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Identification of Psk2, Skp1, and Tub4 as trans-acting factors for uORF-containing *ROK1* mRNA in *Saccharomyces cerevisiae*

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Rok1, a DEAD-box RNA helicase, is involved in rRNA processing and the control of cell cycle progression in *Saccharomyces cerevisiae*. Rok1 protein expression is cell cycle-regulated, declining at G1/S and increasing at G2. The down-regulation of Rok1 expression in G1/S phase is mediated by the inhibitory action of two upstream open reading frames (uORFs) in the *ROK1* 5′-untranslated region (5′UTR). We identified Psk2 (PAS kinase), Skp1 (kinetochore protein) and Tub4 (γ-tubulin protein) as *ROK1* 5′UTR-interacting proteins using yeast three-hybrid system. A deletion analysis of *PSK2* or inactivation of temperature-sensitive alleles of *SKP1* and *TUB4* revealed that Rok1 protein synthesis is repressed by Psk2 and Skp1. This repression appeared to be mediated through the *ROK1* uORF1. In contrast, Tub4 plays a positive role in regulating Rok1 protein synthesis and likely after the uORF1-mediated inhibitory regulation. These results suggest that 5′UTR-interacting proteins, identified using three hybrid screening, are important for uORF-mediated regulation of Rok1 protein expression.

Keywords: RNA helicase Rok1, uORF, yeast three-hybrid, PAS kinase Psk2, cell-cycle progression, *Saccharomyces cerevisiae*

Introduction

The translation efficiency of eukaryotic mRNAs is often modulated by structural and compositional features of their 5′-untranslated region (5′UTR). The length, secondary structure, and specific sequence elements of the 5′UTR have been shown to affect downstream translation initiation at the main open reading frame (ORF) (Wilkie *et al.*, 2003; Churbanov *et al.*, 2005; Calvo *et al.*, 2009; Wethmar *et al.*, 2014). In particular, the presence of an upstream ORF (uORF), located 5′ of the true translation start site in mRNA, is considered to be a strong negative regulatory signal. Eukaryotic ribosomes usually load onto the 5′-cap of mRNA transcripts and scan for the first AUG start codon (Kozak, 1989; Vilela and McCarthy, 2003). Accumulating evidence indicates that a ribosome encounter with a uORF can either trigger mRNA decay or decrease translation from the main ORF (Gaba *et al.*, 2005; Hinnebusch, 2014).

Translation of the yeast *GCN4* gene is the best-studied example of uORF-mediated regulation (Hinnebusch, 2005, 2014). Phosphorylation of the translation initiation factor eIF2α during amino acid starvation allows the ribosome to bypass the uORFs and translate the *GCN4* ORF. In mammals, translation of *ATF4*, *ATF5*, or *CHOP* mRNAs is also regulated by phosphorylation of eIF2α during stress conditions (Dey *et al.*, 2010; Palam *et al.*, 2011). Accumulating evidence indicates that a dynamic interplay among translation initiation factors and other regulatory factors regulates selection of the proper start codon and efficient translation.

Rok1, a DEAD-box RNA helicase, plays a role in rRNA processing and the control of cell cycle progression in *Saccharomyces cerevisiae* (Venema *et al.*, 1997; Oh and Kim, 1999). The *ROK1* 5′UTR contains two uORFs that exert inhibitory influence on *ROK1* translation (Jeon and Kim, 2010). Rok1 protein expression is cell cycle-regulated, declining at G1/S and increasing at G2. The down-regulation of Rok1 protein expression in G1/S phase is mediated by the inhibitory action of uORFs. Here, to understand the mechanism of uORF-mediated regulation of Rok1 protein expression, we sought to identify trans-acting factors that might interact with the 5′UTR of *ROK1* mRNA using three-hybrid analysis, an assay based on the RNA-protein interactions. Three candidate factors, Psk2, Skp1, and Tub4, were identified and characterized with respect to uORF-mediated regulation of Rok1 protein expression.

Materials and Methods

Yeast strains and plasmids

The *S. cerevisiae* strains and plasmids used in this study are listed in Table 1. The *psk2* deletion mutation was introduced in JK406 or JK407 using a polymerase chain reaction (PCR)-based gene disruption method (Lorenz *et al.*, 1995), generating JK410 and JK411, respectively. *ROK1-myc* or *uAAG1-ROK1* was integrated into the yeast genome as previously described (Toulmay and Schneiter, 2006; Jeon and Kim, 2010). Strains JK450, JK453, and JK472 were derived from JK429, JK452, and JK471, respectively, by transformation of a 3-kb *Cla*I fragment from pJI376 that contained the *URA3* gene inserted at the 5′UTR of myc-tagged *ROK1*. *URA3* was removed by culturing in 5-fluoroorotic acid (5-FOA) media. Strains JK473, JK474, and JK476 were derived from JK472,

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Table 1. Strains and plasmids used in this study

Strains	Genotype	References
JK406	*MATa ura3-52 leu2-3,112 trp1-1 ade2 cyh^r ROK1::myc*	This laboratory
JK407	*MATa ura3-52 leu2-3,112 trp1-1 ade2 cyh^r uAAG1-ROK1::myc*	This laboratory
JK410	*MATa ura3-52 leu2-3,112 trp1-1 ade2 cyh^r psk2::LEU2 ROK1::myc*	This study
JK411	*MATa ura3-52 leu2-3,112 trp1-1 ade2 cyh^r psk2::LEU2 uAAG1-ROK1::myc*	This study
JK419	*MATa ura3-52 leu2-3,112 trpΔ63 his4-303(ATT) Δtif11* pRS314-*TIF11 <TRP1 CEN>*	This laboratory
JK420	*MATa ura3-52 leu2-3,112 trpΔ63 his4-303(ATT) Δtif11* pRS314-*tif11::S125A <TRP1 CEN>*	This laboratory
JK421	*MATa ura3-52 leu2-3,112 trpΔ63 his4-303(ATT) Δtif11* pRS314-*tif11::S125D <TRP1 CEN>*	This laboratory
JK429 (YV413)	*MATα tub4-Y445D::TRP1 ura3 trp1 his3*	Kitagawa *et al.* (2003)
JK450	*MATα tub4-Y445D::TRP1 ura3 trp1 his3 ROK1::myc*	This study
JK452 (YPH1161)	*MATa ura3-52 lys2-801 lys2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 skp1Δ::TRP1 skp1-L146S::LEU2 CFIII(CEN3.L.YPH983) HIS3 SUP11*	Sobel and Snyder (1995)
JK453	*MATa ura3-52 lys2-801 lys2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 skp1Δ::TRP1 skp1-L146S::LEU2 CFIII(CEN3.L.YPH983) HIS3 SUP11 ROK1::myc*	This study
JK470 (YV427)	*MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ1/trp1Δ1 his3Δ200/his3Δ200*	Kitagawa *et al.* (2003)
JK471	*MATα ura3-52 lys2-801 ade2-10 trp1Δ1 his3Δ200*	This study
JK472	*MATα ura3-52 lys2-801 ade2-10 trp1Δ1 his3Δ200 ROK1::myc*	This study
JK473	*MATα ura3-52 lys2-801 ade2-10 trp1Δ1 his3Δ200 uAAG1-ROK1::myc*	This study
JK474	*MATα tub4-Y445D::TRP1 ura3 trp1 his3 uAAG1-ROK1::myc*	This study
JK476	*MATa ura3-52 lys2-801 lys2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 skp1Δ::TRP1 skp1-L146S::LEU2 CFIII(CEN3.L.YPH983) HIS3 SUP11 uAAG1-ROK1::myc*	This study
L40-*ura3*	*MATa ura3-52 leu2-3,112 his3*Δ200 trp1Δ1 ade2 LYS::(LexA-op)4-HIS3, *ura3::(LexA-op)8-lacZ*	Invitrogen
Plasmids	**Genotype**	**References**
pJI376	*URA3 flanked by ROK1 5′UTR ROK1::myc URA3 CEN*	This laboratory
pJI382	*LexA-MS2 coat LEU2 2μ*	This study
pJI383	*MS2-ROK1 URA3 2μ*	This study
pRH3′	*MS2 RNA (at 3') URA3 2μ*	Invitrogen
pRH5′	*MS2 RNA (at 5') URA3 2μ*	Invitrogen
pRH3′/IRE	*IRE-MS2 URA3 2μ*	Invitrogen
pYESTrp3	*VP16 AD TRP1 2μ*	Invitrogen
pYESTrp3/IRP	*VP16 AD-IRE TRP1 2μ*	Invitrogen
pHybLex/Zeo-MS2	*LexA-MS2 coat Zeo^r 2μ*	Invitrogen
pRS425	*2μ ARS LEU2*	Christianson *et al.* (1992)

JK450, and JK453, respectively, using a 1.7-kb *URA3-uAAG1-ROK1-myc* cassette, which was PCR amplified from plasmid pJI376 using uAAG1-mutagenic primers R443 and R840 (Table 2). JK471 is a wild-type strain derived from the sporulation of JK470.

The plasmid pJI382 was constructed by cloning a 3.1-kb *Ava*II fragment containing a LexA-MS2 coat hybrid from pHybLex/Zeo-MS2 (Invitrogen) into the *Sma*I and *Nae*I sites of the *2μ*-based plasmid pRS425 (Christianson *et al.*, 1992). *ROK1-MS2* RNA hybrids, in plasmids pJI383 and pJI384, were constructed by cloning a 157-bp PCR-amplified fragment of the *ROK1* 5′UTR region into the *Sma*I site of pRH5′ and pRH3′ (Invitrogen), respectively. Primers R619 and R620 were used to amplify the *ROK1* 5′UTR region. The correct orientation of *ROK1-MS2* hybrids was confirmed by DNA sequencing analysis.

Table 2. Primers used in this study

Primer name	Sequence (5′→3′)
R443	TTTCCTTCCCATCTCTTCGCAAAAAAT
R840	CAGCTATCACAGCAGATCTAGG
R1052	CTGCGACCATTCCATCCAAT
R1053	ATTTGCTGCTTCCTTGTGGC
R1054	CTGCCGGTATTGACCAAACT
R1055	CGGACATAACGATGTTACCG

Yeast three-hybrid assay

To identify trans-acting factors that interact with the *ROK1* 5′UTR, we transformed a yeast genomic DNA library in the activating domain vector pJG4-5 (Park *et al.*, 2002) into the yeast L40-*ura3* strain carrying the plasmids pJI382 and pJI383. Colonies positive for *HIS3* expression were subsequently assayed for β-galactosidase activity using a filter assay. Of the initial ~1,200 yeast transformants selected on SC (synthetic

complete) plates lacking leucine, uracil, tryptophan, and histidine, 28 colonies expressed β-galactosidase activity and were further analyzed by sequencing. Strains carrying pJI382, pRH5′ and pYESTrp3 or pJI382, pRH3′/IRE, and pYESTrp3/IRP were used for negative and positive controls, respectively.

The β-galactosidase filter assay was carried out as follows. Briefly, 10 μl of exponentially growing cells were spotted onto a nylon membrane (Amersham Biosciences) placed on an SC plates lacking leucine, uracil, and tryptophan. Following incubation at 30°C for 1 day, filters were removed and air dried for 5 min. The filters were incubated at -70°C for 1 h and then thawed on Whatman 3 MM paper at room temperature for 1 min. The filters were then placed on 3 MM paper that had been soaked with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgSO_4$), without β-mercaptoethanol, containing 1 mg/ml Xgal (Duchefa Biochemie) and incubated at 30°C until blue color developed.

Cell cycle analysis

Strains were grown in SC medium to early-exponential phase and then treated with 5 μM α-factor for 2 h. Cell synchronization was monitored by observation of cell morphology under a light microscope. Cells were washed twice with pre-warmed media and then resuspended in pre-warmed media to allow cell cycle progression. Samples were collected periodically for analysis of Rok1 protein and transcript expression.

Western blot analysis

Protein preparation and Western blot analysis were carried out as previously described (Park *et al.*, 2006). Rok1-myc was detected with anti-c-myc antibody (Roche). The blot was also probed with α-tubulin antibody (Santa Cruz Biotech), used as a loading control. Horseradish peroxidase-conjugated anti-mouse antibodies (Amersham Biosciences) were utilized as secondary antibodies.

Total RNA extraction and qRT-PCR

Total RNA was prepared as described previously (Park *et al.*, 2002). cDNA was synthesized from total RNA by reverse transcription (RT) using an M-MLV cDNA synthesis kit (Enzynomics). *ROK1* mRNA levels were measured by quantitative real-time PCR (qRT-PCR) using SYBR green I (GenetBio). All values were normalized to the level of *ACT1* (actin) mRNA. *ROK1*-specific primers (R1052 and R1053) and *ACT1*-specific primers (R1054 and R1055) were used to amplify 100-bp fragments of each coding sequence.

Results

Identification of ROK1 5′UTR-interacting proteins using three-hybrid screening

To identify trans-acting factors that interact with the 5′UTR of *ROK1* mRNA, we conducted three-hybrid screening assays against an yeast library (SenGupta *et al.*, 1996; Bernstein *et al.*, 2002). The bait RNA was the 148-nucleotide *ROK1* 5′UTR, which was fused downstream of the MS2 sequence as an RNA hybrid. The hybrid protein, LexA-MS2 coat, consists of a LexA DNA-binding domain and an MS2 RNA-binding domain. The yeast strain, carrying a LexA-MS2 coat hybrid and an MS2-ROK1 RNA hybrid was transformed with library DNA for the activation-domain fusion. Upon formation of a stable three-hybrid complex, transcription of the

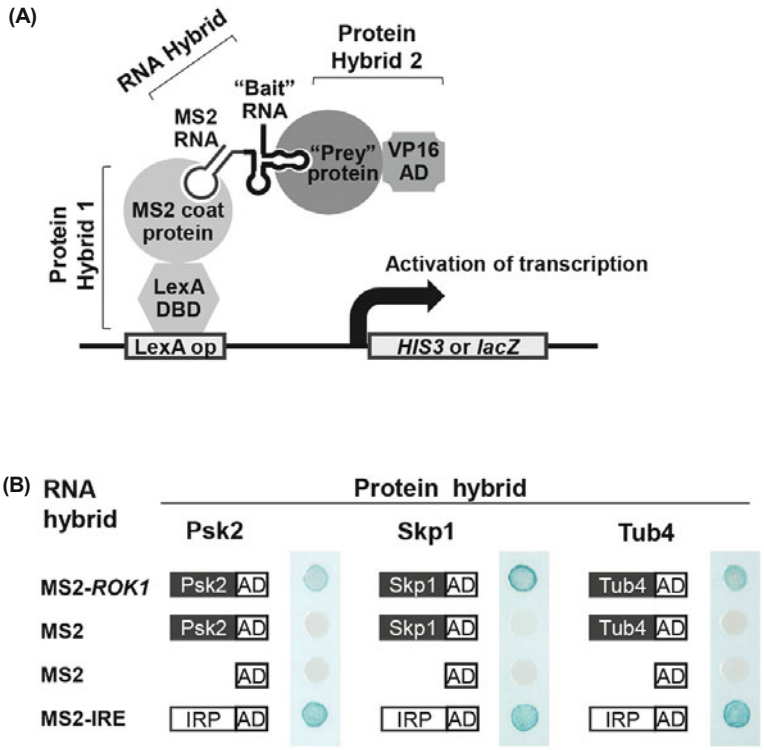

Fig. 1. Identification of *ROK1* 5′UTR-interacting proteins by yeast three-hybrid assay. (A) Schematic representation of the yeast three-hybrid assay to detect RNA-protein interaction. A hybrid protein containing a DNA-binding domain (LexA) with RNA-binding domain (MS2 coat protein) localizes to the promoter of a reporter gene. A second hybrid protein containing a transcriptional activation domain (VP16-AD) with RNA-binding protein will activate transcription of the reporter gene. A hybrid RNA containing sites recognized by the two RNA-binding proteins links the two hybrid proteins to one another. The tripartite complex results in detectable expression of the reporter gene (*lacZ* or *HIS3*). (B) Interactions between the *ROK1* 5′ UTR and Psk2, Skp1 or Tub4 were monitored by β-galactosidase activity. Yeast strain (L40-*ura3*) transformed with plasmids encoding the indicated hybrid RNA and activation domain (AD) fusion protein were assayed by colony color assay. Strains carrying MS2 (pRH5′) and AD (pYESTrp3) were used as a negative control and strains containing MS2-IRE (pRH/IRE) and IRP-AD (pYESTrp3-IRP) were used as a positive control.

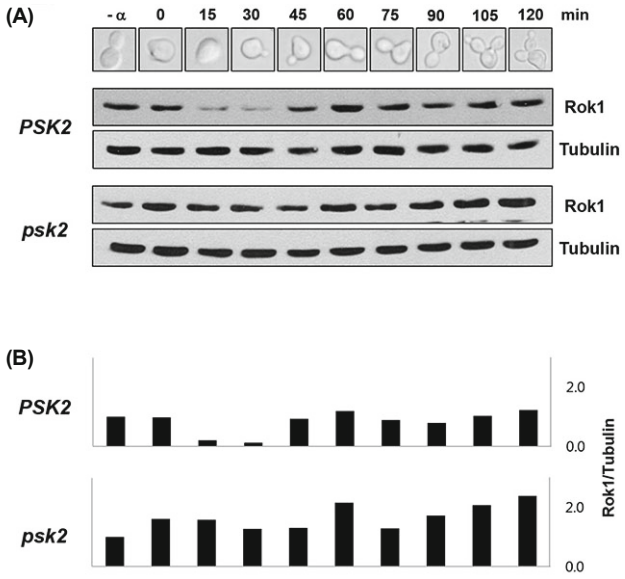

Fig. 2. Cell cycle-dependent expression of Rok1 protein in wild-type and *psk2* deletion mutant strains. The wild-type (JK406) and *psk2* deletion mutant (JK410) strains were arrested in G1 by treatment with α-factor and then released from G1 arrest by resuspending in fresh media. Samples were collected periodically, and their cell morphology (microscopy) and Rok1 protein levels (Western blotting) were analyzed (A). Tubulin was used as a loading control. Rok1 protein levels were normalized to tubulin protein levels (B).

HIS3 and *lacZ* genes is activated. Of the initial 28 clones positive for both *HIS3* and *lacZ* expression, ten were confirmed by sequence analysis.

The binding specificity of interacting proteins for the *ROK1* 5′UTR was further analyzed using a re-transformation procedure. Psk2, Tub4, and Skp1 were identified as candidates showing specific binding to the *ROK1* 5′UTR (Fig. 1). Psk2 is a PAS kinase, a serine/threonine kinase containing a PAS (Per-Arnt-Sim) domain (Rutter *et al.*, 2002). Tub4, γ-tubulin protein, is a microtubule organizing center (MTOC) component and is located at the spindle pole body (SPB). Skp1 is known to be a component of the Skp1-Cullin-F-box (SCF) ubiquitin ligase complex as well as a kinetochore protein (Sobel and Snyder, 1995; Connelly and Hieter, 1996).

Cell-cycle dependent regulation of *ROK1* expression was abolished in a *psk2* deletion mutation

Psk2, a member of a PAS kinase family, has been shown to phosphorylate three translation factors, Caf20, eIF1A, and Sro9, as well as Ugp1 (UDP-glucose pyrophosphorylase), which is involved in glycogen synthesis (Rutter *et al.*, 2002; Grose *et al.*, 2009). To determine whether Psk2 plays a critical role in Rok1 protein synthesis, we introduced a *psk2* deletion mutation in the *ROK1-myc* strain. Synchronized cultures of wild-type and *psk2* deletion strains were analyzed for Rok1 protein level during cell cycle progression. As reported previously (Jeon and Kim, 2010), Rok1 protein levels oscillated during the cell cycle in wild-type cultures, declining at G1/S phase and then increasing during S/G2 phase (Fig. 2). We found that *psk2* deletion mutation relieved the repression of Rok1 synthesis at G1/S phase, resulting in a constant level of Rok1 protein throughout the cell cycle. These results demonstrate that Psk2 plays an inhibitory role in Rok1 expression during the G1/S phase.

The effects of *psk2* deletion on Rok1 protein synthesis are very similar to those of a substitution mutation at uATG1 (uATG1 → uAAG1) of *ROK1*, which results in a significant increase in Rok1 protein level during logarithmic culture and relieves the down-regulation at G1/S phase during synchronized culture (Jeon and Kim, 2010). To investigate whether the inhibitory role of Psk2 is dependent on uORF1-mediated repression of Rok1 protein synthesis, we introduced a *psk2* deletion mutation into the *uAAG1-ROK1* strain. The *uAAG1-ROK1* and *psk2* deletion mutations led to 1.8- and 1.51-fold increases, respectively (Fig. 3A). The *psk2* deletion mutation

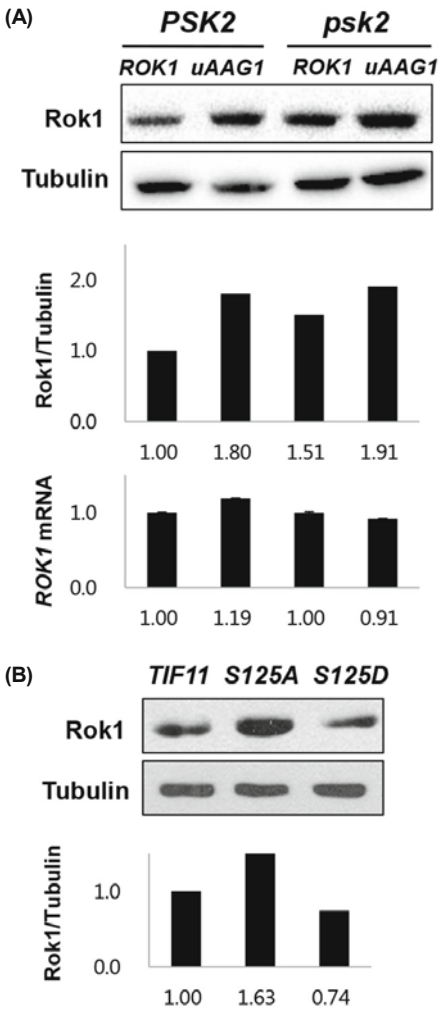

Fig. 3. Analysis of the effects of *psk2* deletion and *uAAG1-ROK1* mutations on Rok1 protein expression. (A) Western blotting and qRT-PCR analysis of *psk2* deletion and *uAAG1-ROK1* strains. Rok1 protein levels were detected using monoclonal anti-c-myc antibodies. Rok1 protein levels were normalized to tubulin protein levels. Relative mRNA levels were determined as the ratio *ROK1*/*ACT1*. The error bars indicate the standard errors of duplicate real-time PCR determination of three independent cDNA reactions. (B) Western blotting analysis of *TIF11* (JK419), *tif11-S125A* (JK420), and *tif11-S125D* (JK421) strains.

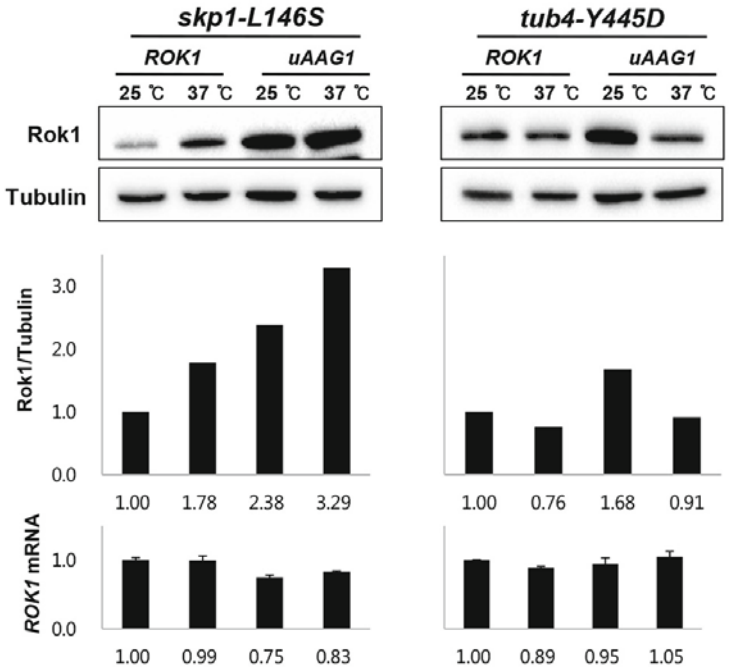

Fig. 4. Analysis of the effects of *skp1-L146S*, *tub-Y445D*, and *uAAG1-ROK1* mutations on Rok1 protein expression. Western blotting and qRT-PCR analysis were carried out. Rok1 protein levels were detected using monoclonal anti-c-myc antibodies. Rok1 protein levels were normalized to tubulin protein levels. Relative mRNA levels were determined as the ratio *ROK1*/*ACT1*. The error bars indicate the standard errors of duplicate real-time PCR determination of three independent cDNA reactions.

in the *uAAG1-ROK1* strain showed a 1.9-fold increase, an effect similar to that of *uAAG1-ROK1* mutation. These results suggest that Rok1 protein synthesis is repressed by Psk2 and this repression may be mediated through the *ROK1* uORF1.

Phosphorylation of eIF1A is required for the repression of Rok1 protein synthesis

The translation initiation factor eIF1A, encoded by the essential, single-copy gene *TIF11* in *S. cerevisiae*, promotes preinitiation complex (PIC) assembly, scanning, and start codon selection (Fekete *et al.*, 2005). eIF1A is one of three translation factors that have been identified as Psk2 kinase substrates (Rutter *et al.*, 2002). The Psk2 phosphorylation site in eIF1A has been mapped to serine 125 in the C-terminal region.

To determine whether eIF1A phosphorylation by Psk2 is required for the regulation of Rok1 protein synthesis, we constructed eIF1A mutant plasmids carrying a phosphorylation-defective (*tif11-S125A*) or phosphorylation-mimetic (*tif11-S125D*) mutation at the Ser125 site of eIF1A, respectively. As shown in Fig. 3B, the *tif11-S125A* mutation caused an increase in Rok1 protein level as compared to the wild-type strain; in contrast, the *tif11-S125D* mutation caused a large decrease in the level of Rok1 protein. These results suggest that phosphorylation of eIF1A, possibly mediated by Psk2 kinase, is required for the repression of Rok1 protein synthesis.

Roles of Skp1 and Tub4 in uORF1-mediated regulation of Rok1 protein expression

To investigate the roles of Skp1 and Tub4 in uORF1-mediated regulation of Rok1 protein synthesis, we employed temperature-sensitive mutant alleles of *SKP1* and *TUB4*. Skp1, a kinetochore protein and component of the SCF ubiquitin ligase complex, is essential for cell cycle progression (Connelly and Hieter, 1996; Kitagawa *et al.*, 2003). The *skp1-L146S* mutant strain arrests predominantly as large budded cells with a G2 DNA content. The temperature-sensitive *tub4-Y445D* mutation maps to the C-terminal DSYL motif, a conserved domain required for proper microtubule organization (Vogel *et al.*, 2001). The *tub4-Y445D* mutant also arrests as large budded cells with short bipolar spindles. The *ROK1-myc* and *uAAG1-ROK1-myc* constructs were introduced into the *skp1-146S* and *tub4-Y445D* mutant strain using a chromosome integration procedure (Materials and Methods).

To inactivate Skp1 or Tub4 protein, *skp1-L146S* or *tub4-Y445D* mutant strains cultured at the permissive temperature (25°C) were shifted to non-permissive temperature

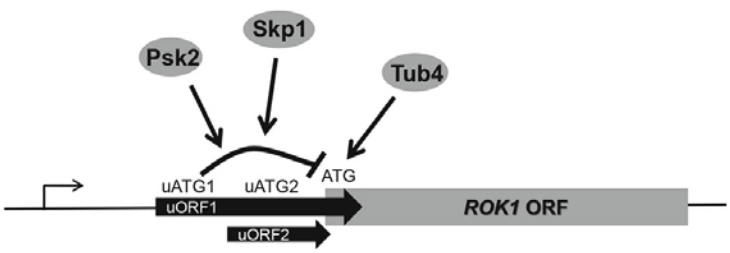

Fig. 5. Translational regulation of uORF-containing *ROK1* mRNA. The *ROK1* 5′UTR contains uORF1 and uORF2 which are inhibitory to *ROK1* translation. Psk2 and Skp1 repress *ROK1* mRNA translation via a uORF1-dependent inhibitory pathway. Tub4 plays a positive role in regulating *ROK1* mRNA translation.

(37°C) for 4 h. This temperature shift had no significant effect on Rok1 protein levels in the wild-type strain (data not shown). Rok1 protein expression in the *skp1-L146S* mutant strain was increased by ~1.8-fold at 37°C compared with the levels at 25°C (Fig. 4). The *uAAG1* mutation in *skp1-L146S* mutant strain further increased Rok1 levels at 37°C to ~3.3-fold. The effects of mutations *uAAG1* and *skp1-L146S* appeared to be additive. These results indicate that Rok1 protein synthesis is repressed by Skp1. This repression may be mediated through the *ROK1* uORF1 or possibly work in parallel.

The *tub4-Y445D* mutation resulted in a decrease in Rok1 protein expression after 4-h temperature shift (Fig. 4). These effects were opposite those observed with the *psk2* deletion mutation and *skp1* point mutation. Interestingly, the derepression of Rok1 protein synthesis caused by the *uAAG1* mutation was abolished in the *tub4-Y445D* mutant strain at 37°C. The 1.7-fold increase in Rok1 protein level was reduced to 0.9-fold after the temperature shift. *ROK1* transcript expressions appeared to be constitutive. These results suggest that Tub4 positively regulates Rok1 protein synthesis and functions after the uORF-mediated regulatory pathway.

Discussion

The yeast three-hybrid system, which assays RNA-protein interactions through simple phenotypes, enables the identification of proteins that bind known RNA sequences. In this study, Psk2 (PAS kinase), Tub4 (γ-tubulin protein), and Skp1 (kinetochore protein) were identified as *ROK*1 5′UTR-interacting proteins. A deletion analysis of *PSK2* and inactivation of temperature-sensitive alleles of *SKP1* and *TUB4* revealed that Rok1 protein synthesis is repressed by Psk2 and Skp1. This repression appeared to be mediated through the *ROK1* uORF1. In contrast, Tub4 plays a positive role in Rok1 protein synthesis and functions after the uORF1-mediated regulatory pathway.

The *S. cerevisiae* genome contains two PAS kinase homologs, Psk1 and Psk2, which share an overall homology to one another of 71% (Rutter *et al.*, 2002; Grose *et al.*, 2009). We observed that the *psk2* deletion caused an increase in Rok1 protein level (Figs. 2 and 3). This phenotype was also observed in the *psk1* deletion strain, albeit to a lesser degree (data not shown). The phenotype of the double-deletion *psk1Δpsk2Δ* was very similar to that of the *psk2* deletion (data not shown). From this we infer that Psk2 exerts the major inhibitory effect on Rok1 protein synthesis.

Psk2 has been shown to phosphorylate the translation factors, Caf20, eIF1A, and Sro9, as well as the glycogen synthesis-related protein, thereby coordinately regulating translation and sugar flux (Grose *et al.*, 2009). We observed that phosphorylation site mutants of *eIF1A*, *tif11-S125A* and *tif11-S125D* showed altered levels of Rok1 protein (Fig. 3B). A previous report has shown that Psk2 phosphorylates UDP-glucose pyrophosphorylase, causing a conformational change without affecting catalytic activity. These changes allow Ugp1 to be targeted to the cell periphery, where its product UDP-glucose is generated in proximity to the site of glucan synthesis (Smith and Rutter, 2007). In a manner similar to Ugp1 phosphorylation, the translation factor eIF1A, phosphorylated by Psk2, could regulate uORF-mediated *ROK1* translation. In mammals, the translational elongation factor eEF1A1 is phosphorylated by the PAS kinase, PASKIN, at threonine 432 in the C-terminal region (Eckhardt *et al.*, 2007). eEF1A1 is a GTP-binding protein that catalyzes the binding of charged aminoacyl-tRNA to the A-site of the ribosome. It is related to yeast eIF1A, which mediates the transfer of initiator Met-$tRNA_i^{Met}$ to the 40S subunit of the ribosome. Although the phosphorylation of eIF1A has been established, little is been known about the regulatory consequences of eIF1A phosphorylation.

Based on the results of mutational analyses of *PSK2*, *SKP1*, and *TUB4*, we hypothesize a possible mechanism for Rok1 mRNA translation (Fig. 5). According to this model, Rok1 protein synthesis is repressed by Psk2 and Skp1, an effect that is associated with the inhibitory role of uORF1. In contrast, Tub4 is required for Rok1 protein synthesis, but its regulatory role is uORF1-independent and likely after the uORF-mediated regulatory pathway. Understanding in detail how Skp1 and Tub4 participate in the translational mechanism will require further investigations, including analyses of direct and/or indirect interactions of trans-acting proteins with *ROK1* mRNA.

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References

Bernstein, D.S., Buter, N., Stumpf, C., and Wickens, M. 2002. Analyzing mRNA-protein complexes using a yeast three-hybrid system. *Methods* **26**, 123–141.

Calvo, S.E., Pagliarini, D.J., and Mootha, V.K. 2009. Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans. *Proc. Natl. Acad. Sci. USA* **106**, 7507–7512.

Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H., and Hieter, P. 1992. Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**, 119–122.

Churbanov, A., Rogozin, I.B., Babenko, V.N., Ali, H., and Koonin, E.V. 2005. Evolutionary conservation suggests a regulatory function of AUG triplets in 5′-UTRs of eukaryotic genes. *Nucleic Acids Res.* **33**, 5512–5520.

Connelly, C. and Hieter, P. 1996. Budding yeast *SKP1* encodes an evolutionarily conserved kinetochore protein required for cell cycle progression. *Cell* **86**, 275–285.

Dey, S., Baird, T.D., Zhou, D., Palam, L.R., Spandau, D.F., and Wek, R.C. 2010. Both transcriptional regulation and translational control of *ATF4* are central to the integrated stress response. *J. Biol. Chem.* **285**, 33165–33174.

Eckhardt, K., Troger, J., Reissmann, J., Katschinski, D.M., Wagner,

K.F., Stengel, P., Paasch, U., Hunziker, P., Borter, E., Barth, S., *et al.* 2007. Male germ cell expression of the PAS domain kinase PASKIN and its novel target eukaryotic translation elongation factor eEF1A1. *Cell Physiol. Biochem.* **20**, 227–240.

Fekete, C.A., Applefield, D.J., Blakely, S.A., Shirokikh, N., Pestova, T., Lorsch, J.R., and Hinnebusch, A.G. 2005. The eIF1A C-terminal domain promotes initiation complex assembly, scanning and AUG selection *in vivo*. *EMBO J.* **24**, 3588–3601.

Gaba, A., Jacobson, A., and Sachs, M.S. 2005. Ribosome occupancy of the yeast *CPA1* upstream open reading frame termination codon modulates nonsense-mediated mRNA decay. *Mol. Cell* **20**, 449–460.

Grose, J.H., Sundwall, E., and Rutter, J. 2009. Regulation and function of yeast PAS kinase: a role in the maintenance of cellular integrity. *Cell Cycle* **8**, 1824–1832.

Hinnebusch, A.G. 2005. Translational regulation of *GCN4* and the general amino acid control of yeast. *Annu. Rev. Microbiol.* **59**, 407–450.

Hinnebusch, A.G. 2014. The scanning mechanism of eukaryotic translation initiation. *Annu. Rev. Biochem.* **83**, 779–812.

Jeon, S. and Kim, J. 2010. Upstream open reading frames regulate the cell cycle-dependent expression of the RNA helicase Rok1 in *Saccharomyces cerevisiae*. *FEBS Lett.* **584**, 4593–4598.

Kitagawa, K., Abdulle, R., Bansal, P.K., Cagney, G., Fields, S., and Hieter, P. 2003. Requirement of Skp1-Bub1 interaction for kinetochore-mediated activation of the spindle checkpoint. *Mol. Cell* **11**, 1201–1213.

Kozak, M. 1989. The scanning model for translation: an update. *J. Cell Biol.* **108**, 229–241.

Lorenz, M.C., Muir, R.S., Lim, E., McElver, J., Weber, S.C., and Heitman, J. 1995. Gene disruption with PCR products in *Saccharomyces cerevisiae*. *Gene* **158**, 113–117.

Oh, J.Y. and Kim, J. 1999. ATP hydrolysis activity of the DEAD box protein Rok1p is required for *in vivo ROK1* function. *Nucleic Acids Res.* **27**, 2753–2759.

Palam, L.R., Baird, T.D., and Wek, R.C. 2011. Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance *CHOP* translation. *J. Biol. Chem.* **286**, 10939–10949.

Park, Y.U., Hwang, O., Hur, H., Ka, M., and Kim, J. 2002. Two-hybrid cloning and characterization of *OSH3,* a yeast oxysterol-binding protein homolog. *Biochem. Biophys. Res. Comm.* **293**, 733–740.

Park, Y.U., Hur, H., Ka, M., and Kim, J. 2006. Identification of translational regulation target genes during filamentous growth in *Saccharomyces cerevisiae*: regulatory role of Caf20 and Dhh1. *Eukaryot. Cell* **5**, 2120–2127.

Rutter, J., Probst, B.L., and McKnight, S.L. 2002. Coordinate regulation of sugar flux and translation by PAS kinase. *Cell* **111**, 17–28.

SenGupta, D.J., Zhang, B., Kraemer, B., Pochart, P., Fields, S., and Wickens, M. 1996. A three-hybrid system to detect RNA-protein interactions *in vivo*. *Proc. Natl. Acad. Sci. USA* **93**, 8496–8501.

Smith, T.L. and Rutter, J. 2007. Regulation of glucose partitioning by PAS kinase and Ugp1 phosphorylation. *Mol. Cell* **26**, 491–499.

Sobel, S.G. and Snyder, M. 1995. A highly divergent gamma-tubulin gene is essential for cell growth and proper microtubule organization in *Saccharomyces cerevisiae*. *J. Cell Biol.* **131**, 1775–1788.

Toulmay, A. and Schneiter, R. 2006. A two-step method for the introduction of single or multiple defined point mutations into the genome of *Saccharomyces cerevisiae*. *Yeast* **23**, 825–831.

Venema, J., Bousquet-Antonelli, C., Gelugne, J.P., Caizergues-Ferrer, M., and Tollervey, D. 1997. Rok1p is a putative RNA helicase required for rRNA processing. *Mol. Cell. Biol.* **17**, 3398–3407.

Vilela, C. and McCarthy, J.E. 2003. Regulation of fungal gene expression via short open reading frames in the mRNA 5′ untranslated region. *Mol. Microbiol.* **49**, 859–867.

Vogel, J., Drapkin, B., Oomen, J., Beach, D., Bloom, K., and Snyder, M. 2001. Phosphorylation of gamma-tubulin regulates microtubule organization in budding yeast. *Dev. Cell* **1**, 621–631.

Wethmar, K., Barbosa-Silva, A., Andrade-Navarro, M.A., and Leutz, A. 2014. uORFdb–a comprehensive literature database on eukaryotic uORF biology. *Nucleic Acids Res.* **42**, D60–67.

Wilkie, G.S., Dickson, K.S., and Gray, N.K. 2003. Regulation of mRNA translation by 5′- and 3′-UTR-binding factors. *Trends Biochem. Sci.* **28**, 182–188.