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ORIGINAL PAPER

Ishita Chatterjee · Stephane R. Gross
Terri Goss Kinzy · Kuang Yu Chen

Rapid depletion of mutant eukaryotic initiation factor 5A at restrictive temperature reveals connections to actin cytoskeleton and cell cycle progression

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Abstract Eukaryotic initiation factor 5A (eIF5A) is the only protein in nature that contains hypusine, an unusual amino acid derived from the modification of lysine by spermidine. Two genes, *TIF51A* and *TIF51B*, encode eIF5A in the yeast *Saccharomyces cerevisiae*. In an effort to understand the structure–function relationship of eIF5A, we have generated yeast mutants by introducing plasmid-borne *tif51A* into a double null strain where both *TIF51A* and *TIF51B* have been disrupted. One of the mutants, tsL102A strain (*tif51A L102A tif51a*Δ *tif51b*Δ) exhibits a strong temperature-sensitive growth phenotype. At the restrictive temperature, tsL102A strain also exhibits a cell shape change, a lack of volume change in response to temperature increase and becomes more sensitive to ethanol, a hallmark of defects in the *PKC/WSC* cell wall integrity pathway. In addition, a striking change in actin dynamics and a complete cell cycle arrest at G1 phase occur in tsL102A cells at restrictive temperature. The temperature-sensitivity of tsL102A strain is due to a rapid loss of mutant eIF5A with the half-life reduced from 6 h at permissive temperature to 20 min at restrictive temperature. Phenylmethyl sulfonylfluoride (PMSF), an irreversible inhibitor of serine protease, inhibited the degradation of mutant eIF5A and suppressed the temperature-sensitive growth arrest. Sorbitol, an osmotic stabilizer that complement defects in *PKC/WSC* pathways, stabilizes the mutant eIF5A and suppresses all the observed temperature-sensitive phenotypes.

Keywords eIF5A · Hypusine · Temperature sensitivity · Actin · Cell wall integrity

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I. Chatterjee · K. Y. Chen
Department of Chemistry and Chemical Biology,
Rutgers—The State University of New Jersey, Piscataway,
NJ 08854-8087, USA

S. R. Gross · T. G. Kinzy
Department of Molecular Genetics, Microbiology
and Immunology, UMDNJ Robert Wood Johnson Medical
School, Piscataway, NJ, USA

K. Y. Chen (✉)
Department of Chemistry, Rutgers University,
610 Taylor Road, Piscataway, NJ 08854-8087, USA
E-mail: KYCHEN@rutchem.rutgers.edu
Tel.: +1-732-4453739
Fax: +1-732-4455312

Introduction

Eukaryotic initiation factor 5A (eIF5A) is present in eukaryotes and archae, but not in eubacteria. It is the only protein in nature that contains hypusine, an unusual amino acid derived from the modification of a specific lysine residue by deoxyhypusine synthase and deoxyhypusine hydroxylase (Chen and Liu 1997; Park et al. 1997; Chen and Jao 1999). The activity of hypusine formation correlates positively with cell proliferation and inhibition of deoxyhypusine synthase in cells leads to growth arrest and cell death (Jakus et al. 1993; Park et al. 1994; Chen et al. 1996; Chen and Chen 1997). Moreover, the disruption of the genes encoding either eIF5A or deoxyhypusine synthase in the yeast *Saccharomyces cerevisiae* leads to lethal phenotype (Schnier et al. 1991; Sasaki et al. 1996).

The amino acid sequence of eIF5A is conserved from archae to human, suggesting that eIF5A from different species share a similar structure. X-ray crystal analysis of archaea aIF5A precursor protein reveals an N-terminal domain (I) and a C-terminal domain (II), connected via a short hinge (Peat et al. 1998; Kim et al. 1998; Yao et al. 2003). The side chain of hypusine, located at residue 51 in domain I of yeast eIF5A, resembles spermidine and spermine, both of which bind specifically to tRNA (Rochon and Cohen 1972). Domain II resembles the oligonucleotide-binding fold found in the bacterial cold shock protein A (CspA) (Murzin 1993). We have reported that human eIF5A can bind to Rev-response element (RRE) and U6 snRNA and that hypusine is required for the binding (Liu et al.

1997). We have also demonstrated that eIF5A binds to a SELEX-enriched RNA in a sequence-specific and hypusine-dependent manner (Xu and Chen 2001). When compared with other bimodular RNA-binding proteins, we noticed the presence of a leucine-rich stretch in eIF5A (aa 90–103 in *S. cerevisiae* eIF5A), similar to that present in the effector domain of Rev and NS1 (Liu et al. 1997), suggesting that eIF5A may also be capable of interacting with other cellular proteins via domain II. In this regard, eIF5A has been reported to interact with CRM1 (Rosorius et al. 1999), exportin4 (Lipowsky et al. 2000), recently syntenin (Li et al. 2004), and recently translating 80S ribosome (Jao and Chen 2005). Whether and how any of these interactions may be relevant to the essential function of eIF5A remains to be determined.

The yeast *S. cerevisiae* contains two highly homologous genes, *TIF51A* and *TIF51B*, encoding two eIF5A isoforms (Schnier et al. 1991). Since the deletion of either *TIF51A* or both *TIF51A* and *TIF51B* produces a lethal phenotype (Schnier et al. 1991; Whol et al. 1993), it is not possible to use a knockout approach to study the function of eIF5A. An alternative strategy is to generate conditional mutant yeast strains to probe the structure–function relationship and biological events downstream of eIF5A. To this end, several temperature-sensitive mutants of the *TIF51A* gene have been described in the literature (Zuk and Jacobson 1998; Valentini et al. 2002). These mutants were isolated and characterized by complementation of certain temperature-sensitive phenotypes, raising concerns with regard to the effect of the presence of *TIF51B*, the other eIF5A-encoding gene. To address these concerns, we created yeast eIF5A mutants by introducing a plasmid-borne *tif51a* allele into yeast strain where both *TIF51A* and *TIF51B* have been disrupted to ensure that the *tif51a* allele is the only eIF5A-encoding gene in the mutant strain.

Among the mutant strains that we generated using this approach, a yeast strain, termed tsL102A, with the L102A mutation showed that this single amino acid mutation conferred a temperature-sensitive growth defect phenotype and an altered morphology characteristic of weakened cell wall integrity. In addition, the tsL102A strain exhibited a striking change in actin dynamics and a complete cell cycle arrest at G1 phase. The temperature sensitivity of tsL102A was due to a rapid loss of mutant eIF5A rather than any change in structure–function relationship at restrictive temperature. Stabilization or the inhibition of the degradation of mutant eIF5A protein by phenylmethyl sulfonylfluoride or sorbitol led to the suppression of the observed temperature-sensitive phenotypes.

Expermental procedures

Yeast strains and bacterial strains

The genotypes and sources of *S. cerevisiae* strains used or constructed in this work are described in Table 1. Yeast was grown in YPED (Yeast Extract, Peptone, Dextrose), or defined complete medium (C or C-) supplemented with 2% dextrose as carbon source (Sherman et al. 1986). Yeast cells were transformed by lithium acetate method (Gietz et al. 1992). For sporulation, cells were streaked from YPED plates onto sporulation media (1% potassium acetate) and incubated for 3 days. Spores were dissected and the tetrads analyzed by their ability to grow on complete media with 5-fluoro-orotic acid (Boeke et al. 1987). Experiments were carried out at 25°C unless otherwise mentioned. For agar plates containing 1 M sorbitol, 20% glucose, or 0.2 M NaCl, each reagent was added to YPED media before autoclaving. For YPED plates with 0.2 mM PMSF (phenylmethyl sulfonyl fluoride), 50 nM TPA (12-O-tetradecanoylphorbol-13-acetate) or 6% ethanol, each reagent was added to the media after autoclaving. Growth rate in liquid media was determined by measuring absorbance at 600 nm.

Cloning the *TIF51A* gene in pRS416 and pRS414

The sequences of *TIF51A* and *TIF51B* have been assigned the GenBank accession numbers M63541 and M63542, respectively. *TIF51A* (0.5 kb ORF, 0.7 kb 5′UTR and 1.3 kb of 3′UTR) was amplified by PCR using oligo primers P1 (CGC GGA TCC GCG AGA AGG CAA CTA GAC CAG A) and P2 (CCG CTC GAG CGG GTT AGA GTA CGA GGA GAC AAT GCG). The sequences of oligos used in this study are listed in Table 2. Both pRS416 and pRS414 are centromere-based yeast plasmid vector with selective markers *URA3* and *TRP1*, respectively. The *TIF51A* PCR product and the vector pRS414 were digested with *BamH*1 and *Xho*1. The digested product was purified and the *TIF51A* fragment (2.5 kb) was cloned into the *BamH*1 and *Xho*1 sites of pRS414 to generate the plasmid pKCB20. The same enzyme sites in the plasmid pRS416 were used to clone the *TIF51A* PCR fragment, resulting in plasmid pKCB51. All the plasmids used in this study are listed in Table 3.

Mutagenesis of *TIF51A*

Mutagenesis was carried out by the QuikChange site-directed mutagenesis kit using the manufacture's protocol (Stratagene). Oligonucleotide primers containing the desired mutation and complementary to the opposite strands of the vector was used to amplify *TIF51A* from pKCB20 (*TIF51A* in pRS414). The resulting vector was then transformed into competent *E. coli* strain and the mutation was confirmed by sequencing.

Construction of *TIF51A* and *TIF51B* deletion mutant strains

The wildtype diploid strain KCY15 was used for the deletion of the *TIF51A* and *TIF51B* genes. PCR-based

Table 1 Yeast strains used in this study

Strain	Genotype	Reference
KCY15	*MATa/a ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-63/ trp1-63 his3-200/his3-200 leu2-1/leu2-1*	Carr-Schmid et al. (1999)
KCY13	*MATa ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1*	Carr-Schmid et al. (1999)
KCY14	*MATα ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1*	Carr-Schmid et al. (1999)
KCY26	*MATα ura3-52 lys2-801 ade2-101 trp1-63 his3-200 LEU2+*	Carr-Schmid et al. (1999)
KCY30	*MATa/a tif51a::LEU2/TIF51A ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-63/ trp1-63 his3-200/his3-200*	This study
KCY31	*MAT a tif51a::LEU2 tif51b::KanMX ura3-52 lys2-801 ade2-101 trp1-63 his3-200 Kan^R [KCB51]*	This study
KCY34	*MAT a tif51a::LEU2 ura352 lys2-801 ade2-101 trp1-63 his3-200Kan^R*	This study
KCY32	*MAT α tif51a::LEU2 ura3-52 lys2-801 ade2-101 trp1-63 his3-200 [KCB51]*	This study
KCY117	*MAT α tif51a::LEU2 tif51b::KanMX ura3-52 lys2-801 ade2-101 trp1-63 his3-200 Kan^R [KCB51]*	This study
KCY118	*MAT α tif51b::KanMX ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1 Kan^R*	This study
KCY158 (tsL102A)	*MAT α tif51a::LEU2 tif51b::KanMX ura3-52 lys2-801 ade2-101 trp1-63 his3-200 Kan^R [KCB25]*	This study
KCY159	*MAT α tif51a::LEU2 ura3-52 lys2-801 ade2-101 trp1-63 his3-200 [KCB25]*	This study
KCY164	*MAT α tif51a::LEU2 ura3-52 lys2-801 ade2-101 trp1-63 his3-200 [pKCB20]*	This study
KCY174 WT)	*MAT α tif51a::LEU2 tif51b::KanMX ura3-52 lys2-801 ade2-101 trp1-63 his3-200 Kan^R [KCB20]*	This study
KCY161 tsL124A)	*MAT α tif51a::LEU2 tif51b::KanMX ura3-52 lys2-801 ade2-101 trp1-63 his3-200 Kan^R [KCB26]*	
SS330	*MATa ade2-101 his3-200 tyr1 ura3-52*	Zuk and Jacobson (1998)
ts1159	*MATa ade2-101 his3-200 tyr1 ura3-52 tif51a [S149P (CCC)]*	Zuk and Jacobson (1998)

gene disruption method (Eberhardt and Hohmann, 1995) was used. Chromosomal *TIF51A* gene was replaced by *LEU2* gene in the strain KCY15. The resulting diploid yeast strain with *tif51a::LEU2 TIF51B* (KCY30) was transformed with pKCB51 (*TIF51A URA3*), sporulated, and dissected. Tetrads were streaked on plates with 5-FOA, and two of the four haploid spores had the chromosomal *TIF51A* disrupted and the rescuing plasmid pKCB51, producing the haploid yeast strain KCY32 (*tif51a::LEU2 TIF51B pKCB51*). Using the same PCR-based gene disruption method, the *TIF51B* gene was disrupted in KCY30 and replaced with *KanMX* gene, which confers resistance to the drug Geneticin. Sporulation and tetrad dissection of the *tif51B::KanMX TIF51A* diploid yeast strain produced haploid yeast strain KCY118 (*TIF51A tif51b::KanMX*) and haploid strain KCY117 (*tif51a::LEU2 tif51b::KanMX pKCB51*) (see Table 1).

Table 2 Plasmid used in this study. *TIF51A*, *tif51A*, or *TIF51B* was cloned into yeast centromere-based plasmid (Ycp) pRS414 with *Trp1* marker or pRS416 with *Ura3* marker

Plasmid	Description	Reference
PKCB 20	*TIF51A* in pRS414 (Ycp-*TRP1*)	This study
pKCB 51	*TIF51A* in pRS416 (Ycp-*URA3*)	This study
pKCB 52	*TIF51B* in pRS416 (Ycp-*URA3*)	This study
pKCB 25	*tif51A* (**L102A**) in pRS414	This study
PKCB26	*tif51A* (**L124A**) in pRS414	This study

Construction of yeast strains expressing *tif51A* alleles

The plasmid-shuffling technique was employed to introduce plasmid-borne *TIF51A TRP1*-based plasmids into strain KCY32 (*tif51a::LEU2*) or KCY117 (*tif51a::LEU2 tif51b::KanMX*) via 5-FOA selection. Two plasmids, pKCB20 (pRS414 containing *TIF51A*) and pKCB25 (pRS414 containing *tif51a-L102A*) (see Table 2), were used for transformation. The resulting strains KCY159 and KCY158 express mutant eIF5A (L102A) in the genetic background of KCY32 and KCY117, respectively. Strains expressing wildtype eIF5A in KCY32 and KCY117 are KCY164 and KCY174, respectively.

Western Blot analysis

For biochemical experiments involving temperature shift, yeast cells were grown to logarithmic phase ($OD_{600} = 1$) at 25°C. The culture was centrifuged and the cell pellet was suspended in pre-warmed YPED and incubated at 37°C. For determining the half-life of eIF5A, yeast cells were grown at 25°C to log phase. The cultures were briefly centrifuged and the pellet was suspended in fresh media containing 100 μg/ml cycloheximide and incubated at 25°C for 1 h. An aliquot was taken and the rest of the culture was shifted to 37°C. Cells were harvested at the designated times and lysates prepared for protein determination and gel electropho-

Table 3 Oligonucleotides used in this study

Oligo	Sequence (5′-3′)
L102A[a]	G GAC ATT GAT GAC GGT TTC **GCT** TCT TTG ATG AAC ATG GAC GG (TTG)
L124A[a]	GAA GGT GAA TTG GGT GAC AGT **GCT** CAA ACT GCT TTT GAT GAA GG (TTG)
S149A[a]	GGT GAA GAA GCC GCC ATC **GCT** TTC AAG GAA GCT GC (TCC)
S149P[a]	GGT GAA GAA GCC GCC ATC **CCC** TTC AAG GAA GCT GC (TCC)
5′HYP2	CCC AAA CAC ACA CAA ATA CCA ACT C
3′HYP2	CAT TTA TAT CCC ATG CCA TGA TGT TAA CCG
5′ANB1	CCT ATT TCA TTC ACA CAC TAA AAC
3′ANB1	CGC GTA TTT TGT AAG CTA TAG AAA TC
5′TDH3	CGA ATA AAC ACA CAT AAA CAA AC
3′TDH3	CTA AGT CAT AAA GCT ATA AAA AG
P1	CGC GGA TCC GCG AGA AGG CAA CTA GAC CAG A
P2	CCG CTC GAG CGG GTT AGA GTA CGA GGA GAC AAT GCG

[a]Amino acid substitution was created in *TIF51A* by using Quikchange mutagenesis kit. The numbering represents the position of the mutated amino acid residue. The codon of the original amino acid residue is indicated in parenthesis underneath the codon of mutated amino acid which is typed in bold

resis (15% SDS/PAGE). Western Blot analysis was performed using a rabbit anti-yeast eIF5A antibody (1:1,000 dilution) and enhanced chemiluminescence detection (Amersham Biosciences, UK).

RNA preparation and RT-PCR

RNA was extracted using the RNeasy Kit (Qiagen, CA, USA) and treated with Rnase-free DNase to remove contaminating DNA. Reverse transcription was performed using Superscript TM II or M-MLV reverse transcriptase. PCR was performed on the RT product using oligo primers specific for *TIF51A* (5′HYP2 and 3′HYP2) or *TIF51B* (5′ANB1 and 3′ANB1) transcripts (see Table 3). The PCR products were electrophoresed in 1% agarose gel using the level of α-GAPDH mRNA as a control.

Microscopy and actin phalloidin staining

Yeast strains were grown in YPED media with or without 1 M sorbitol for 16 h in log phase by continual dilution at 24°C before shifting to 37°C for 2 days. Cell morphologies were observed using Nomarski light microscopy. Cells were scored for the size of either 2.5, 5 or 10 mm. Averages of cell size were calculated by adding the percentages of the three different categories and dividing by the total number of cells in the population. For actin staining, fixation of cells was performed by adding formaldehyde and Triton X100 to a final concentration of 4 and 0.5%, respectively, and incubated for 30 min at room temperature. Yeast pellets were resuspended in PBS buffer with 0.5 mM $MgCl_2$ and 4% formaldehyde for further fixation for 90 min at room temperature. Cells were then washed once with PBS prior to phalloidin addition to a final concentration of 0.6 mM in PBS and incubated for 60 min at room temperature in the dark. Yeast samples were subsequently washed three times with PBS and resuspended in 1× mounting media (90% glycerol, 0.1% PBS, 92 mM p-phenylmediane). Images were captured with an I×70 inverted fluorescence microscope (Olympus) equipped with a HiQ fluorescein filter set (excitation wavelength: 450–492 nm); a Planapochromatic 100× oil immersion objective lens; and a 100 W Hg lamp. Images were collected and analyzed with a Princeton Instruments 5 MHz MicroMax cooled CCD camera, a shutter and controller unit, and IPLab software (version 3.5, Scanalytics).

Flow cytometry

Yeast strains were grown in YPED media with or without 1 M sorbitol for 16 h in log phase by continual dilution at 24°C before shifting to 37°C for 2 days. Fixation was performed by adding ethanol to a final concentration of 70% and incubation at 4°C for an hour. Yeast pellets were resuspended in 50 mM Sodium citrate (pH 7.0), and digested with RNAse 1 (0.25 mg/ml) for 1.5 h at 50°C. Samples were washed three times with 50 mM Sodium citrate (pH 7.0) before suspension in this buffer with 16 mg/ml propidium iodide. Samples were analyzed on a Coulter Cytomics FC500 Flow Cytometer.

Results

Generation of eIF5A mutation and yeast mutant strains

Yeast *S. cerevisiae* has two eIF5A-encoding genes, *TIF51A* on chromosome V and *TIF51B* on chromosome X. The disruption of both genes produces lethal phenotype (Schnier et al. 1991; Wohl et al. 1993). In order to study the structure–function relationship of eIF5A, we have used the plasmid-shuffling method to introduce plasmid-borne *tif51a* alleles into yeast null mutants where either one or two of the eIF5A encoding genes were disrupted. Mutation sites were chosen to target at

conserved residues within the putative RNA- and protein-binding regions based on previous motif analysis (Liu et al. 1997). In this study we have focused on the yeast mutant strain KCY158 (*ptif51a-L102A tif51aΔ bΔ*), which harbors the L102A mutation on the plasmid-borne *tif51a* allele with both chromosomal *TIF51A* and *TIF51B* disrupted. L102 is a strictly conserved residue located within the putative protein-binding motif in domain II. Figure 1 shows the relative position of L102 within the 3-D ribbon structure of an archae aIF5A. As a comparison, a mutant strain expressing L124A, a conserved L residue located outside the putative protein-binding motif was also analyzed.

Growth phenotype of yeast mutant strain

Figure 2a compares the growth phenotypes of the mutant strain KCY158 (*ptif51a-L102A tif51aΔ bΔ*) with the corresponding isogenic wildtype strain KCY174 (*pTIF51A tif51aΔ bΔ*) at three different temperatures. The slow growth phenotype of KCY158 strain was apparent at 30°C and became prominent at 37°C. In contrast, another mutant strain KCY159 (*ptif51a-L102A tif51aΔ TIF51B*), which has the same plasmid-borne *tif51a-L102A* mutant but with only *TIF51A* disrupted, exhibited a less severe temperature-sensitive growth phenotype, probably due to the presence of the remaining chromosomal *TIF51B*. For the subsequent studies, the strain KCY158 was referred to as tsL102A and the corresponding isogenic wildtype KCY174 was referred to as WT strain. Next we tested whether the temprature-dependent growth arrest is reversible or not. Figure 2b shows that tsL102A cells, maintained at 37°C for over 4 days, resumed growth after shifting back to permissive temperature, indicating that no permanent damage was done to the cells and that the temperature-dependent growth arrest of tsL102A was reversible. In contrast to tsL102A, the tsL124A strain, which has the L124A mutation in a plasmid-borne *TIF51A*, did not exhibit strong temperature-sensitive growth phenotype (Fig. 2b).

eIF5A protein but not mRNA levels are reduced in tsL102A at the restrictive temperature

To determine whether the temperature-sensitive phenotype of tsL102A reflects a structure–function defect, we examined the protein level of wildtype and mutant eIF5A before and after a temperature shift. Figure 3a shows that the levels of wildtype and mutant eIF5A were comparable at the permissive temperature. But at restrictive temperature, the mutant eIF5A protein in tsL102A became almost non-detectable. The rapid loss of the mutant eIF5A in tsL102A at restrictive temperature was due to a striking change in the half-life of the protein, from ~6 h at permissive temperature to only 15~20 min after shifting to restrictive temperature (Fig. 3b). It can be noted that under either permissive or restrictive temperatures, the wildtype eIF5A was very stable with a half-life longer than 24 h. Thus, a single amino acid mutation at L102 changed the stability of mutant eIF5A and that the effect became very prominent at restrictive temperature. Figure 3c shows that the level of *tif51a* transcript in tsL102A was comparable to

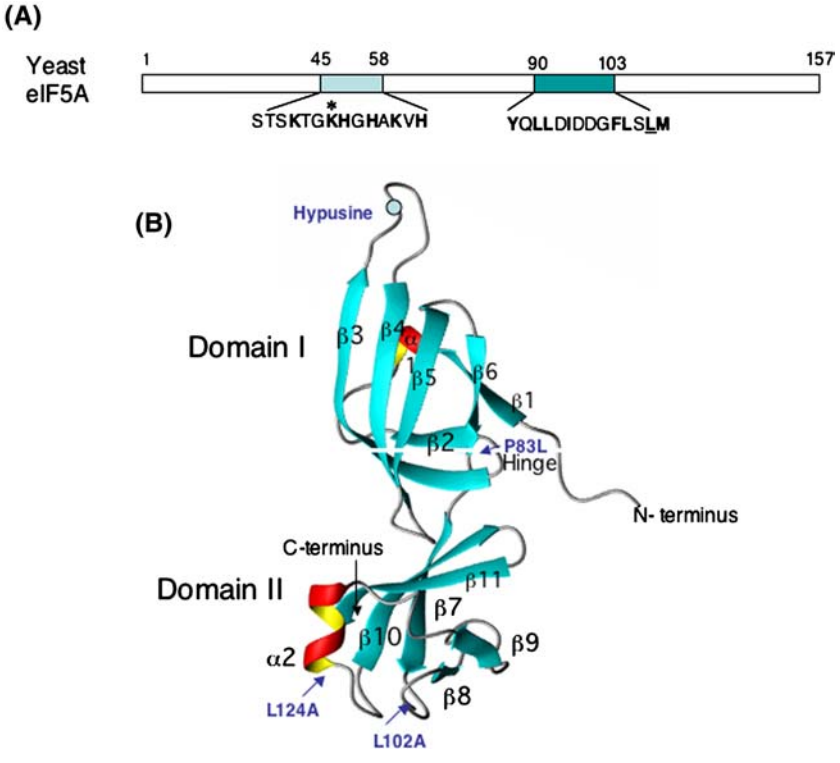

Fig. 1 **a** Motif analysis of yeast eIF5A. The hypusination site (K51) of eIF5A is marked with an *asterisk*. The basic residues in amino acids 45–58 and the hydrophobic residues in amino acids 90–103 are indicated in *boldface*. The hydrophobic amino acid residues in the putative effector domain of eIF5A are shown with *bold face* and the critical leucine residue is *underlined*. **b** X-ray diffraction structure of aIF5A from *Methanococcus jannashii*. The protein is bimodular with N-terminal half (domain I) and C-terminal half (domain II) connected by a short flexible hinge. The positions of mutated residues are indicated

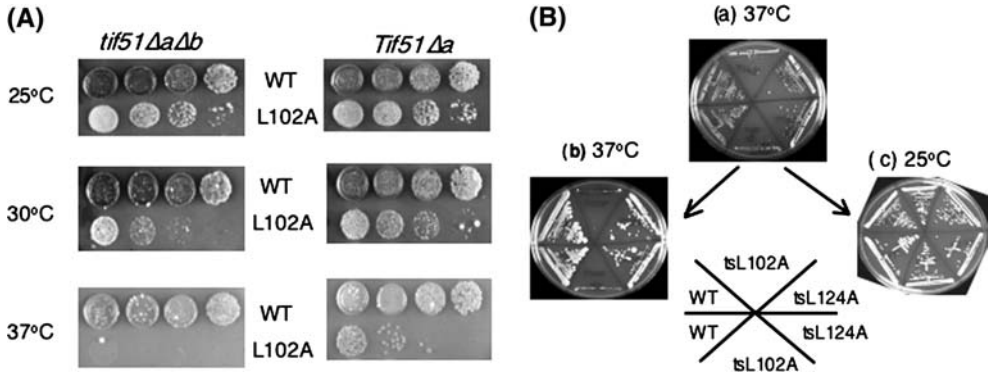

Fig. 2 **a** Temperature-sensitive growth phenotype of KCY158 (*ptif51a-L102A tif51aΔ bΔ*) and KCY159 (*ptif51a-L102A tif51aΔ TIF51B*). A tenfold serial dilution of KCY158 and KCY159 and their corresponding isogenic wildtype strains, KCY174 (*pTIF51A tif51aΔ bΔ*) and KCY164 (*pTIF51A tif51aΔ TIF51B*) were spotted on YPED plates and incubated at indicated temperatures. KCY158 and KCY174 are referred to as tsL102A and WT, respectively, in this study. **b** Reversibility of the temperature-sensitive growth phenotype. **a** Growth of the strain WT, tsL102A (KCY158) and tsL124A (KCY161) at 37°C. Each strain was streaked on YPED plates and incubated at 37°C for 4 days. **b** Growth at 37°C after re-streaking. Each strain was re-streaked on fresh YPED plate and incubated at 37°C. **c** Growth at 25°C after re-streaking. Each strain from plate incubated at 37°C was re-streaked on fresh YPED plate and incubated at 25°C. Growth of tsL102A resumed at 25°C, but not at 37°C

TIF51A in the WT strain (lane 4 vs. lane 2) and remained unchanged at restrictive temperature for over 19 h (lanes 6, 8 vs. lane 4). As expected, we did not find any detectable *TIF51B* transcript in either WT or tsL102A cells (lanes 3, 5, 7, and 9), since both strains lack chromosomal *TIF51B*.

eIF5A depletion alters cell size and morphology

Cell size homeostasis represents a fine balance between cell growth and cell division (Neufeld and Edgar 1998). For the budding yeast *S. cerevisiae*, one of these processes deals with polarized growth and the other

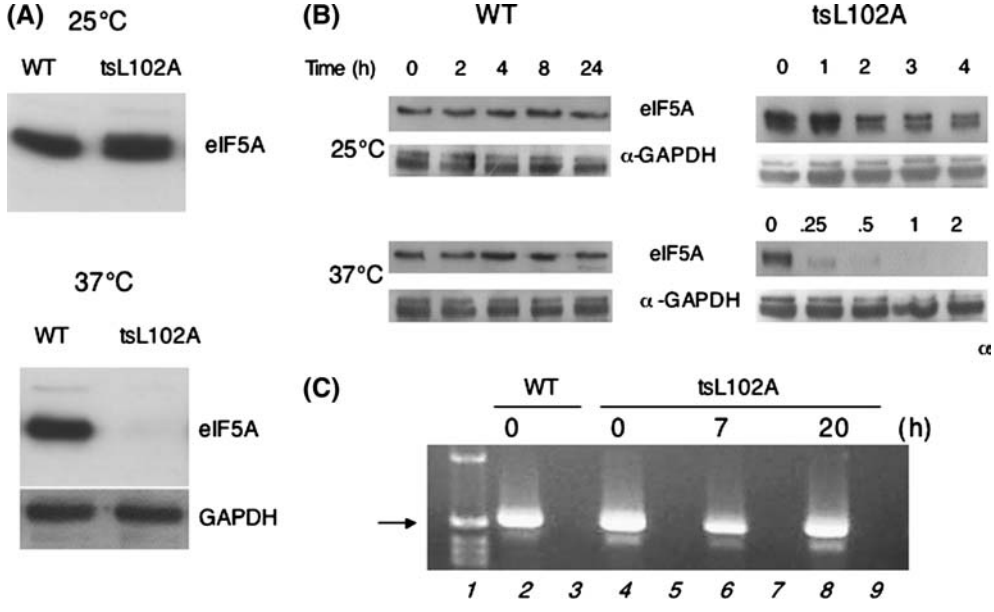

Fig. 3 The levels of eIF5A mRNA and protein in wildtype (WT) and mutant tsL102A strains. **a** Western Blot analysis of the eIF5A protein level in WT and tsL102A strain. **b** Half-life of wildtype and mutant eIF5A at permissive and restrictive temperature. The yeast strains tsL102A and WT were grown in YPED to mid-logarithmic phase at 25°C. Cells were treated with cycloheximide (100 μg/ml) and split into two separate cultures, one incubated at 37°C and the other kept at 25°C. Aliquots were then taken out at indicated times. Total protein was isolated and 10 μg of protein was electrophoresed in 15% SDS/PAGE for western Blot analysis using anti-eIF5A antibody. Western Blot using anti- α-GAPDH antibody was used as a loading control. **c** RT-PCR analysis of the level of eIF5A mRNA. The tsL102A strain was grown at 25°C to log phase and then shifted to 37°C. At indicated time, aliquots were removed for RNA extraction and RT-PCR. RNA (1 μg) was reverse transcribed and the corresponding cDNA was amplified using oligonucleotide primers specific for *TIF51A* or *TIF51B*. The PCR product was separated on a 1% agarose gel. *Lane 1* size marker; *lane 2 TIF51A* transcript level in WT; *lane 3 TIF51B* transcript level in WT; *lanes 4, 6 and 8* are *tif51A* transcript of tsL102A; *lanes 5, 7 and 9* are transcript level of*TIF51B*

cytokinesis, and both are also related to the establishment of cell morphology (Madden and Snyder 1998). Gene deletion has proved to be a powerful means to identify genes involved in the regulation of the yeast size and shape (Giaever et al. 2002). This approach, however, cannot be applied to eIF5A because deletion of eIF5A gives lethal phenotype. In this regard, the mutant tsL102A offers a conditional null system to examine how the depletion of eIF5A affects cell size and shape. At permissive temperature, there was no significant difference in cell shape between WT and tsL102A cells, but the size of tsL102A was slightly smaller than WT cells (Fig. 4a). However, at restrictive temperature the size difference between WT and tsL102A became prominent as early as 2 h after temperature shift (Fig. 4a, b). This large difference in size is primarily due to the fact that WT strain showed a gradual increase in cell size after temperature shift, whereas the tsL102A cells actually exhibited a slight decrease in cell size at 37°C (Fig. 4b). In addition to the lack of cell size change at the restrictive temperature, the morphology of tsL102A cells also suggested that the cell wall integrity might be compromised. To determine whether this is the case, we tested the sensitivity of tsL102A strain to ethanol, since hyper-sensitivity to ethanol is a hallmark for weakened cell wall integrity (Zu et al. 2001). Figure 4c shows that indeed tsL102A was at least one or two orders of magnitude more sensitive to ethanol in comparison with WT strain.

Osmotic stabilizer such as sorbitol can alleviate the defects in cell wall integrity caused by deletion of *PKC/WSC* pathway genes (Levin and Bartlett-Heubusch 1992; Paravincini et al. 1992; Verna and Ballester 1999). To further ascertain that the temperature-sensitive growth arrest and morphology change of tsL102A are due to cell wall defect, we examined whether sorbitol might suppress these phenotypes. Figure 5 shows that the addition of sorbitol almost completely restored the growth of tsL102A at restrictive temperature. Other reagents that increase the medium osmolarity, including glucose and NaCl, also significantly suppressed the temperature-sensitive growth arrest (Fig. 5, lower panels). We then examined whether sorbitol can also restore the cell size and morphology of tsL102A at restrictive temperature. Interestingly, we found that the presence of sorbitol not only suppressed the morphological change (Fig. 6a) but also restored the ability of tsL102A cells to increase in size in response to temperature shift to the same degree as that of the WT cells (Fig. 6b).

Sorbitol and PMSF blocked the temperature-sensitive degradation of eIF5A

The fact that hyper-osmolarity could suppress the temperature-sensitive growth phenotype and morphological change of tsL102A suggests that genes involved in cell wall integrity may compensate for the function of eIF5A. Alternatively, hyper-osmolarity may block the rapid depletion of mutant eIF5A in tsL102A and thus restore the level of eIF5A for growth at restrictive temperature. To distinguish these two possibilities, we have examined the effect of sorbitol on the level of mutant eIF5A in tsL102A before and after temperature shift. The addition of sorbitol did restore the level of mutant eIF5A in tsL102A at restrictive temperature (Fig. 7a). It appears that sorbitol stabilized the mutant eIF5A by increasing the half-life of the protein at

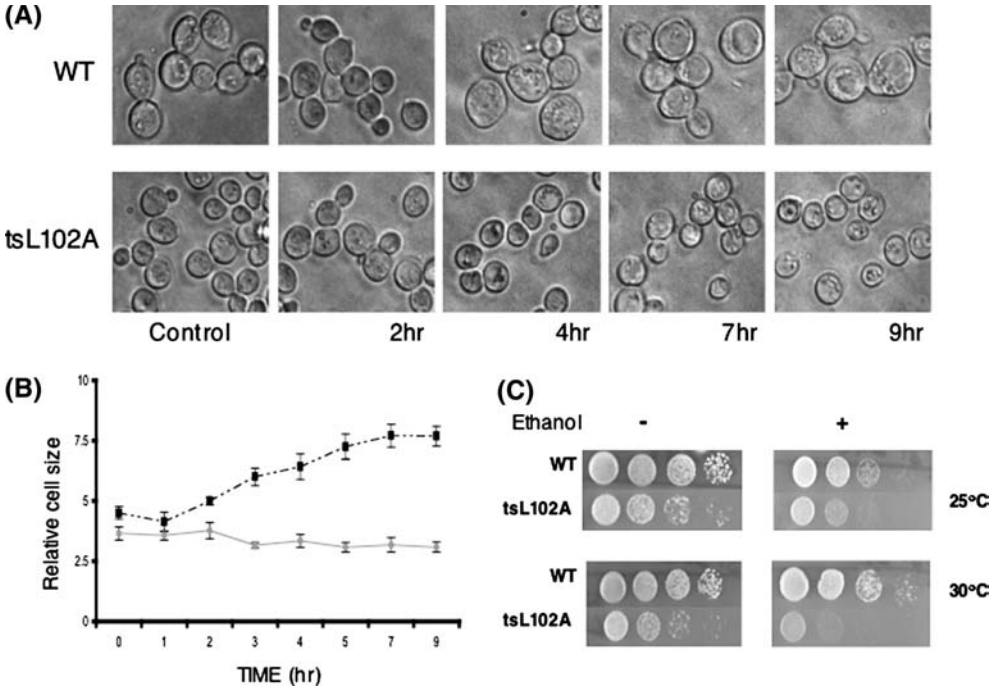

Fig. 4 **a** Cell morphology and **b** cell size of WT and tsL102A yeast cells grown in YPED media in log phase at 25°C before shifting to 37°C for the time indicated. Cell morphologies were observed using Nomarski light microscopy and size determined as determined in methods. *Solid square*: WT; *gray circle*: tsL102A. **c** tsL102A is sensitive to ethanol. Tenfold serial dilution of WT and tsL102A were spotted on YPED plates with or without 6% ethanol. The plates were incubated at 25 and 30°C

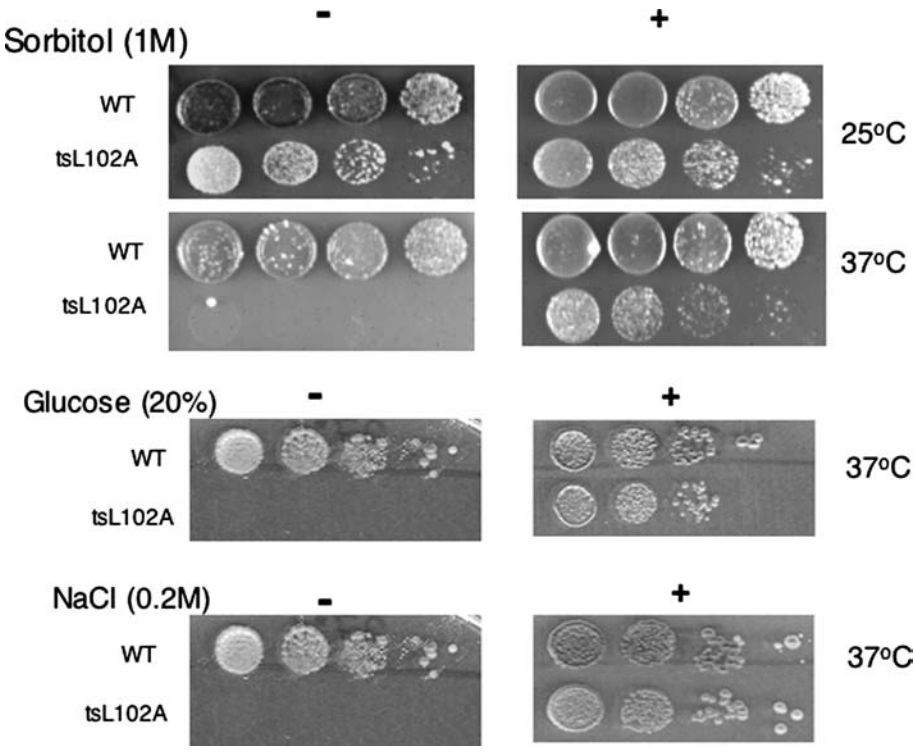

Fig. 5 Sorbitol, glucose, and NaCl suppress the temperature-sensitive growth phenotype of tsL102A. WT and tsL102A cells were grown in YPED at 25°C. Tenfold serial dilutions were spotted on plates with YPED or YPED with 1 M Sorbitol, 20% glucose, or 0.2 M NaCl. The plates were incubated at 25 or 37°C and grown for 3 days

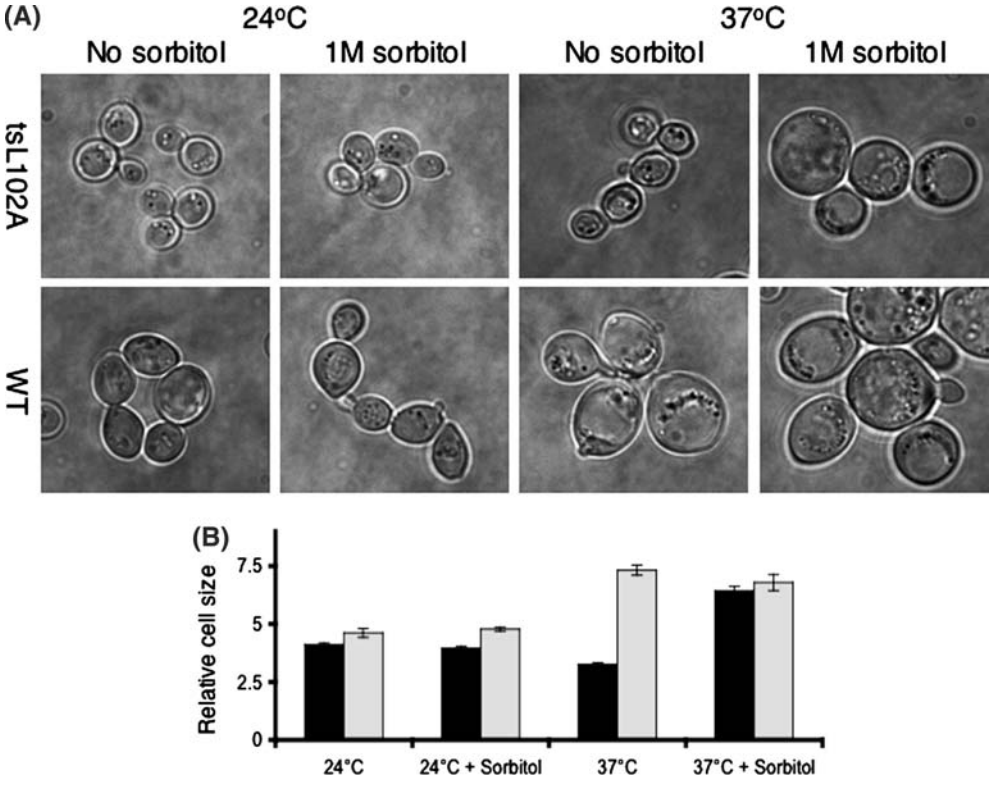

Fig. 6 **a** Cell morphology of WT and tsL102A yeast cells grown in YPED media at permissive or restrictive temperatures with or without sorbitol. Yeast strains were grown in YPED media with or without 1 M sorbitol for 16 h by continual dilution at 24°C before shifting to 37°C for 2 days. Cell morphologies were observed using Nomarski light microscopy. **b** Quantitative estimate of the cell size in WT and tsL102A cells. Cells were scored for the size of either 2.5, 5 or 10 μm. Averages of cell size were calculated by adding the percentages of the three different categories and dividing by the total number of cells in the population. *Black bar*: tsL102A; *gray bar*: WT

restrictive temperature (Fig. 7b). It can be noted that the effect of sorbitol appears almost immediately after temperature shift, suggesting that the stabilization of eIF5A occurs earlier than other biological effects. Thus, the change of the stability of the mutant eIF5A could explain the suppression effects of sorbitol on the temperature sensitive phenotypes of tsL102A. The fact that mutant eIF5A has a very short half-life and that sorbitol can change this half-life prompted us to determine whether any protease may be involved in the rapid loss

of eIF5A at restrictive temperature. As a preliminary effort, we tested the effect of PMSF, a serine protease inhibitor, on the eIF5A protein level in tsL102A at restrictive temperature. As shown in Fig. 7c, PMSF inhibited the degradation of mutant eIF5A. More importantly, PMSF suppressed the temperature-sensitive growth arrest phenotype (Fig. 7d). Taken together, these results suggest that mutant eIF5A is functional at restrictive temperature and that the temperature-sensitivity of tsL102A was suppressed by sorbitol via stabilizing the mutant eIF5A.

TsL102A confers temperature-sensitive change of the actin cytoskeleton

The actin cytoskeleton plays a key role in cell morphogenesis (Karpova et al. 1998). In yeast, the branched actin filaments and non-branched actin filaments serve as the basis for the formation of actin patches and actin cables, respectively (Narumiya 2003). In addition, the structure of the actin cytoskeleton has also been implicated in the organization of the translation machinery (Stapulionis et al. 1997; Hesketh 1994; Bassell and Singer 1997). Given that eIF5A depletion led to morphological change (Fig. 6) and eIF5A may have a role in translation (Kang and Hershey 1994), we decided to examine the effect of eIF5A depletion on the actin cytoskeleton structure. The actin cytoskeleton from WT and tsL102A strains was stained using rhodamine phalloidin and compared by fluorescence microscopy (Fig. 8). Both WT and tsL102A strains exhibited similar actin cytoskeleton organization at permissive temperature, with actin cables extended from the buds and along the cytoplasm between cortical actin patches. This pattern of actin patch distribution was not affected significantly by the presence of sorbitol. At restrictive temperature, we observed a striking change in actin cytoskeleton organization in the strain tsL102A, but not in strain WT. The cortical patches apparently disappeared and, instead, clustered at one end of the cell as an aggregate. Concomitantly, all the actin cables all but disappeared. Thus, tsL102A cells at restrictive temperature have gone through a dramatic attenuation of actin dynamics due to the depletion of eIF5A. Again, sorbitol blocked this change of actin dynamics in tsL102A cells, indicating the tight coupling between eIF5A and actin dynamics. The loss of actin cables, which are cortically located, could explain the weakened cell wall integrity of tsL102A at restrictive temperature. Because of the dramatic changes in actin dynamics that we observed in tsL102A cells at restrictive temperature, we further examined the time course of these changes to determine how early this event might occur. Figure 9 shows that the change in actin cytoskeleton organization became apparent as early as 4 h after temperature shift, suggesting that actin organization is an early downstream event affected by eIF5A.

Effect of eIF5A depletion on cell cycle arrest

The fact that eIF5A is essential and tightly coupled to proliferation suggests that it may have a role in the cell cycle. However, previous studies using pharmacological approach have yielded conflicting data. For example, it was reported that the inhibition of deoxyhypusine synthase reduces the G1 population of CHO-K1 cells (Shi et al. 1996), while another group found that the inhibition of deoxyhypusine hydroxylase blocks the lymphoblastoid cells at G1/S (Hanauske-Abel et al. 1995). In both cases, the specificity of the inhibitors used and the depletion of eIF5A have not been fully established. Thus, the temperature-sensitive mutant tsL102A strain offers an ideal system to determine how the depletion of eIF5A may affect the cell cycle progression. Figure 10 shows that at restrictive temperature almost all tsL102A cells were arrested at G1 phase. In contrast, WT cells were distributed in both G1 and G2 phases. Sorbitol reversed the G1 arrest of tsL102A cells and pushed more than 60% of cells into G2 phase. It can be noted that although tsL102A cells were represented both in the G1 and G2 phase at permissive temperature, a partial G1 accumulation was observed as compared to the WT cells.

Discussion

One useful approach in probing the physiological function of eIF5A is to develop temperature-sensitive yeast mutants. In an effort to establish the structure–function relationship of eIF5A in yeast, we have employed the plasmid-shuffling technique to construct a set of yeast mutants harboring different plasmid-borne *tif51a* alleles in a genetic background where either or both of *TIF51A* and *TIF51B* genes have been disrupted. Among the mutants we have generated, the tsL102A strain exhibited a strong and reversible temperature-sensitive growth phenotype (Fig. 2) and was used for further characterization in this study. The temperature sensitivity of tsL102A was not related to a structure–function change, but rather, it was due to the loss of mutant eIF5A at restrictive temperature, resulted from the dramatic change of its half-life from 6 h to 15 min after temperature shift (Fig. 3). Since PMSF inhibited the loss of the mutant eIF5A and also suppressed the temperature-sensitive growth defect (Fig. 7c, d), we concluded that at restrictive temperature, the mutant eIF5A was functional, albeit it became more susceptible to protease digestion. Although the detailed mechanism for this enhanced sensitivity of mutant eIF5A to protease digestion is not clear, our finding suggested that the depletion of eIF5A via protease digestion is the cause, rather than the effect, of the temperature sensitivity that we observed with tsL102A.

The lack of size change and the irregular shape of tsL102A cells in response to temperature shift point to

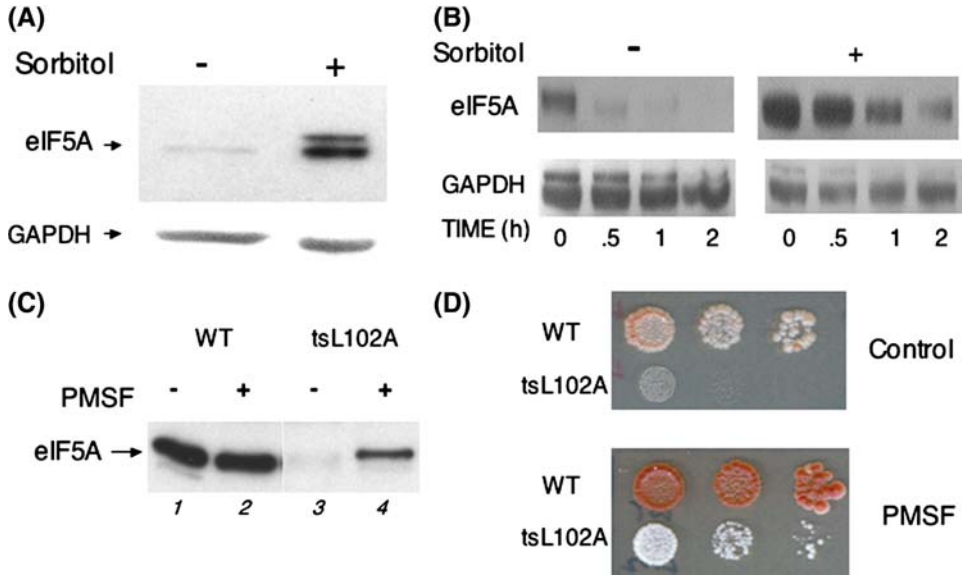

Fig. 7 **a** Effect of sorbitol on eIF5A protein level. tsL102A was grown in YPED with and without 1 M sorbitol at 25°C to log phase and then shifted to 37°C in fresh media. At time points indicated an aliquot was taken out Western Blot analysis. Cell extracts containing 10 μg protein were electrophoresed in 15% SDS/PAGE, blotted onto a nylon membrane and probed with anti-eIF5A antibody. The same blot was washed and probed again with yeast α-GAPDH antibody as a loading control. **b** The effect of sorbitol on the half-life of mutant eIF5A at restrictive temperature. The yeast strain tsL102A was grown in YPED to mid-logarithmic phase at 25°C. Cells were treated with cycloheximide (100 μg/ml) and split into two separate cultures with and without 1 M sorbitol at 37°C. Aliquots were then taken out at indicated times. Total protein was isolated and 10 μg of protein was electrophoresed in 15% SDS/PAGE for Western Blot analysis using anti-eIF5A antibody. Western Blot using anti-α-GAPDH antibody was used as a loading control. **c** Suppression of temperature-sensitive growth phenotype of tsL102A by PMSF. Tenfold serial dilution of log phase WT and tsL102A were spotted on YPED with 0.2 mM PMSF and incubated at 37°C for 3 days. **d** The effect of PMSF on eIF5A in WT and tsL102A at restrictive temperature. WT and tsL102A were grown at 37°C with 0.2 mM PMSF (*lanes 2 and 4*) for 5 h. Protein from each sample was extracted for electrophoresis and Western Blot analysis performed as described in Experimental Procedures

weakened cell wall integrity (Fig. 4a, b). This notion is supported by the finding that tsL102A is sensitive to ethanol at restrictive temperature (Fig. 4c), a hallmark of weakened cell wall integrity. Cell wall integrity of the yeast *S. cerevisiae* is regulated and maintained via a complicated *WSC/PKC1* signaling pathway (Verna et al. 1997; Heinisch et al. 1999). Defects in *WSC/PKC1* pathway lead to weakened cell wall integrity, which can be stabilized or suppressed by osmotic stabilizer such as sorbitol (Levin and Bartlett-Heubusch 1992). Sorbitol suppressed the temperature-sensitive growth defect of tsL102A (Fig. 5) and restored the size change of tsL102A in response to temperature shift (Fig. 6). These observations would suggest that the *WSC/PKC1*-signaling pathway and eIF5A are functionally linked. However, the findings that sorbitol stabilizes the mutant eIF5A and restores its level in tsL102A (Fig. 7a, b) suggest that mutant eIF5A is fully functional at

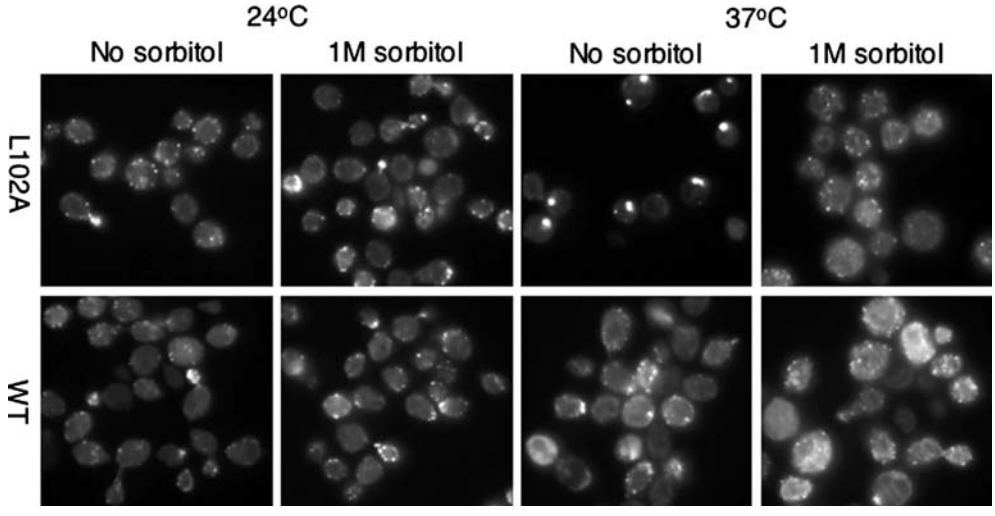

Fig. 8 Actin cytoskeleton of WT and tsL102A yeast cells grown in YPED media with or without sorbitol. Yeast strains were grown in YPED media with or without 1 M sorbitol for 16 h in log phase by continual dilution at 24°C before shifting to 37°C for 2 days before fixation and staining with Rhodamine phalloidin prior to mounting. The images were collected and analyzed as described in Experimental Procedures

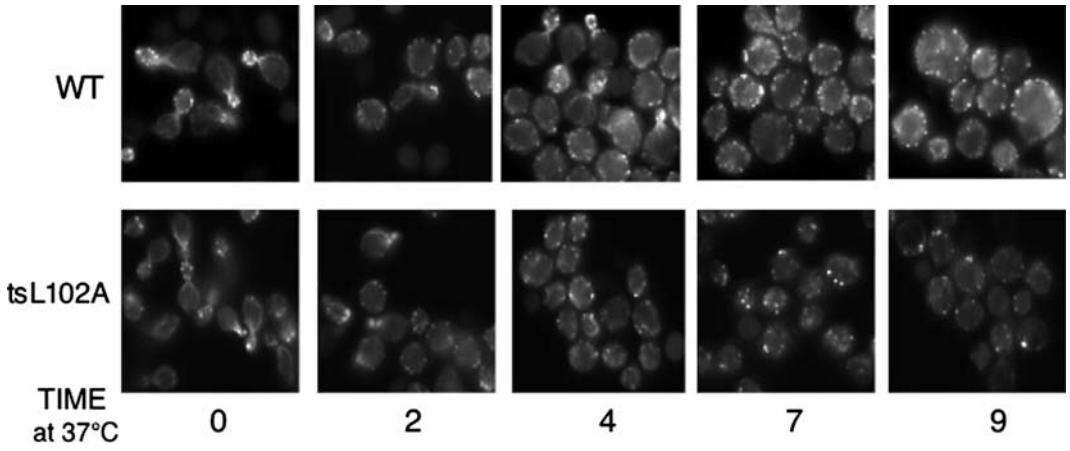

Fig. 9 Time course of changes in actin organization. WT and tsL102A yeast cells were grown in YPED media in log phase at 24°C before shifting to 37°C for the time indicated. Yeast cells were fixed and stained. The images were collected and analyzed as described in Experimental Procedures

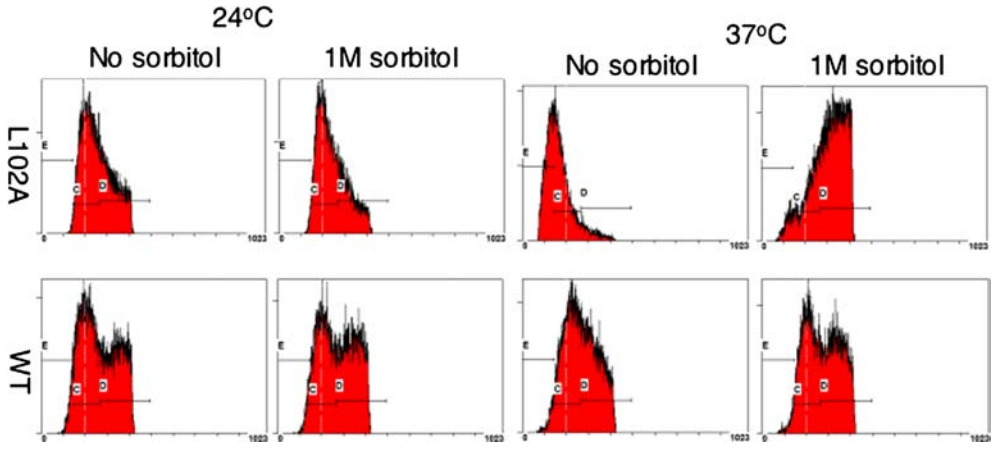

Fig. 10 Cell cycle distribution of WT and tsL102A yeast cells. DNA samples collected from WT or tsL120A yeast strains grown for 16 h in liquid YPED with or without 1 M sorbitol at 24°C before shifting to 37°C for 2 days were analyzed by flow cytometry after propidium iodide staining using a Coulter Cytomics FC500 Flow Cytometer

restrictive temperature and thus argue against the notion that *WSC*/*PKC1*-signaling pathway can complement the function of eIF5A by replacing the mutant eIF5A. Thus, we conclude that instead of functional complementation, sorbitol suppresses temperature sensitivity of tsL102A by restoring the protein level of mutant eIF5A. However, previous study with other temperature-sensitive eIF5A mutations has suggested that a functional complementation of eIF5A depletion by *PKC*/*WSC* pathway molecules (Valentini et al. 2002). This discrepancy could be due to a difference in the genetic background of the strains. Our mutant strain tsL102A was constructed in a background where both chromosomal eIF5A alleles were disrupted. In contrast, the ts mutants used in previous study still retain one functional and intact eIF5A allele (i.e., *TIF51B*). Further study is needed to resolve this discrepancy.

Actin in eukaryotes is organized into different pools and these pools are highly dynamic in response to cell physiology and external environment. In budding yeast the filamentous actin exists in two morphologically distinct pools, termed patches and cables, that are formed from branched and non-branched filaments, respectively (Pollard 2002). Actin patches in budding yeast contain more than 30 different proteins, including Arp2/3 complex that regulates the polymerization of branched actin filaments (Pruyne and Bretscher 2000). The depletion of eIF5A in tsL102A at the restrictive temperature leads to a striking change of actin dynamics, where actin cables completely disappear and actin patches cluster into a single aggregate (Fig. 8, 9). Although the causal relationship between eIF5A depletion and changes in actin organization remains to be established, it can be noted that these changes occur shortly after the depletion of intracellular eIF5A (Fig. 9). The lack of actin cables and cortical actin patches could be the direct cause for the weakened cell wall in tsL102A after temperature shift. In addition, in light of the critical role of actin cytoskeleton in polarity growth and cytokinesis, it is not surprising that the eIF5A depletion also leads to cell cycle-specific arrest phenotype (Fig. 10). It has been reported that Pkc1p is an upstream regulator of the SBF transcription factor that regulates the G1-to-S phase progression in yeast (Madden et al. 1997). It is therefore of interest to know whether eIF5A is involved in this pathway. *PKC*/*WSC*-

signaling pathway has also been implicated in ribosome biogenesis (Li et al. '). Many studies have shown that defects in ribosome biogenesis lead to cell cycle arrest, mostly stalled at G1 phase. It can be noted that eIF5A has been indirectly linked to large ribosomal subunit assembly and export (Stage-Zimmermann et al. 2000). Thus, the depletion of eIF5A may either interfere with the *PKC*/*WSC* pathway or affect ribosome formation and thus lead to G1 arrest. We have recently shown by tadem affinity purification that eIF5A interacts physically with the translation active 80S ribosome in an RNA- and hypusine-dependent manner (Jao and Chen 2005). We have also shown that eIF5A binds to synthetic RNA in a sequence-dependent manner (Xu and Chen 2001) and that eIF5A binds specifically to a certain mRNAs in vitro (Xu et al. 2004). It is therefore possible that eIF5A, through its binding with the 80S ribosome via specific mRNA species, may control the G1/S transition by regulating the translation of mRNAs that are critical for the transition.

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