A, S432A</td>
<td></td>
</tr>
<tr>
<td>In SUP35 gene:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sup35-21</td>
<td>Q422 → stop</td>
<td>Cosson et al. (2002)</td>
</tr>
<tr>
<td>sup35-228</td>
<td>R372→stop</td>
<td>Chabelskaya et al. (2004)</td>
</tr>
</tbody>
</table>

Strain D1631 is the autodiploid derivative of 1a-D1628 (Le Goff et al. 2002) induced by the YRp-HO plasmid, which contains the homothallism (HO) gene. Strain 1a-D1628 is the isogenic derivative of strain GT81 (Chernoff et al. 2000). The diploid strains D1640 (sup35::HIS3) and D1641 (sup45::HIS3) are derivatives of the strain 10560 (obtained from H-U. Mosch) in a Σ1278b background (Rupp et al. 1999). The diploid strain D1639 was obtained by mating 10560-23c and 10560-4a. This strain was further used for construction of D1640 (sup35::HIS3) and D1641 (sup45::HIS3) using the PCR-targeting procedure (Longtine et al. 1998). HIS3 cassettes were amplified by PCR from plasmid pFA6a-His3MX6 with a pair of primers that included 40 bp upstream and downstream of the SUP35 or SUP45 open reading frame and integrated into the genome by homologous recombination. The haploid strains 8a-D1640 and 9a-D1641 were recovered from the meiotic progeny of D1640 and D1641, respectively.

**Yeast plasmids**

The centromeric (CEN) plasmids pRS315/SUP45, pRS315/sup45 and pRS316/SUP45 (Moskalenko et al. 2003) express SUP45 or its mutant alleles from its own promoter. Construction of pRSU1/sup35-21 was described earlier (Chabelskaya et al. 2004), pRSU1/sup35-228 plasmid was constructed by replacing the 1.3-kb PstI-NcI fragment of SUP35 from pRSU1 by the PstI-NcI fragments from pGEMT/sup35-228 plasmid. The presence of sup35 mutation was verified by sequencing. Plasmids bearing mutations affecting the phosphorylation sites of eRF1 or different sup45 antisuppressor mutations (Table 2) were described earlier. Plasmid pDB843 encodes eRF1 protein lacking the C-terminal 19 amino acids (Kallmeyer, Keeling and Bedwell 2006). Derivatives of pGP564 contain segments of the yeast S. cerevisiae genome (Yeast Genomic Tiling Collection, Open Biosystems) (Jones et al. 2008).

**Genetic and microbiological procedures**

Standard rich YPD medium, synthetic complete SC medium and selective media lacking individual components of SC were used (Sherman, Fink and Hicks 1986). Yeast strains were grown at 25°C. Synthetic low-ammonia dextrose medium (SLAD) used to induce PH development was prepared as described (Gimeno et al. 1992). Strains scored for PH filamentation were streaked on SLAD plates, and representative colonies were photographed after 5 days of growth at 25°C. Haploid invasive growth was assayed as described (Roberts and Fink 1994). Haploid strains were grown on YPD medium for 3 days. Surface cells were washed off, and the plate was incubated for 30 h at 25°C. The plate-washing assay was performed as previously described (Roberts and Fink 1994).

Yeast transformation was performed by lithium acetate procedure (Gietz et al. 1995). For E. coli transformation, the high-efficiency procedure was performed (Inoue, Nojima and Okayama 1990). The haploid strain (sup::HIS3 [CEN URA3 SUP45]) was transformed with [CEN LEU2 sup45] plasmids. Transformants, selected on −Ura−Leu medium, were replica plated onto 5-FOA medium (1 mg ml⁻¹, purchased from Sigma), which counterselects against URA3 plasmids and thus against wild-type SUP45 gene (Boeke, LaCroute and Fink 1984).

For quantitative characterization of nonsense suppression, β-galactosidase reporter system (Stansfield, Akhmaloka and Tuite 1995) was employed. In this the vector-based assay, we used plasmids that were not subjected to NMD (Wang et al. 2001). Previously, we also showed that the steady-state levels of wild-type and nonsense-containing lacZ transcripts were nearly identical and proportional to the amount of actin mRNA (Cosson et al. 2002). Yeast strains were transformed with UAA, UAG or UGA plasmids carrying TAA, TAG or TGA (termination codons), respectively, cloned in frame with lacZ and with control LacZ plasmid containing the lacZ gene without the stop codon (all plasmids were a gift of S. Feltz and W. Wang). Only in the case of nonsense suppression could active β-galactosidase be synthesized. Efficiencies of suppression were calculated as a ratio of β-galactosidase activity in cells harboring lacZ with premature termination codon to that in cells with a normal allele of lacZ. Values for liquid β-galactosidase assay represent the mean of at least three assays from each of three independent transformants.

Quantitative assessment of intracellular cAMP levels was conducted using iodine staining of intracellular glycerone (Chester 1968; Enjalbert et al. 2000), which gives inverse proportional estimation of cAMP (Kubler et al. 2000). For this purpose, cells were spotted on YNB plates supplemented with uracil. After 6 days of incubation, plates were inverted over iodine crystals for 1 min, removed for 15 s and exposed again for 2 min.

**Photomicroscopy**

For calcofluor staining, cultures were grown on YPD or SC liquid medium to the mid-logarithmic phase (OD600 ~ 0.6–0.8). About 10⁶ cells were harvested, washed and mixed with 20 μl of calcofluor white (Sigma) solution (1 mg ml⁻¹). After 10 min of incubation, cells were washed three times in 1× PBS, mounted in 50% glycerol/PBS and viewed using Zeiss AxioLab system (Zeiss). Fluorescent images were acquired at 100× magnification with Canon Powershot 7.2 camera. Budding pattern analysis was performed as described (Cali et al. 1998) with minor alterations. Cells with four bud scars were scored. At least 200 cells were scored for each strain.

Pearson product moment correlation coefficient (r) was calculated using Microsoft Excel software.
Reverse transcription of RNA from yeast cells
For extraction of RNA, cultures were grown in SC liquid medium to the mid-logarithmic phase (OD600 ~ 0.6–0.8). Cells were harvested, washed and further grown in SLAD liquid medium during 8 h. Total RNA was extracted from cultures with the Ambion RiboPure-Yeast (Applied Biosystems) according to the manufacturer’s instructions. Purified RNA was reverse transcribed with iScript Select kit (Bio-Rad).

Quantitative polymerase chain reaction
The expression of target genes was analyzed by quantitative PCR (qPCR) with IQ SYBR Green supermix (Bio-Rad) according to the manufacturer’s instructions. The following primers were used:

- ACT1-F (TGGACAAGAAATGCAACGCTGC), ACT1-R (TGACATTGACCATCTGGAAGTTCGAGG)
- FLO11-F (TATTGACCTGAAGTATCTAGG), FLO11-R (TATTGACGCGCCACTACCT).

Duplicate qPCRs were performed for each sample. The ΔΔCT method (Livak and Schmittgen 2001) was used to measure the relative fold quantification.

Protein isolation
Protein isolation, SDS-PAGE electrophoresis and western blotting were described previously (Chabelskaya et al. 2004). Polyclonal rabbit antibodies (SE-45-2; Kiktev et al. 2009) were used to detect the Sup45 protein.

RESULTS
Mutations in the SUP45 gene impair formation of pseudohyphae in diploid strains
All sup45-n (nonsense) and sup45-m (missense) mutations used in this study were selected by the effect of simultaneous suppression of nonsense mutations his7-1 (UAAs) and lys9-A21 (UAAs) (Moskalenko et al. 2003), and therefore affect the main function of the Sup45 protein—termination of translation. We compared the induction of PH growth among sup45-n or sup45-m mutants with that in yeast strains bearing either mutations that disrupt eRF1 phosphorylation (Kallmeyer, Keeling and Bedwell 2006) or mutations that improve the efficiency of translation termination (Hatin et al. 2009). For this purpose, we used yeast strains with Σ1278b and GT81 genetic backgrounds. Derivatives of Σ1278b are traditionally used to study PH growth, while derivatives of GT81 have not previously been used for this purpose. The full list of sup45 mutations studied is given in Table 2: among them were five nonsense mutations (sup45-101, sup45-102, sup45-104, sup45-105 and sup45-107) and two missense mutations (sup45-103 and sup45-113) described in our previous studies (Moskalenko et al. 2003, 2004), as well as two mutations that disrupt eRF1 phosphorylation (Kallmeyer, Keeling and Bedwell 2006) and five mutations that improve the efficiency of translation termination (Hatin et al. 2009).

Strains D1631 (GT81 background) and D1643 (Σ1278b background) are diploid strains homozygous for deletion of the SUP45 gene. Viability of the strains is maintained by a CEN plasmid with a URA3 marker bearing the wild-type SUP45 gene. A plasmid shuffle technique (see the section ‘Materials and Methods’) was used to substitute this plasmid with a CEN plasmid with a LEU2 marker bearing a mutant allele of the SUP45 gene. Since only MATα/α diploids are able to shift to PH form, we monitored the heterozygosity of strains for mating type locus by crossing the strains with haploid testers. This was necessary because sup45 mutations increase chromosomal instability (Borchsenius, Tchorouikova and Inge-Vechtomov 2000) and loss of MAT heterozygosity would lead to the blocking of PH growth. PH growth was evaluated in low-ammonia medium (SLAD) only for non-mating colonies. The diploid strain D1643 bearing any sup45 nonsense mutations or missense mutation sup45-103 did not form pseudohyphae (Fig. 1A, upper panel). Similar results were obtained for the strain D1631 (Fig. 1A, lower panel). Missense mutation sup45-113 did not completely abolish pseudohyphae formation in either of the genetic backgrounds.

To detect the amount of the Sup45 protein in the sup45 mutants in different conditions, we used western blot hybridization (Fig. S1, Supporting Information). In accordance with previous data (Moskalenko et al. 2003), cells bearing nonsense mutations sup45-104 and sup45-105 contained decreased amount of full-length Sup45p accompanied by truncated fragments with molecular mass consistent with the predicted one (31.5 and 35.0 kDa, respectively). Western blot analysis did not reveal the presence of truncated protein in lysates prepared from the sup45-107 strain (Fig. S1, Supporting Information). Failure to detect such truncated protein with a predicted molecular mass of 35.2 kDa can be explained by antigen-binding specificities of antibodies. Relative to the level of Sup45p in the wild-type strain (set to 1), the amount of full-length Sup45p in the sup45-113 (missense), sup45-104, sup45-105, sup45-107 strains were 0.90, 0.28, 0.20, 0.26 on YPD medium and 0.80, 0.15, 0.18, 0.24 in SLAD medium, respectively. Thus, inability of sup45 mutants to undergo PH growth cannot be explained by a decreased amount of Sup45 protein in low-ammonia medium.

Double amino acid substitutions that prevent Sup45 phosphorylation (S421A/S432A) or mimic constitutive phosphorylation (S421D/S432D) did not lead to impairment of PH growth in diploids with a different genetic background (Fig. 1B, left). Mutations in the SUP45 gene that increase translation termination efficiency had different effects on PH differentiation (Fig. 1B, right). Mutations A2 and B1 diminished the ability of strains of both Σ1278b and GT81 backgrounds to form pseudohyphae. Mutation C6 abolished PH growth in the strain of GT81 but not the Σ1278b background. Mutation C11 did not influence PH growth of either strain.

Prolonged incubation of haploid strains on the rich medium provokes them to penetrate the agar; hence, this growth type is known as invasive growth. We found that wild-type haploid strains of the GT81 genetic background are not able to invade the agar (data not shown). A haploid strain 9a-D1641 of Σ1278b genetic background developed a strong invasive phenotype and was employed in a plasmid shuffle experiment to check the effects of mutant sup45 alleles. Two tested nonsense mutations, sup45-102 and sup45-104, and a missense mutation, sup45-103, reduced the ability of the haploid strain to invade the agar (Fig. 2).

Transcription profiles were previously built for wild-type strains and strains bearing sup45-103 mutation grown in low-ammonia medium compared to the same strains grown in standard SD medium using the Affymetrix Genechip WT Sense Target Labeling Assay (Petrova et al., unpublished). These data will be presented elsewhere. Amount of sup45-103 mRNA was not changed in SLAD medium compared with SD medium. Thus, defect in PH growth of this mutant cannot be explained by decreased level of the SUP45 mRNA. FLO11 transcript was not detected in the global transcription assay, so FLO11 expression was checked by a quantitative real time PCR. ACT1 was used as a reference gene. A small but reproducible increase in the FLO11
Figure 1. Yeast cells with sup45 nonsense and missense mutations are defective in PH development. Diploid strains D1643 (Σ1278b background) and D1631 (GT81 background) bearing SUP45 (WT) or plasmids with sup45 mutations were induced to undergo PH growth in a nitrogen-limiting SLAD medium; colony morphology was estimated after 5 days at 30 °C. (A) Mutations selected as nonsense suppressors. (B) Mutations affecting Sup45p phosphorylation or leading to antisuppression.

mRNA levels was detected in the strains bearing sup45-102 (1.27 ± 0.085) or sup45-103 (2.95 ± 0.326) mutation. Thus, a lack of PH and invasive growth is not likely to be due to a low level of Flo11p.

To learn more on how reduced and expressed level on these genes affects PH growth and budding, we have tested whether high copy expression of some of the genes suppresses the observed phenotypes. Overexpression of TAO3, BUD8, PHD1, DFG16 and GPI1 in sup45 mutants had no effect on PH growth (data not shown).

Suppressor mutations in the SUP45 gene lead to a change in the budding pattern in diploid and haploid strains

Upon nitrogen source depletion, vegetative diploid cells with bipolar budding acquire an axial budding pattern and form long chains of cells (Gimeno et al. 1992; Kron, Styles and Fink 1994). We hypothesized that the inability of sup45 mutants for PH growth can be attributed to changes in budding pattern. We analyzed the distribution of budding sites in the D1631 derivatives bearing different sup45 mutant alleles (we could not apply a similar analysis to the strain D1643 due to the strong flocculation of all D1641 derivatives that prevented accurate counting of the bud scars).

The presence of sup45 suppressor mutations led to a significant decrease in cells with bipolar budding while the proportion of cells with the random budding was 3–5-fold higher compared to the wild-type strain (Fig. 3A). Double amino acid substitutions that prevent Sup45p phosphorylation (S421A/S432A) or mimic constitutive phosphorylation (S421D/S432D) led to an increase in the proportion of cells with an axial budding type and to a complementary decrease in the number of cells with bipolar budding without changing the proportion of cells with random budding (Fig. 3B). Strains bearing sup45 mutations that increase translation termination efficiency had a higher proportion of cells with a random type of budding, although to a lesser extent than strains with mutations that decrease the efficiency of translation termination (Fig. 3C). Apparently, the altered budding pattern in the sup45-n and sup45-m mutant strains is one of the factors that reduce the capacity for PH growth.

In the haploid state, yeast cells typically exhibit axial budding type as detected for the strain 1a-D1628 (Fig. 4A). The same strain bearing mutant sup45 alleles had a significant (7–20%) decrease in the proportion of cells with axial budding, and had a complementary increased number of cells with random and bipolar budding pattern. This effect was most pronounced in the presence of nonsense mutations sup45-104, sup45-105 and sup45-107. The strain bearing weak missense mutation
sup45-113 showed a budding pattern identical to the wild type. Supporting these data, strain 9a-D1641 bearing mutations sup45-102, 103 and 105 had a significant decrease in the proportion of cells with an axial budding pattern and a higher number of randomly budding cells compared to the wild-type strain (Fig. 4B). We also tested the budding pattern in different sup45 antisuppressor mutants. As shown in Fig. S2 (Supporting Information), the character of budding was not changed compared to the wild-type cells.

To test the relationship between the budding pattern and efficiency of nonsense suppression, we measured stop codon readthrough in the same derivatives of the strain 1a-D1628 (Fig. 4A) grown under the same conditions that were used for budding analysis. For this purpose, a vector-based assay system for quantification of nonsense codon readthrough in vivo was employed (Stansfield, Akhmaloka and Tuite 1995). In the omnipotent mutants tested, nonsense suppression levels of all stop codons varied between 2 and 8% for UAA, between 3 and 13% for UAG and between 3 and 15% for UGA (Fig. 4C). A strong positive correlation (r = 0.9) between stop codon readthrough at UAG codon and random budding was observed with lower positive correlation for other two stop codons (0.5 and 0.77 for UAA and UGA, respectively) (Fig. 4C).

Previously we have performed transcriptome analysis to find global gene expression differences between sup45 mutants and wild-type cells (Moskalenko et al., unpublished). These data will be presented elsewhere. Using these data we identified few genes, participating in budding and downregulated in sup45 mutants. We chose three of them which are known as involved in bud-site selection by different ways: RSR1 (Chant and Pringle 1995), BUD8 (Zahner, Harkins and Pringle 1996) and BUD32 (Ni and Snyder 2001; Kato et al. 2011). To check the effect of these genes overexpression on character of budding in sup45 mutants, we transformed sup45-102 and sup45-103 mutants with derivatives of pGP564 which contain corresponding segments of the yeast S. cerevisiae genome (Jones et al. 2008). Presence of multiplicity plasmids bearing RSR1 or BUD32 did not significantly change budding in the sup45 mutants (Fig. S3, Supporting Information). In the meantime, overexpression of BUD8 in diploid strain bearing sup45-102 mutation decreased proportion of cells with axial budding in the same way as a wild copy of SUP45. However, derivatives of D1631 strain bearing sup45-102 mutation transformed by plasmid pGP564/BUD8 still were not able to form pseudohyphae (see the previous section). No obvious effect of BUD8 overexpression on PH growth in sup45 mutants may be due to insufficient restoration of bipolar budding compared to the wild-type SUP45.

Mutations in SUP35 do not affect PH and invasive growth

The effect of sup45 mutations on PH growth may be mediated by the non-specific damage of translation termination. To address this possibility, we tested the effects of mutations in the SUP35 gene that encodes a functional partner of Sup45p needed for the efficient translation termination. For this purpose, we used two sup45 mutations, which decreased an accuracy of translation termination (Chabelskaya et al. 2004). Previously, one of them, nonsense mutation sup35-21, was characterized as a strong suppressor (Cosson et al. 2002). Missense mutation sup35-228 is also an efficient suppressor (Chabelskaya et al. 2004) comparable with sup35-21 (Fig. S4, Supporting Information).

We used diploid strain D1642 that had chromosomal SUP35 genes deleted. Viability of the strain was supported by plasmids bearing a wild type or mutant alleles of the SUP35 gene. D1642 derivatives proven to be heterozygous by MAT locus were used for the analysis of the capacity for PH growth. Both tested mutant alleles did not inhibit filamentous growth (Fig. 5A) and did not affect the invasive growth of 8a-D1640 (Fig. 5B).

cAMP restores the ability of sup45 mutants to form pseudohyphae

One of the key components of the signaling cascade that controls the transition to the filamentous form of growth is cAMP (Rupp et al. 1999). An increase in cAMP levels in response to environmental changes, including nitrogen starvation, triggers FLO11 expression, which is essential for PH and invasive growth. We found that the addition of cAMP to a final concentration of 10 mM in low-ammonium medium partly restored PH growth of D1631 derivatives bearing sup45 mutant alleles (Fig. 6), suggesting that sup45 mutants are defective in the cAMP-dependent cascade inducing pseudohyphae formation.

To estimate the level of intracellular cAMP in vivo, we used iodine staining of the intracellular glycogen (Chester 1968; Enjalbert et al. 2000). This method is used for detection of the defects in the Ras-cAMP pathway (Kubler et al. 1997). We did not find significant difference in staining between sup45 mutants compared to the wild-type strain (Fig. S5, Supporting Information).

Figure 2. SUP45 is required for invasive growth. Haploid strain 9a-D1641 (Y1278b background) bearing pRS315/SUP45 (WT) or pRS315/sup45 plasmids was patched on a YPD plate and incubated for 5 days at 30°C. The plate was photographed before (total growth) and after (invasive growth) washing the cells off the agar surface.
High levels of cAMP led to a higher rate of random budding pattern in both diploid wild type and sup45-101 mutant strains with a reduction of bipolar budding (Fig. S6A, Supporting Information). The sup45-102 mutant was characterized by high percentage of cells forming branched chains when grown in the presence of cAMP (Fig. S6B, Supporting Information). Thus, cAMP does not restore the bipolar budding in sup45 mutants.

Inability of sup45 mutants to form pseudohyphae is connected with a defect in translation but not with the interaction eRF1–eRF3

The main function of Sup45p is participation in translation termination. To address the question of whether translation termination efficiency is involved in filamentation in yeast, we checked the PH growth of a wild-type strain D1639 (Sigma1278b background) in a low-ammonia medium supplemented with translational antibiotic hygromycin B, or cycloheximide, or paromomycin. The ability of yeast to form pseudohyphae was repressed by all three drugs (Fig. 7A) at concentrations that still allowed strains to grow (Fig. 7B). Thus, filamentous growth of yeast can be abolished not just by a general inhibition of translation caused by cycloheximide but also by drugs affecting translation accuracy such as paromomycin or hygromycin B.

In order to check whether the influence of eRF1 on PH formation depends on its interaction with eRF3, we used a C-terminal deleted variant of eRF1 (eRF1-CΔ19). It was shown previously that deletion of the last 19 amino acids importantly decreases the interaction of eRF1 with eRF3 and leads to an enhancement of nonsense suppression (Eurwilaichitr et al. 1999; Kallmeyer, Keeling and Bedwell 2006). We compared the PH growth of diploid strains D1643 and D1631 containing full-length Sup45p with derivatives of the same strains but bearing the pDB843 plasmid encoding truncated version of Sup45p, and found that deletion of the C-terminal part of eRF1 did not lead to impairment of PH growth; moreover, strains bearing eRF1-CΔ19 actually had an enhanced hyphae formation (Fig. 7C). Thus, impaired filamentous growth is specific to sup45 mutants and could not be explained by defects in translation termination.

DISCUSSION

The main function of Sup45 and Sup35 proteins is to participate in termination of protein synthesis. Numerous phenotypes of sup45 and sup35 mutations imply that multiple intracellular...
systems are modified by low efficiency in the translation termination or that these proteins participate in processes other than translation termination. In this study, we show that filamentous growth of both haploid and diploid yeast strains is diminished by mutations in the SUP45 gene, and it was restored, at least partly, by the high external concentration of cAMP.

Figure 4. Suppressor mutations in SUP45 change bud-site selection in haploid strains. Budding patterns of derivatives of haploid strains 1a-D1628 (A), 9a-D1641 (B) bearing sup45 suppressor mutations, grown at 25 °C, were determined after calcofluor staining. A total of 200–300 cells were counted for each strain in three independent sets of experiments. The average percentage of each budding pattern is shown (SD < 2%); axial (black bar), bipolar (white bar) and random (gray bar). (C) Stop codon readthrough of haploid strains showed on panel A. Results are the means of at least three separate experiments. Pearson product moment correlation coefficients for readthrough efficiency and type of budding are given, a—axial, b—bipolar, r—random. Statistically significant relationships are shown by one or two asterisks (P < 0.05 and P < 0.01, respectively).

Figure 5. Mutations in SUP35 do not affect PH and invasive growth. (A) Derivatives of diploid strain D1642 (Σ1278b background) bearing sup35 mutations were induced to undergo PH growth in a nitrogen-limiting SLAD medium; colony morphology was estimated after 5 days at 30 °C. (B) Haploid strain 8a-D1640 (Σ1278b background) bearing pRSU1/SUP35 (WT) or pRSU1/sup35 plasmids was patched on a YPD plate and incubated for 5 days at 30 °C. The plate was photographed before (total growth) and after (invasive growth) washing the cells off the agar surface. Three independent transformants were tested in each case.

Mutations in the SUP45 gene, used in this study, were selected as spontaneous reversions to prototrophy for histidine and lysine in the strains carrying the UAA nonsense mutations in the HIS7 and LYS9 genes (Moskalenko et al. 2003). All mutants, except for the missense mutant sup45-113, are omnipotent nonsense suppressors. Additionally, all nonsense but not...
missense mutants are characterized by a decreased amount of full-length eRF1 ranging from 8 to 32% compared to the amount of eRF1 in the wild-type strain (Moskalenko et al. 2003). Previous studies revealed a correlation between a low amount of eRF1 and increased nonsense suppression (Valouev, Kushnirov and Ter-Avanesyan 2002; Moskalenko et al. 2003). Missense mutations in the SUP45 gene do not influence the amount of eRF1 and do not break its interaction with eRF3; therefore, nonsense suppression is likely to be caused by changes in the spatial structure of eRF1 (Moskalenko et al. 2004).

Mutations in the SUP45 gene lead to a multitude of pleiotropic effects (Inge-Vechtomov, Zhouravleva and Philippe 2003). It was
shown that at least some of the eRF1-specific phenotypes such as cell length, cell diameter and caffeine sensitivity are unlinked to translation termination and may be caused by defects in non-translational functions of eRF1 (Merritt et al. 2010). In this work, we used several approaches to clarify the role of eRF1 in the regulation of PH differentiation. Despite the fact that the filamentous growth in yeast is controlled by several regulatory cascades that overlap only partially and activate various transcription factors, inactivation of solely the FLO8 gene is sufficient to disrupt the ability of the strain to form hyphae (Liu, Styles and Fink 1996). Many laboratory yeast strains, including strain S288C, contain a nonsense mutation in the FLO8 gene, which leads to the formation of non-functional protein. Strains with a Σ1278b background, commonly used for the study of filamentous growth, do not contain this mutation (Liu, Styles and Fink 1996). Strain D1631, used in our work to study the PH differentiation, had a different genotypic background than the derivatives of the S288C or Σ1278b strains. The FLO8 gene sequencing confirmed the absence of mutation and allowed the Flo8 protein dysfunction to be excluded as a cause of the absence of filamentous growth in D1631 strain derivatives (Zhouravleva and Petrova 2010).

Also a lack of PH growth of sup45 mutants could not be explained by repression of FLO11 because level of its mRNA measured by qPCR is not decreased in mutants and overexpression of FLO11 does not restore PH growth.

The analysis of the budding pattern of strains carrying different sup45 mutant alleles showed that both nonsense and missense mutations used in this experiment led to a change in the proportion of cells manifesting different budding types in both diploid and haploid strains. Suppression efficiencies of UAG and UGA codons in sup45-a and sup45-m mutants correlate with an increase in random budding: the Pearson correlation coefficients are 0.90 and 0.77, respectively, both above the 95% confidence level (0.71). Derivatives of the diploid strain D1631 bearing sup45 mutant alleles and retaining heterozygosity at the MAT locus had an increased proportion of cells with a random budding pattern that, as was shown earlier, impedes PH growth (Gimeno et al. 1992).

Nonsense and missense mutations in the SUP45 gene have a strong effect on the accuracy of translation termination and cell viability in general (Inge-Vechtomov, Zhouravleva and Philippe 2003). If the absence of filamentous growth is a direct consequence of the translation defects, the sup45 mutations that do not disturb the eRF1 function in translation termination should not affect the ability of mutant strains for filamentous growth. We showed that mutations that cause a loss of eRF1 phosphorylation (Kallmeyer, Keeling and Bedwell 2006) do not affect the ability of strains to form pseudohyphae and did not promote random budding. However, some mutations that increase the accuracy of translation termination (Hatin et al. 2009) impaired PH differentiation. Thus, the filamentous growth defect caused by mutations in the SUP45 gene may be explained by alternate non-translational functions of eRF1.

Translation termination factor eRF3 is a partner of eRF1 in translation termination. The SUP35 gene, as well as the SUP45 gene, is essential, and mutations in these genes have the same or a very similar manifestation. Pleiotropic effects of sup45 and sup35 mutations are similar in the most of the described cases. The resemblance of sup35 and sup45 phenotypes is in good agreement with the fact that translation termination factors eRF1 and eRF3, the products of these two genes, interact in translational termination and possibly participate in several other cellular processes (Inge-Vechtomov, Zhouravleva and Philippe 2003). If the defects in filamentous growth in the sup45 mutants are caused by decreased efficiency in translation termination, it is logical to assume that an impairment of the eRF3 function will cause a similar effect. However, in this study we did not detect a defect in filamentous growth in the presence of sup35 mutant alleles selected as strong omnipotent suppressors. Moreover, deletion of the eRF3 interacting domain of eRF1 does not affect PH differentiation. Thus, the altered efficiency of translation termination is not the reason for defects in filamentous growth in sup45 mutants and suggests a specific role of eRF1 in its regulation. Influence of several other eRF1-interacting proteins on invasive and PH growth was tested previously. Deletions of non-essential genes NAM7 (UPF1) or ITT1 had no effect on either phenotypes (Ryan et al. 2012), while overexpression of essential DBP5 increased filamentous growth (Jin et al. 2008).

A variety of evidence indicates a role of efficient protein synthesis in the control of PH differentiation in yeast. In the early studies of the developmental switch from vegetative to PH growth, the inhibitory effect of cycloheximide on PH growth was noted (Kron, Styles and Fink 1994). It was shown that sublethal concentrations of the translation inhibitor cycloheximide inhibit PH growth (Cutler et al. 2001) and adhesion of yeast cells (Ross, Saxena and Altmann 2012). Our data show that translation inhibition by hygromycin B, cycloheximide and paromomycin blocks the conversion of wild-type diploid yeast cells to PH growth. However, inability of sup45 mutants to form pseudohyphae cannot be explained by decreased rate of overall protein synthesis because this model is not supported by the observation that in the presence of exogenic cAMP sup45 mutants restore the ability to form pseudohyphae. Previously, it was shown that overall protein synthesis is not impaired in different sup45 mutants in permissive conditions (Surguchov et al. 1980; Stansfeld et al. 1997).

Several genes implicated in translation are also able to influence PH growth. It was shown that proper regulation of translation initiation by the eIF4E binding proteins is required for the PH response (Ibrahimo, Holmes and Ashe 2006). Mutation in the SUP70 gene (sup70-65) which encodes the tRNA<sup>CUG</sup> (Gln) induces PH growth even on a rich medium (Murray et al. 1998; Beeser and Cooper 1999). Recently, it was proposed that the sup70-65 mutation alters the efficiency of translation of the mRNA encoding a negative regulator of PH growth. The authors speculated that gene encoding such a regulator contains CAG codons towards the 5’ end of its open reading frame which will be efficiently read by CAG-decoding tRNA (Kemp et al. 2013).

Interestingly, the deletion of DOM34 gene leads to defects in PH growth and budding in diploid cells (Davis and Engbrecht 1998). Dom34p of S. cerevisiae consists of three domains, two of which (M and C) are homologous to the corresponding domains in eRF1, while the N-terminal domain of Dom34p is different from that of eRF1 and is probably necessary for the recognition of the mRNA stem (Graille, Chaillet and van Tilbeurgh 2008). We have shown that deletion of C-terminal 19 aa of eRF1 has no effect on PH growth; however, similar effects of DOM34 deletion and SUP45 mutations on PH growth prompted us to propose the existence of a common mechanism/or factor including both Dom34p or eRF1.

The level of cAMP is a signal in the cascades that regulate the cell cycle, sporulation, cell growth and stress response (see Santangelo 2006). Increased cAMP induces PH and invasive growth in response to a change in the amount of nutrients in the environment (Rupp et al. 1999). Impaired signal transduction due to improper function of one or more cascade components may lead to the loss of sensitivity for the changes in cAMP.
levels and, in turn, to the absence of PH growth. We showed that strains bearing sup45 mutant alleles form pseudohyphae on the medium supplemented with 10 mM of cAMP. Partial restoration of PH growth by addition of exogenous cAMP assumes that sup45 mutants are defective in the CAMP-dependent pathway that control filament formation. However, we found no differences between mutant and wild-type cells in the amount of intracellular cAMP. Thus, the effect of sup45 mutations on PH development could not be due to the decreased level of intracellular cAMP, but defects in other components of Ras/cAMP pathway cannot be excluded. Further studies will be required to find the molecular mechanism of this effect.

Nonetheless, our data imply a possibility to affect a PH growth of fungi through decreased efficiency of translation termination that can be achieved by some antibiotics, such as hygromycin B and paromomycin. These drugs inhibit cell growth through an arrest of protein biosynthesis as a result of the drugs binding to translating ribosomes. The biggest difficulty in treating fungal infections is that fungi are eukaryotic organisms that share the same antibiotic that affects human cells. Our results prove that the filamentation of fungi can be prevented by some known translational antibiotics in concentrations that do not have a lethal effect on cells, and that can be used as a therapeutic approach.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSYR online.

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Conflict of interest. None declared.

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