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Protein Kinase A Is Part of a Mechanism That Regulates Nuclear Reimport of the Nuclear tRNA Export Receptors Los1p and Msn5p

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The two main signal transduction mechanisms that allow eukaryotes to sense and respond to changes in glucose availability in the environment are the cyclic AMP (cAMP)/protein kinase A (PKA) and AMP-activated protein kinase (AMPK)/Snf1 kinase-dependent pathways. Previous studies have shown that the nuclear tRNA export process is inhibited in *Saccharomyces cerevisiae* deprived of glucose. However, the signal transduction pathway involved and the mechanism by which glucose availability regulates nuclear-cytoplasmic tRNA trafficking are not understood. Here, we show that inhibition of nuclear tRNA export is caused by a block in nuclear reimport of the tRNA export receptors during glucose deprivation. Cytoplasmic accumulation of the tRNA export receptors during glucose deprivation is not caused by activation of Snf1p. Evidence obtained suggests that PKA is part of the mechanism that regulates nuclear reimport of the tRNA export receptors in response to glucose availability. This mechanism does not appear to involve phosphorylation of the nuclear tRNA export receptors by PKA. The block in nuclear reimport of the tRNA export receptors appears to be caused by activation of an unidentified mechanism when PKA is turned off during glucose deprivation. Taken together, the data suggest that PKA facilitates return of the tRNA export receptors to the nucleus by inhibiting an unidentified activity that facilitates cytoplasmic accumulation of the tRNA export receptors when glucose in the environment is limiting. A PKA-independent mechanism was also found to regulate nuclear tRNA export in response to glucose availability. This mechanism, however, does not regulate nuclear reimport of the tRNA export receptors.

Eukaryotes use several signaling pathways to sense and respond to changes in environmental or cellular nutrient levels. During nutrient deprivation, the signaling pathways enable cells to reduce cellular processes, including those involved in translation and transcription, and to arrest the cell cycle in G_1 to accommodate a decrease in energy availability (1, 2). Cellular response to glucose availability involves the conserved cyclic AMP (cAMP)/protein kinase A (PKA) and Snf1p/AMP-activated protein kinase (AMPK) pathways (3, 4). In *Saccharomyces cerevisiae*, the presence of glucose results in activation of the Ras1p/Ras2p and Gpr1p/Gpa2p pathways (5–7). Activation of these two independent pathways leads to activation of the Cyr1p adenylate cyclase and increased production of cAMP (8). cAMP binds to the PKA regulatory subunit Bcy1p, causing Bcy1p to release the three partially redundant PKA catalytic subunits Tpk1p, Tpk2p, and Tpk3p and causing activation of PKA (9–12). Phosphorylation of downstream targets, such as the transcription factors Msn2p and Msn4p, by PKA results in the repression of stress response genes and an increase in the expression of genes associated with metabolic processes such as ribosome biogenesis (3). This allows cells to exit G_1 and progress through the cell cycle (13). However, a decrease in the glucose level results in activation of the intrinsic GTPase activity of Ras by the GTPase-activating proteins Ira1p and Ira2p (5). Reduction in the cAMP level allows Bcy1p to inhibit PKA by binding to the catalytic subunits (14–16). In contrast to the PKA pathway, the Snf1p pathway is activated by the depletion of glucose or growth on a nonfermentable carbon source (3). A decrease in the glucose level stimulates the kinases Sak1p, Elm1p, and Tos3p to phosphorylate Snf1p on its activation loop within the kinase domain (17, 18). Binding of the regulatory subunit Snf4p to the regulatory domain of Snf1p fully activates the catalytic kinase domain (17, 19, 20). Interaction of one of the three scaffolding proteins, Gal83p, Sip1p, or Sip2p, with active Snf1p regulates its localization, and therefore the specific targets. For instance, Gal83p directs Snf1p to the nucleus, where it phosphorylates transcription factors such as Mig1p, causing derepression of glucose-repressed genes (21, 22). Expression of these genes allows the cells to survive under poor carbon conditions. Upon addition of glucose, the regulatory protein Reg1p directs the protein phosphatase Glc7p to Snf1p, allowing dephosphorylation and inactivation of Snf1p (23). Furthermore, the Glc7p-Reg1p complex is inhibited under glucose-limiting conditions by phosphorylation of Reg1p by active Snf1p (18).

Nuclear tRNA export in *S. cerevisiae* has recently been added to the list of processes regulated by nutrient availability (24–30). The mechanism responsible for tRNA export in *S. cerevisiae* is complex, involving several pathways. The *S. cerevisiae* tRNA export process begins in the nucleolus by aminoacylation quality assurance of mature tRNAs derived from extensive processing of intronless and intron-containing precursor tRNAs (pre-tRNAs) (31–34). tRNAs made from both classes of pre-tRNAs that are deemed functional by aminoacylation are collected from the aminoacyl-tRNA synthetases in the nucleolus by Utp8p and delivered to the nuclear tRNA export receptors Los1p and Msn5p in the nucleoplasm and at the nuclear pore complex (NPC) using a channeling mechanism (33). While Utp8p is required to deliver functional tRNAs made from both intronless and intron-containing pre-tRNAs, Utp8p depends on the function of Utp9p to deliver functional tRNAs made from intron-containing precursors to Msn5p, but not Los1p (26), and on the function of Utp22p to

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deliver functional tRNAs made from both classes of pre-tRNAs to Los1p, but not Msn5p (35). In addition to delivering tRNAs to the export receptors, Utp8p facilitates formation of the Los1p export complex, consisting of Los1p, tRNA, and the Ran GTPase Gsp1p bound to GTP (33), whereas Utp8p and Utp9p are both needed to form the Msn5p-tRNA-Gsp1p-GTP export complex (26). However, Utp22p does not appear to be required for formation of the Los1p-tRNA-Gsp1p-GTP export complex (35). Once the export complexes are formed, the Utp proteins return to the nucleolus and the export complexes translocate across the NPC by the export receptors interacting with components of the NPC termed nucleoporins. This step in mammalian cells is facilitated by Xpo-t and Xpo-5, the orthologues of Los1p and Msn5p, respectively (36–39). Los1p and Msn5p are not the only tRNA export receptors in *S. cerevisiae*, as loss of the functions of both Los1p and Msn5p has no effect on cell growth or viability (40). Moreover, it is presently not known whether the functions of Utp8p and Utp9p are required in mammalian cells, as the mammalian orthologues of these proteins are not known. The function of Utp22p may be necessary in mammalian cells to deliver tRNAs to the nuclear tRNA export receptors, as Utp22p is conserved from yeast to mammals.

The final step of the nuclear tRNA export process is disassembly of the export complex, which is thought to occur at the cytoplasmic face of the NPC (41, 42). This step requires activation of the GTPase activity of Gsp1p by the activating protein Rna1p and the Gsp1p binding protein Yrb1p (43). However, recent studies indicate that the conserved N-terminal kinase-like domain protein Cex1p, but not Yrb1p, is involved in this process (41). Cex1p has been shown to recruit Rna1p to the tRNA export complex at the NPC and enables Rna1p to gain access to Gsp1p of the tRNA export complex (41). Hydrolysis of GTP to GDP by Gsp1p is thought to result in dissociation of the tRNA from the export receptors (26, 36–39, 41). However, other studies have shown that the function of Cex1p is also required for release of the aminoacyl-tRNAs from the receptors (42). Cex1p is thought to collect the tRNA from the receptors after dissociation of Gsp1p-GDP and delivery of the tRNA to eukaryotic elongation factor 1A (eEF-1A) for utilization in protein synthesis (42). This step in mammalian and plant cells is facilitated by Scyl1 and CTEXP, the orthologues of Cex1p (44, 45).

Nuclear export of tRNA has been shown to be inhibited in *S. cerevisiae* deprived of glucose (30, 46). This could to be due to regulation of the function of the tRNA export receptors by accumulation of the receptors in the cytoplasm, as cytoplasmic accumulation of several export receptors, including Los1p and Msn5p, has been shown to occur under limiting glucose conditions (47). Here, we provide evidence that nuclear reimport of the tRNA export receptors Los1p and Msn5p is controlled by glucose availability. Analyses of mutant strains of components of the Snf1p signaling pathway indicate that activation of the function of Snf1p during glucose deprivation is not responsible for cytoplasmic accumulation of the tRNA export receptors and inhibition of nuclear tRNA export. PKA appears to be involved in the mechanism that facilitates the return of the nuclear tRNA export receptors to the nucleus, as glucose deprivation had no effect on nuclear tRNA export or localization of the receptors to the nucleus in mutant strains with constitutively active PKA. However, return of the tRNA export receptors to the nucleus when glucose is available is not due to phosphorylation of the receptors by PKA. The data suggest that inactivation of PKA during glucose deprivation results in activation of a mechanism that blocks nuclear reimport of the nuclear tRNA export receptors. Based on these data, we propose that PKA plays a role in inhibiting an unidentified mechanism that facilitates cytoplasmic retention of the tRNA export receptors when glucose in the environment is limiting. A second mechanism that is not dependent on PKA activity is also involved in regulating nuclear tRNA export in response to glucose availability, based on the finding that nuclear tRNA export in an *msn2 msn4* strain devoid of PKA activity is affected only when the mutant strain is deprived of glucose. However, this PKA-independent mechanism does not play a role in regulating nuclear reimport of the nuclear tRNA export receptors.

MATERIALS AND METHODS

Plasmids. The plasmids pYX242-LOS1, pYX242-UTP8, and pYX242-CEX1 were described previously (33, 42, 48). pYX242-MSN5 was constructed using the pYX242 vector, obtained from Novagen, containing the *LEU2* selection marker and the triose-phosphate isomerase promoter and transcription termination sequence. The *MSN5* open reading frame (ORF) was amplified by PCR from *S. cerevisiae* genomic DNA and inserted into the NcoI and SmaI sites of pYX242. The plasmids YCp50, YCp50-RAS2, and YCp50-RAS2^{Val19} were provided by E. Martegani (University of Milan-Bicocca, Milan, Italy). Preparation of pET19b-LOS1, pET23d-MSN5, pET19b-UTP8, and pET19b-CEX1 was described previously (26, 33, 42). pRS416-LOS1-mCherry was constructed using the pRS416 vector with the URA3 selection marker obtained from Stratagene; the BamHI-SalI fragment containing the ORF of mCherry was PCR amplified from pFA6a-mCherry and introduced into pRS416. The *LOS1* ORF was PCR amplified from *S. cerevisiae* genomic DNA, along with 1 kb of upstream sequence including the endogenous promoter and a GDGAG linker to improve the mobility of the mCherry tag, and inserted into the NotI and BamHI sites of pRS416-mCherry.

Strains and growth conditions. The *S. cerevisiae* strains used in this study are listed in Table 1. Yeast strains were grown in complete synthetic (CS) medium containing 2% (wt/vol) glucose to mid-logarithmic phase at 30°C. The cells were washed twice with sterile water and incubated in synthetic medium lacking glucose for 60 min. Following the 60-min starvation, the medium was resupplemented with glucose at a final concentration of 2% (wt/vol) glucose, and the culture was then incubated at 30°C for an additional 20 min.

Fluorescent *in situ* hybridization in *S. cerevisiae*. Fluorescent *in situ* hybridization (FISH) for localization of mature tRNA was performed as previously described (32). Briefly, cells were fixed in growth medium with 4% formaldehyde for 15 min at 30°C and then in 4% formaldehyde, 0.1 M potassium phosphate buffer at room temperature for 3 h. Hybridization was performed at 37°C in hybridization buffer (4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50% formamide, 10% dextran sulfate, 125 μg/ml *Escherichia coli* tRNA, 500 μg/ml salmon sperm DNA, 1 mM dithiothreitol [DTT], 0.4 U/μl RNAsin) containing 5 pmol of 5′-end Cy3-labeled probe specific for mature tRNA^{Tyr} (5′-GCGAGTCGAACGCCCG ATCTCAAGATTTACAGTCTTGCGCCTTAAACCAACTTGGCTACC-3′), mature tRNA^{Gly} (5′-CGAACCGGGGGCCCAACGATGGCAACGT TGGATTTTACC-3′), or mRNA [30-mer poly(dT)]. The slides were mounted with Mowial, sealed with coverslips, and viewed under a 100× objective lens of a Nikon Eclipse 6600 microscope. Images were captured using a Coolsnapfx monochrome charge-coupled device (CCD) digital camera (Roper Scientific) and processed using the Metamorph program (Universal Imaging).

Western blot analysis of the levels of Los1p and Msn5p during glucose deprivation. Los1p and Msn5p levels during glucose starvation were determined by preparing total cell lysate from 25 ml of culture at specified times. The cells were resuspended in 75 μl of NP-40 buffer (15 mM Na_2HPO_4, pH 7.2, 10 mM NaH_2PO_4, 2.0% NP-40, 150 mM NaCl, 2 mM

TABLE 1 Yeast strains used in this study

Strain	Genotype	Source
BY4742	*MAT*α *his3 leu2 lys2 ura3*	Open Biosystems
BY4743	*MAT***a**/*MAT*α *his3/his3 leu2/leu2 met15/MET15 LYS2/lys2 ura3/ura3*	Open Biosystems
SNF1-HA	*MAT*α *his3 leu2 lys2 ura3 SNF1*::*HAkanMX6*	G. van der Merwe
sak1	*MAT*α *his3 leu2 lys2 ura3 sak1*::*kanMX6*	Open Biosystems
snf1	*MAT*α *his3 leu2 lys2 ura3 snf1*::*kanMX6*	Open Biosystems
snf4	*MAT*α *his3 leu2 lys2 ura3 snf4*::*kanMX6*	Open Biosystems
sch9	*MAT*α *his3 leu2 lys2 ura3 sch9*::*kanMX6*	Open Biosystems
elm1	*MAT*α *his3 leu2 lys2 ura3 elm1*::*kanMX6*	Open Biosystems
rim15	*MAT*α *his3 leu2 lys2 ura3 rim15*::*kanMX6*	Open Biosystems
UTP8-GFP	*MAT***a** *his3 leu2 met15 ura3 UTP8*::*GFPHIS3*	Invitrogen
SP1	*MAT***a** *his3 leu2 ura3 trp1 ade1 can1*	I. Sadowski
S18-1D	*MAT***a** *his3 leu2 ura3 trp1 ade1 can1 tpk1w1 tpk2*::*HIS3 tpk3*::*TRP1 BCY1*	M. Hall
RS13-58A-1	*MAT***a** *his3 leu2 ura3 trp1 ade1 can1 tpk1w1 tpk2*::*HIS3 tpk3*::*TRP1 bcy1*	I. Sadowski
bcy1/bcy1	*MAT***a**/*MAT*α *his3/his3 leu2/leu2 met15/MET15 LYS2/lys2 ura3/ura3 bcy1/bcy1*::*kanMX6*	Open Biosystems
msn2 msn4	*MAT*α *his3 leu2 lys2 ura3 msn2*::*hygB msn4*::*kanMX6*	G. van der Merwe
ASY63	*MAT***a** *leu2 trp1 ura3 his3 ade8 tpk1*::*URA3 tpk2*::*HIS3 tpk3*::*TRP1 msn2*::*HIS3 msn4*::*LEU2*	S. Garrett

EDTA, 50 mM NaF, 0.1 mM Na_3VO_4 containing Roche protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and lysed by vortexing 4 times for 30 s each time in the presence of acid-washed glass beads (Sigma). The lysate was clarified by centrifugation for 10 min at 15,000 rpm. Protein from 20 μg of total lysate was separated on a 10% polyacrylamide gel, and Western blot analysis was performed using anti-Msn5p (α-Msn5p) or α-Los1p (E. Hurt, University of Heidelberg, Heidelberg, Germany) and α-actin (Abcam).

Immunofluorescence microscopy. Immunofluorescence microscopy was performed as previously described (33). The cells were fixed for 1 h with 4% formaldehyde at 30°C, spheroplasted with Zymolyase-100T (MP Biochemicals), and mounted on poly-L-lysine-coated slides. The cells were dehydrated by plunging the slides for 6 min in ice-cold methanol, followed by 30 s in ice-cold acetone. The slides were dried and rehydrated in 1× PBS, 1% BSA for 1 h at room temperature. The rabbit polyclonal α-Los1p, α-Msn5p, and α-mCherry (Abcam) and the mouse monoclonal α-Nsp1p (MyBioSource) primary antibodies were added at 1:500 dilution in 1× phosphate-buffered saline (PBS)-1% bovine serum albumin (BSA) for 1 h. After washing the slides with 1× PBS, 1% BSA, the cells were incubated with goat α-mouse antibody conjugated to Alexa Fluor 488 (Invitrogen) and goat α-rabbit antibody conjugated with Alexa Fluor 594 (Invitrogen) for 1 h at room temperature. The slides were washed with 1× PBS containing 1% BSA and dried briefly. The slides were mounted with Mowial and viewed with a 100× objective lens on a Nikon Eclipse 6600 microscope. Images were captured using a Coolsnapfx monochrome CCD digital camera (Roper Scientific) and processed using the Metamorph program (Universal Imaging).

Overexpression of proteins involved in nuclear tRNA export. The BY4742 yeast strain was transformed with pYX242, pYX242-LOS1, pYX242-MSN5, pYX242-UTP8, or pYX242-CEX1. Transformants were grown in CS medium containing glucose and lacking Leu and subjected to glucose starvation. FISH was performed on each strain to determine tRNA localization. In addition, cell lysate was obtained from each strain, and Western blot analysis was performed to verify overexpression of each protein.

mRNA Northern blot analysis. The *S. cerevisiae* strains were grown to an optical density at 600 nm (OD_{600}) of ~0.6 to 0.7, and total RNA was extracted as previously described (49). Cells were harvested and resuspended in 0.2 M Tris-HCl, pH 7.4, buffer containing 0.5 M NaCl and 10 mM EDTA. Glass beads (2 g; Sigma) and an equal volume of phenol-chloroform were added to the suspension. The cells were lysed by vortexing the mixture 3 times for 30 s each time, with 30 s on ice in between. The aqueous phase was removed following centrifugation at 3,500 rpm for 10 min, and the phenol was reextracted with an equal volume of lysis buffer. The aqueous phase was extracted again with phenol-chloroform, followed by an extraction with chloroform. The RNA was precipitated with 1/10 volume 3 M lithium acetate and 2.5 volumes 95% ethanol (EtOH) overnight at −20°C. The precipitated RNA was pelleted at 7,000 rpm for 30 min and resuspended in an appropriate volume of Tris-EDTA (TE). For the analysis of the expression level of *HSP12*, 10 μg of total RNA was separated by electrophoresis on a 1.2% formaldehyde agarose gel using 1× MOPS (morpholinepropanesulfonic acid) running buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0) at 80 V for 3 h. The RNAs were capillary transferred to a positively charged nylon membrane (Roche) with 20× SSC overnight and UV cross-linked to the membrane. Prehybridization was performed by incubating the membrane with 15 ml of DIG Easy Hyb buffer (Roche) for 4 h at 50°C. Digoxigenin (DIG)-labeled probes specific for *HSP12* and *ACT1* were generated by PCR from genomic DNA (Novagen) using the Expand DNA polymerase (Roche) with gene-specific primers and DIG DNA labeling mixture containing DIG-11-dUTP. The probes were cleaned using a PCR cleanup kit (Qiagen) and denatured at 95°C for 5 min. The denatured probe was added to the membrane in 5 ml of DIG Easy Hyb buffer and incubated overnight at 50°C. The membrane was washed twice for 10 min each time at room temperature in low-stringency buffer (2× SSC, 0.1% SDS), followed by 4 washes for 15 min at 50°C in high-stringency buffer (0.1× SSC, 0.1% SDS). To detect the DIG-labeled probe, the blot was blocked with blocking reagent (Roche) in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5, for 1 h at room temperature. The membrane was subsequently incubated with α-DIG-alkaline phosphatase (AP) Fab fragments for 1 h and washed twice for 15 min with 0.1 M maleic acid, pH 7.5, 0.15 M NaCl, 0.3% (vol/vol) Tween 20, followed by a 5-min wash in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). Detection was conducted by autoradiography after the membrane was incubated for 5 min with CDP-Star solution (Roche).

Aminoacylation status of tRNA. Cells were starved of glucose for 60 min, and 1 liter of cell culture was harvested at the appropriate time points. Nuclear and postnuclear RNAs were isolated under acidic conditions, as previously described (32). Briefly, the cells were spheroplasted in 1.2 M sorbitol, 10 mM EDTA, 10 mM potassium phosphate, 0.1% 2-mercaptoethanol with 10 mg Zymolyase-100T for 25 min at 30°C. The spheroplasts were lysed using a Dounce homogenizer in 300 mM sodium acetate, pH 5.0, 5 mM magnesium acetate, 0.1% (vol/vol) NP-40, and 250 mM sucrose. The lysed cells were subjected to a sucrose gradient by overlaying it onto a solution containing 500 mM sodium acetate, pH 5.0, 5 mM magnesium acetate, and 500 mM sucrose. After centrifugation at 10,000 rpm for 20 min, total RNA was isolated from the nuclear and postnuclear fractions with several acidic phenol and chloroform washes.

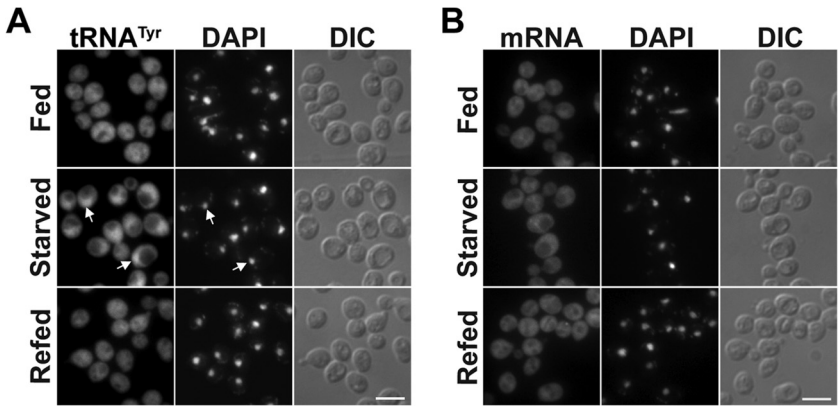

FIG 1 Glucose deprivation affects the efficiency of nuclear export of tRNA, but not nuclear export of mRNA. (A and B) The wild-type BY4742 strain was grown in CS medium to mid-logarithmic phase (Fed) and transferred to medium containing all supplements but lacking glucose for 60 min at 30°C (Starved). A portion of the starved cells were then incubated with glucose at a final concentration of 2% for 20 min at 30°C (Refed). The cellular distribution of $tRNA^{Tyr}$ (A) and mRNA (B) was monitored by FISH. The DNA was visualized with DAPI (4′,6-diamidino-2-phenylindole). The arrows indicate nuclear retention of $tRNA^{Tyr}$. Scale bars, 5 μm. DIC, differential interference contrast.

The RNA was precipitated with ethanol and resuspended in 20 mM sodium acetate, pH 5.0.

Total RNA from the nuclear and postnuclear fractions was separated by electrophoresis on a 6.5% polyacrylamide gel containing 8 M urea at 500 V using 0.1 M sodium acetate buffer, pH 5.0, at 4°C. A marker for deacylated tRNA was made from a sample of RNA from each fraction by incubating total RNA in 200 mM Tris, pH 9.5, at 37°C for 1 h. The separated RNA was transferred onto Nytran Plus membranes (Whatman) and prehybridized for 4 h at 37°C in hybridization buffer (4× SET [1× SET is 30 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM EDTA], 250 μg/ml salmon sperm DNA, 10× Denhardt's solution, and 0.1% SDS). Hybridization was conducted overnight at 37°C in hybridization buffer containing 5′-end ^{32}P-labeled oligonucleotides (1×10^6 to 2×10^6 cpm/ml). The membranes were washed 4 times for 30 min each time with 3× SET, 0.1% SDS at room temperature and subjected to autoradiography. $tRNA^{Tyr}$ and $tRNA^{His}$ were detected with 5′-GCGAGTCGAACGCCCGATCTCAAGA TTTACAGTCTTGCGCCTTAAACCAACTTGGCTACC-3′ and 5′-TCC TAGAATCGAACCAGGGTTTCATCGGCCACAACGATGTGTACTAA CCACTATACTAAG-3′, respectively. To monitor the purity of the nuclear and cytoplasmic fractions, a portion of each fraction was neutralized with 4× NP-40 buffer, and 20 μg of total protein from each fraction was subjected to Western blot analysis. The cytoplasmic fraction was analyzed for nuclear contamination using the nucleolar marker Nop1p with α-Nop1p (Santa Cruz Biotechnology). Cytoplasmic contamination of the nuclear fraction was determined using the cytoplasmic marker Adh1p with α-Adh1p (Rockland).

λ-Phosphatase treatment of protein extracts. Cells from 25 ml of culture were pelleted and lysed in 25 mM Tris-HCl, pH 7.5, buffer containing 2% (vol/vol) NP-40, 150 mM NaCl, 2 mM EDTA, 1 mM $MnCl_2$ and EDTA-free ProteCease inhibitor cocktail (G Biosciences), 1 mM phenylmethylsulfonyl fluoride (PMSF) with glass beads. Total protein (20 μg) was incubated with 40 U of λ-phosphatase (NEB) for 1 h at 30°C. The reaction was stopped by boiling for 10 min after the addition of NuPage loading dye and sample reducing agent.

Overexpression and purification of His-tagged proteins. His affinity-tagged Los1p, Msn5p, Utp8p, and Cex1p were purified as previously described. Briefly, *E. coli* BL21(DE3) Codon Plus RIL (Novagen) harboring the pET expression vectors was grown in 1 liter of 2YT medium (Difco) containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37°C to an OD_{600} of ~0.4 to 0.5. The cells were incubated at 15°C for 15 h with 0.1 to 1.0 mM isopropyl β-D-thiogalactoside (IPTG) and harvested by centrifugation. The cells were resuspended in 30 ml of binding buffer (20 mM NaH_2PO_4, pH 7.5, containing 500 mM NaCl, 5 mM imidazole, protease inhibitor cocktail [EDTA free; Roche]) and lysed with a French press at 70,000 kPa. The lysate was clarified at 18,000 rpm for 30 min before being applied to a 1-ml HisTrap HP column (GE Healthcare). The columns were washed with 20 ml of 20 mM NaH_2PO_4 buffer, pH 7.5, containing 500 mM NaCl and 50 mM imidazole and with 10 ml of 20 mM NaH_2PO_4 buffer, pH 7.5, containing 500 mM KCl to remove tRNA. The proteins were eluted from the column with a gradient of increasing concentrations of imidazole and dialyzed against IPP150 buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20% [wt/vol] glycerol, and 0.1% [vol/vol] Nonidet P-40).

Los1p was further purified by gel filtration using a Superdex 200 16/60 column (GE Healthcare). Cex1p was further purified by loading the proteins dialyzed against 20 mM NaH_2PO_4 buffer, pH 7.5, containing 100 mM NaCl onto a HiTrap Q HP ion exchange column. The proteins were eluted with an increasing gradient of NaCl and dialyzed against IPP150, pH 7.5, containing 20% (wt/vol) glycerol and stored at −80°C.

***In vitro* phosphorylation by PKA.** Yeast recombinant protein was expressed and purified as described above. The catalytic subunit of bovine PKA was obtained from Sigma and reconstituted in 6 mg/ml DTT to a final concentration of 0.5 U/μl (Sigma P2645). The PKA phosphorylation assay was performed by combining 2 μg of purified protein with 1 U of bovine PKA (10 U/μg) and 5 μCi of [γ-^{32}P]ATP in PKA phosphorylation buffer (50 mM potassium phosphate, pH 7.15, 5 mM NaF, 10 mM $MgCl_2$, 4.5 mM DTT, and both ProteCease and PhosphataseArrest-I inhibitors [G Biosciences]) to a total volume of 20 μl. A PKA substrate peptide (Sigma SCP0212) was used as a positive control for the phosphorylation reaction. The reaction mixtures were incubated for 30 min at 25°C before being terminated by the addition of SDS-PAGE sample buffer (NuPAGE; Invitrogen) and boiled for 10 min. The samples were separated on a 10% SDS-PAGE gel. The gel was dried, and protein phosphorylation was visualized using a phosphor screen (Bio-Rad).

RESULTS

Glucose deprivation affects the efficiency of nuclear tRNA export in *S. cerevisiae*, but not the nuclear tRNA aminoacylation quality assurance step. Nuclear export of mature tRNAs made from intronless and intron-containing precursors has been shown to be affected when *S. cerevisiae* is deprived of glucose (30, 46). However, this could be due to a general defect in nuclear RNA export processes. To exclude this possibility, the cellular distribution of mRNA was assessed during glucose deprivation (Fig. 1). FISH analysis of wild-type cells grown in the presence of glucose

showed that mature $tRNA^{Tyr}$ made from an intron-containing precursor (Fig. 1A) and mRNA (Fig. 1B) were uniformly distributed throughout the cell. However, nuclear retention of mRNA was not observed when the cells were deprived of glucose for a 60-min period or after the readdition of glucose. As expected, nuclear retention of $tRNA^{Tyr}$ was detected during glucose deprivation for the same period. Furthermore, addition of glucose to the starved cells for 20 min restored nuclear export of $tRNA^{Tyr}$. The data confirm that glucose deprivation affects the efficiency of nuclear tRNA export and suggest that glucose deprivation specifically affects nuclear export of tRNAs.

The nuclear tRNA aminoacylation quality assurance step is required for efficient export of tRNAs from the nucleus to the cytoplasm in *S. cerevisiae*, as lack of tRNA aminoacylation in the nucleus has been shown to have a dramatic effect on the efficiency of nuclear export of tRNA (31, 32). Previous studies have shown that tRNA aminoacylation in *S. cerevisiae* is not affected during glucose deprivation, suggesting that the reduced efficiency of nuclear tRNA export caused by glucose deprivation is not related to an effect on the efficiency of aminoacylation of tRNAs in the nucleus (30). However, the study reported did not assess whether aminoacylation of nuclear tRNAs was affected during glucose deprivation (30). To exclude this possibility, Northern blot analysis was used to determine whether mature tRNAs made from intronless and intron-containing precursors are aminoacylated in the nucleus during glucose deprivation (Fig. 2). For this analysis, nuclear and cytoplasmic fractions were isolated from total cell lysate prepared under acidic conditions (32) from wild-type cells starved of glucose for 60 min. Total RNA was subsequently isolated under acidic conditions from the cytoplasmic (Fig. 2A) and nuclear (Fig. 2B) fractions (32). Northern hybridization was performed using 5′-end ^{32}P-labeled probes specific for mature $tRNA^{Tyr}$ made from an intron-containing precursor (Fig. 2A and B, top) and $tRNA^{His}$, which is derived from an intronless pre-tRNA (Fig. 2A and B, bottom). To obtain a marker for deacylated tRNAs, total RNA from each fed sample was treated with base to cleave the ester linkage between the tRNA and amino acid (Fig. 2, lanes 1 and 5). The analyses show that aminoacylation of $tRNA^{Tyr}$ and $tRNA^{His}$ in the presence or absence of glucose was not affected in the cytoplasm (Fig. 2A, lanes 2 to 4) or the nucleus (Fig. 2B, lanes 6 to 8). Western blot analyses (Fig. 2C) also suggest that the cytoplasmic and nuclear fractions are essentially pure, based on the observations that very little of the nucleolar protein Nop1p was detected in the cytoplasm (Fig. 2C, top, compare lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6), and that the nuclear fraction contains an insignificant amount of the Adh1p cytoplasmic marker protein (Fig. 2C, bottom, compare lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6). Thus, these findings indicate that the reduction in the efficiency of nuclear tRNA export in response to glucose stress is not likely to be the result of an effect on nuclear tRNA aminoacylation. Moreover, the data suggest that a step after tRNA aminoacylation is affected by glucose deprivation.

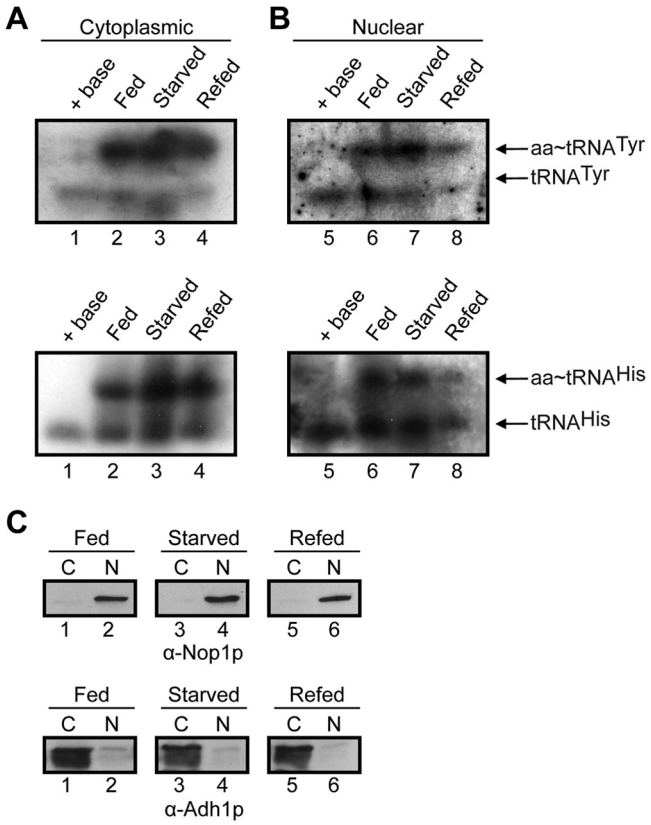

FIG 2 Glucose deprivation does not affect aminoacylation of tRNAs in the nucleus. (A and B) Cytoplasmic (A) and nuclear (B) fractions were isolated from BY4742 under acidic conditions before (Fed, lanes 2 and 6) and after (Starved, lanes 3 and 7) glucose starvation for 60 min or following incubation of the starved cells in medium containing 2% glucose for 20 min at 30°C (Refed, lanes 4 and 8). Total RNA was isolated from nuclear and postnuclear fractions and separated on a 6.5% polyacrylamide gel containing 8 M urea. The separated RNAs were transferred electrophoretically to Nytran Plus membranes for Northern blot analysis to detect the aminoacylation (aa~) status of $tRNA^{Tyr}$ derived from an intron-containing precursor (top) and $tRNA^{His}$ derived from intronless pre-tRNA (bottom). Deacylated tRNA markers were prepared by incubating total RNA from each fraction in 100 mM Tris, pH 9.5 (lanes 1 and 5). (C) The extent of contamination of the cytoplasmic fraction (C) with nuclei (top) and the purity of the nuclear fraction (N) (bottom) were ascertained by using Western blot analysis to monitor the level of the nucleolar protein Nop1p in the cytoplasmic fraction (lanes 1, 3, and 5) and the cytoplasmic protein Adh1p in the nuclear fraction (lanes 2, 4, and 6).

Glucose availability regulates the function of the nuclear tRNA export receptors by affecting the return of the tRNA export receptors to the nucleus. Previous studies have shown that several nuclear export receptors, including the nuclear tRNA export receptors Los1p and Msn5p, accumulate in the cytoplasm of cells exposed to limiting amounts of glucose (47). Therefore, it is possible that regulation of nuclear tRNA export in response to glucose deprivation may, in part, be due to an effect on nuclear reimport of the nuclear tRNA export receptors after a round of tRNA export. To verify that glucose deprivation affects translocation of the tRNA export receptors from the cytoplasm to the nucleus, the cellular locations of Los1p (Fig. 3A) and Msn5p (Fig. 3B) were monitored by immunofluorescence in wild-type cells deprived of glucose. As reported previously, in cells fed with glucose, Los1p is localized primarily at the NPC (50) and Msn5p is found in the nucleoplasm (47), based on colocalization with the nucleoporin Nsp1p. In contrast, cytoplasmic accumulation of both Los1p and Msn5p was detected in a large percentage of cells deprived of glucose (47). Moreover, proper relocalization of both receptors was observed when the starved cells were exposed to glucose for 20 min. The analyses also indicate that localization of Nsp1p to the NPC was not affected during glucose deprivation, as the location of Nsp1p remained unchanged in cells grown in the

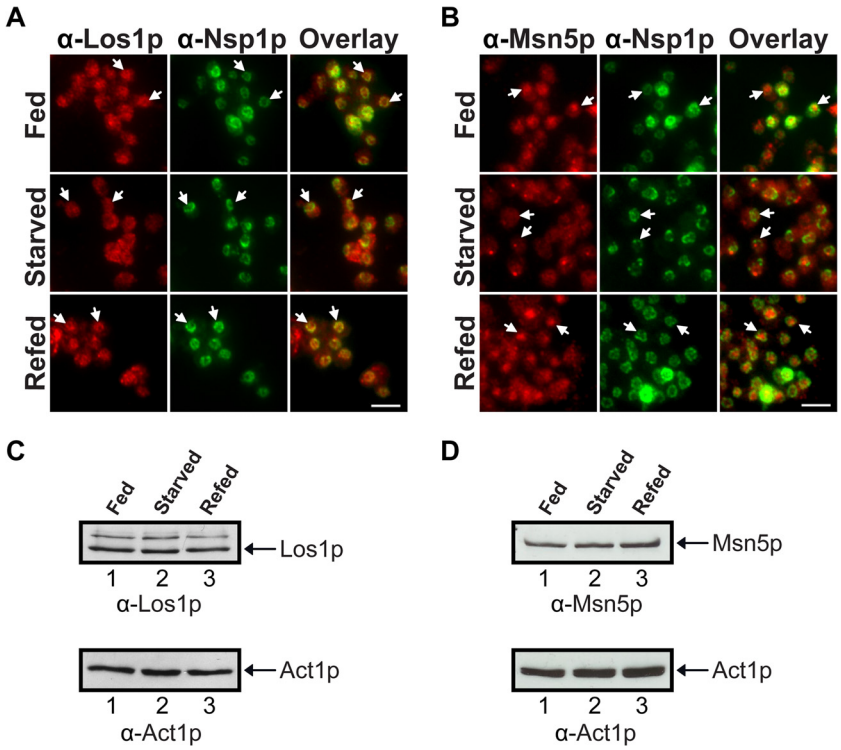

FIG 3 The nuclear tRNA export receptors Los1p and Msn5p accumulate in the cytoplasm during glucose deprivation. (A and B) The BY4742 strain was grown in CS medium (Fed) to mid-logarithmic phase and then transferred to CS medium lacking glucose (Starved) and incubated for 60 min at 30°C. Glucose was added to the starved cells at a final concentration of 2% and incubated at 30°C for 20 min (Refed). The locations of Los1p (A) and Msn5p (B) were monitored by immunofluorescence. Los1p and Msn5p were detected with rabbit polyclonal α-Los1p and α-Msn5p, respectively. Nsp1p served as a marker for the NPC and was detected with α-Nsp1p. The arrows show the locations of Los1p, Msn5p, and Nsp1p. Scale bars, 5 μm. (C and D) The levels of Los1p (C) and Msn5p (D) in total cell lysate prepared from cells grown in glucose (lanes 1), cells starved of glucose for 60 min (lanes 2), or starved cells refed with glucose for 20 min (lanes 3) were monitored by Western blot analysis. Actin levels were monitored to ensure that equal amounts of cell extract were used.

presence or absence of glucose. However, in a small percentage of glucose-starved cells, Nsp1p appears to accumulate in a certain region of the nuclear envelope (Fig. 3B). Similarly, nuclear import and nucleolar localization of Utp8p and Nop5/6p were not affected in cells deprived of glucose (data not shown). Western blot analyses of total cell extract showed that the levels of Los1p (Fig. 3C, top) and Msn5p (Fig. 3D, top) were not affected during glucose starvation (compare lanes 1 and 2) or when the starved cells were exposed to glucose for 20 min (lanes 3). Analyses of the actin level (Fig. 3C and D, bottom) indicate that approximately the same amounts of cell extracts were analyzed. These findings confirmed that glucose deprivation affects translocation of the two tRNA export receptors back to the nucleus, but not the levels of the two proteins, excluding the possibility that the reduced signal of the nuclear tRNA export receptors at the proper location is due to a reduction in the cellular levels of the receptors.

To verify that the effect of glucose deprivation on nuclear tRNA export is caused by regulation of the function of the nuclear tRNA export receptors by an alternative strategy, we investigated whether overexpression of the tRNA export receptor restores nuclear export of tRNAs in wild-type cells deprived of glucose (Fig. 4). For this analysis, FISH was used to monitor the cellular location of $tRNA^{Tyr}$ made from an intron-containing precursor and $tRNA^{Gly}$ derived from an intronless pre-tRNA in wild-type cells harboring pYX242 alone or pYX242 carrying the Los1p, Msn5p, Cex1p, or Utp8p gene. Western blot analyses confirmed that Los1p (Fig. 4B), Msn5p (Fig. 4C), Cex1p (Fig. 4D), and Utp8p (Fig. 4E) are overproduced in BY4742 (Fig. 4B to E, compare lanes 1 and 2). Furthermore, the level of actin indicates that the same amount of cell extract was used. Both $tRNA^{Tyr}$ (Fig. 4A to E, top) and $tRNA^{Gly}$ (bottom) were found primarily in the cytoplasm of the BY4742 pYX242 (Fig. 4A), BY4742 pYX242-LOS1 (Fig. 4B), BY4742 pYX242-MSN5 (Fig. 4C), BY4742 pYX242-CEX1 (Fig. 4D), and BY4742 pYX242-UTP8 (Fig. 4E) strains fed with glucose. A large number of BY4742 pYX242 (Fig. 4A) and BY4742 pYX242-CEX1 (Fig. 4D) cells deprived of glucose showed nuclear accumulation of $tRNA^{Tyr}$ and $tRNA^{Gly}$ (Fig. 4F). In contrast, a low percentage of glucose-deprived BY4742 pYX242-LOS1 (Fig. 4B) and BY4742 pYX242-MSN5 (Fig. 4C) cells showed nuclear retention of the two tRNAs (Fig. 4F). Surprisingly, overexpression of Utp8p during glucose starvation also alleviated nuclear retention of the tRNAs in BY4742 cells (Fig. 4E and F). The data show that overexpression of nuclear components of the nuclear tRNA export process relieved the nuclear tRNA accumulation observed during glucose deprivation, suggesting that these proteins could be the targets of regulation by glucose availability. These findings also suggest that glucose availability affects the function of the tRNA export receptors, most likely by regulating the return of the receptors to the nucleus. This suggestion is supported by data discussed below. In addition, the data suggest that the function of Utp8p in the nucleus, but not Cex1p in the cytoplasm, may be controlled by glucose availability.

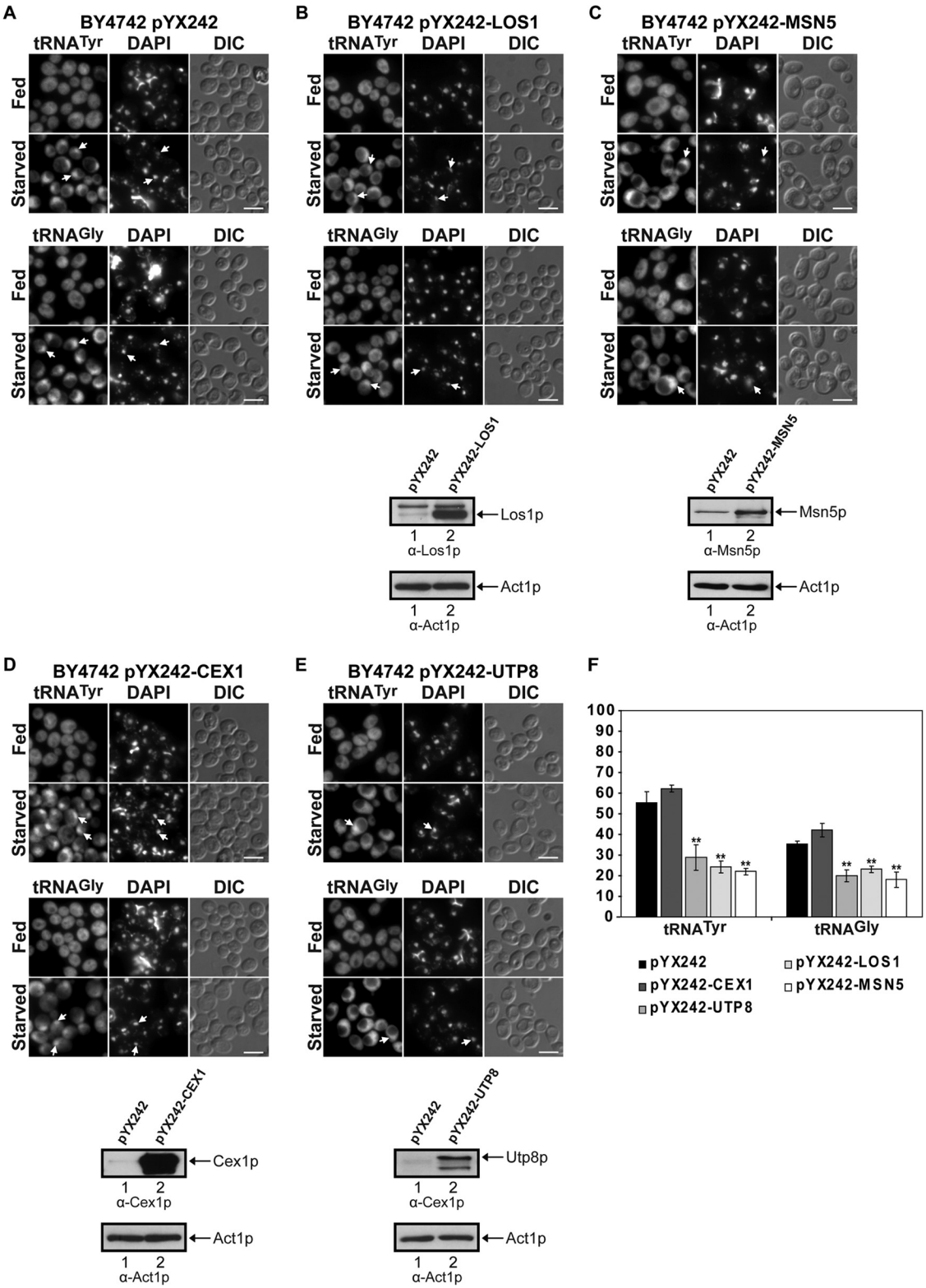

FIG 4 Overexpression of nuclear tRNA export receptors restores nuclear tRNA export in cells deprived of glucose. (A to E) The BY4742 strain harboring pYX242 (A), pYX242-LOS1 (B), pYX242-MSN5 (C), pYX242-CEX1 (D), or pYX242-UTP8 (E) was grown in CS medium lacking Leu to mid-logarithmic phase (Fed) and shifted to CS medium lacking Leu and glucose for 1 h (Starved). The cellular locations of $tRNA^{Tyr}$ and $tRNA^{Gly}$ were monitored by FISH. DNA was visualized by DAPI staining. The arrows indicate cells showing nuclear accumulation of tRNA. Scale bars, 5 μm. Overexpression of the protein in total cell lysate (20 μg of protein) was monitored by Western blot analyses (B to E, bottom, lanes 2). The level of actin (lanes 1 and 2), detected by Western blot analyses, was used to verify that the same amount of cell lysate was used for the analyses. (F) The percentage of cells from 3 independent fields of ~200 cells displaying nuclear accumulation of $tRNA^{Tyr}$ and $tRNA^{Gly}$ after glucose starvation for 60 min. The results from Student's two-tailed *t* test and standard deviations are shown. **, $P \leq 0.005$.

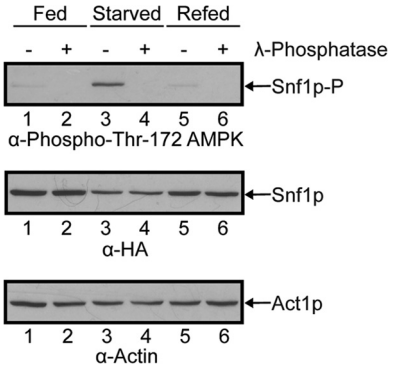

FIG 5 Depletion of glucose results in activation of Snf1p kinase by phosphorylation of Thr-210 in the activation loop. Total cell lysate was prepared from cells grown in CS medium containing glucose (Fed), starved of glucose for 60 min (Starved), and resupplemented with glucose at a final concentration of 2% (Refed) for 20 min. The cell lysates (20 μg of protein) were treated without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) 40 U of λ-phosphatase for 60 min at 30°C. Phosphorylation of Snf1p at Thr-210 was monitored by Western blotting using α-phospho-AMPKα (Thr-172) (top). The total Snf1p-HA present was detected with α-HA (middle). The level of actin was used to verify that the equal amounts of lysate were analyzed (bottom).

Nuclear accumulation of tRNAs and cytoplasmic retention of the nuclear tRNA export receptors in the absence of glucose are not due to activation of the Snf1p signaling pathway. Several signaling pathways are responsible for the cellular response to changes in glucose levels (3). The Snf1p signaling pathway is a major pathway that regulates the derepression of glucose-repressed genes in *S. cerevisiae* (22). The Snf1p kinase is turned on in cells exposed to nonfermentable carbon sources or limiting glucose levels (51). Thus, to understand how the nuclear tRNA export receptors are retained in the cytoplasm when glucose availability is low, we investigated whether activation of the Snf1p pathway when glucose is absent is responsible for inhibition of nuclear tRNA export. Activation of Snf1p requires phosphorylation of threonine residue 210 (Thr-210) in the activation loop by one of the Snf1p-activating kinases: Sak1p, Elm1p, or Tos3p (17). The Snf1p mammalian homolog, AMPK, is also phosphorylated in the activation loop at threonine residue 172 (Thr-172) when turned on during glucose deprivation (52). Previous reports have shown that an antibody to Thr-172 of AMPK is also able to detect phosphorylation of Snf1p at Thr-210 (53). Therefore, to ascertain whether the Snf1p kinase is active during glucose deprivation, the phosphorylation status of Thr-210 in the activation loop of Snf1 was monitored by Western blotting with α-phospho-AMPKα (Thr-172) (Fig. 5). The Snf1p-3HA strain was cultured in CS medium until mid-logarithmic phase before being incubated in CS medium lacking glucose for 60 min. The starved cells were also provided with glucose for 20 min. The cell lysate was extracted by glass bead lysis in the presence of protease and phosphatase inhibitors and separated by SDS-PAGE. Snf1p was phosphorylated to a small degree in glucose-fed cells (Fig. 5, top, lane 1) or when starved cells were resupplemented with glucose (Fig. 5, top, lane 5). In contrast, Snf1p phosphorylation increased after 60 min of glucose starvation (Fig. 5, top, lane 3) compared to Snf1p from fed or refed cells. The authenticity of the phosphorylation was established by treating the cell extract with λ-phosphatase to remove the phosphate moiety from phosphorylated proteins (Fig. 5, top, lanes 2, 4, and 6). Western blot analysis with α-hemagglutinin (HA) showed a decrease in the total amount of Snf1p in the cell lysate from glucose-starved cells (Fig. 5, middle, lanes 3 and 4). The amount of actin loaded was also smaller in the glucose-starved lysate, indicating that the difference in Snf1p protein levels is due to unequal loading (Fig. 5, bottom, lanes 3 and 4). Nevertheless, these findings suggest that Snf1p is activated in cells starved of glucose for 60 min and is turned off when the starved cells are provided with glucose.

To test whether Snf1p is involved in regulating nuclear tRNA export in response to glucose deprivation, FISH was used to monitor the cellular location of tRNAs in mutant strains lacking a component of the Snf1p pathway when deprived of glucose (Fig. 6). The *snf1*, *snf4*, *elm1*, and *sak1* mutant strains were tested. Sak1p and Elm1p are two of the three kinases that are required to activate Snf1p by phosphorylation of the activation loop at Thr-210 within the kinase domain of Snf1p (54, 55); Snf4p is the regulatory subunit that binds to the regulatory domain of Snf1p to facilitate full activation of the Snf1p kinase activity (17). As observed previously, starvation of the wild-type strain over a 60-min period (Fig. 6A) resulted in nuclear retention of mature $tRNA^{Tyr}$ (Fig. 6A, top) and $tRNA^{Gly}$ (bottom). Nuclear export of both $tRNA^{Tyr}$ and $tRNA^{Gly}$ was restored when the starved wild-type cells were supplemented with glucose for 20 min. As with the wild-type strain, nuclear retention of both $tRNA^{Tyr}$ (Fig. 6B to E, top) and $tRNA^{Gly}$ (bottom) was detected in *snf1* (Fig. 6B), *snf4* (Fig. 6C), *sak1* (Fig. 6D), and *elm1* (Fig. 6E) cells deprived of glucose. The percentage of mutant cells exhibiting nuclear retention of $tRNA^{Gly}$ and $tRNA^{Tyr}$ was not significantly different from that of wild-type cells (Fig. 6F). $tRNA^{Tyr}$ was observed in approximately 50% of the glucose-starved cells, whereas approximately 30% of the starved cells retained $tRNA^{Gly}$ in the nucleus (Fig. 6F). Similar to wild-type cells, inhibition of nuclear export of the tRNAs was alleviated when the starved mutant strains were provided with glucose. These findings suggest that the Snf1p signaling pathway may not play a role in controlling nuclear tRNA export by affecting trafficking of the receptors to the nucleus during glucose deprivation.

To show more directly that the Snf1p pathway is not responsible for cytoplasmic accumulation of the nuclear tRNA export receptors, the locations of Los1p and Msn5p were investigated in *snf1* cells deprived of glucose (Fig. 7). Los1p tagged with mCherry (Fig. 7A and B) and Msn5p (Fig. 7C and D) were monitored by immunofluorescence microscopy. As shown for wild-type cells (Fig. 7A and C), localization of the receptors was not affected in glucose-fed *snf1* cells (Fig. 7B and D). A large percentage of *snf1* cells deprived of glucose contain Los1p (Fig. 7B) and Msn5p (Fig. 7D) in the cytoplasm. Quantitative analyses indicated that approximately 12% of wild-type and 19% of *snf1* cells starved of glucose showed normal distribution of Msn5p (Fig. 7E), while about 22% of wild-type and 29% of *snf1* cells deprived of glucose exhibited proper localization of Los1p (Fig. 7E). Localization of the receptors to the nucleus was restored when glucose-starved *snf1* cells were supplemented with glucose (Fig. 7B and D). These results are consistent with the suggestion that the Snf1p pathway is not responsible for cytoplasmic accumulation of the nuclear tRNA export receptors. Moreover, the data suggest that a mechanism becomes activated to block nuclear reimport of the nuclear tRNA export receptors when glucose is limiting.

Nuclear tRNA export and nuclear reimport of the nuclear tRNA export receptors are not affected in glucose-deprived cells with constitutively active PKA. The PKA pathway is another

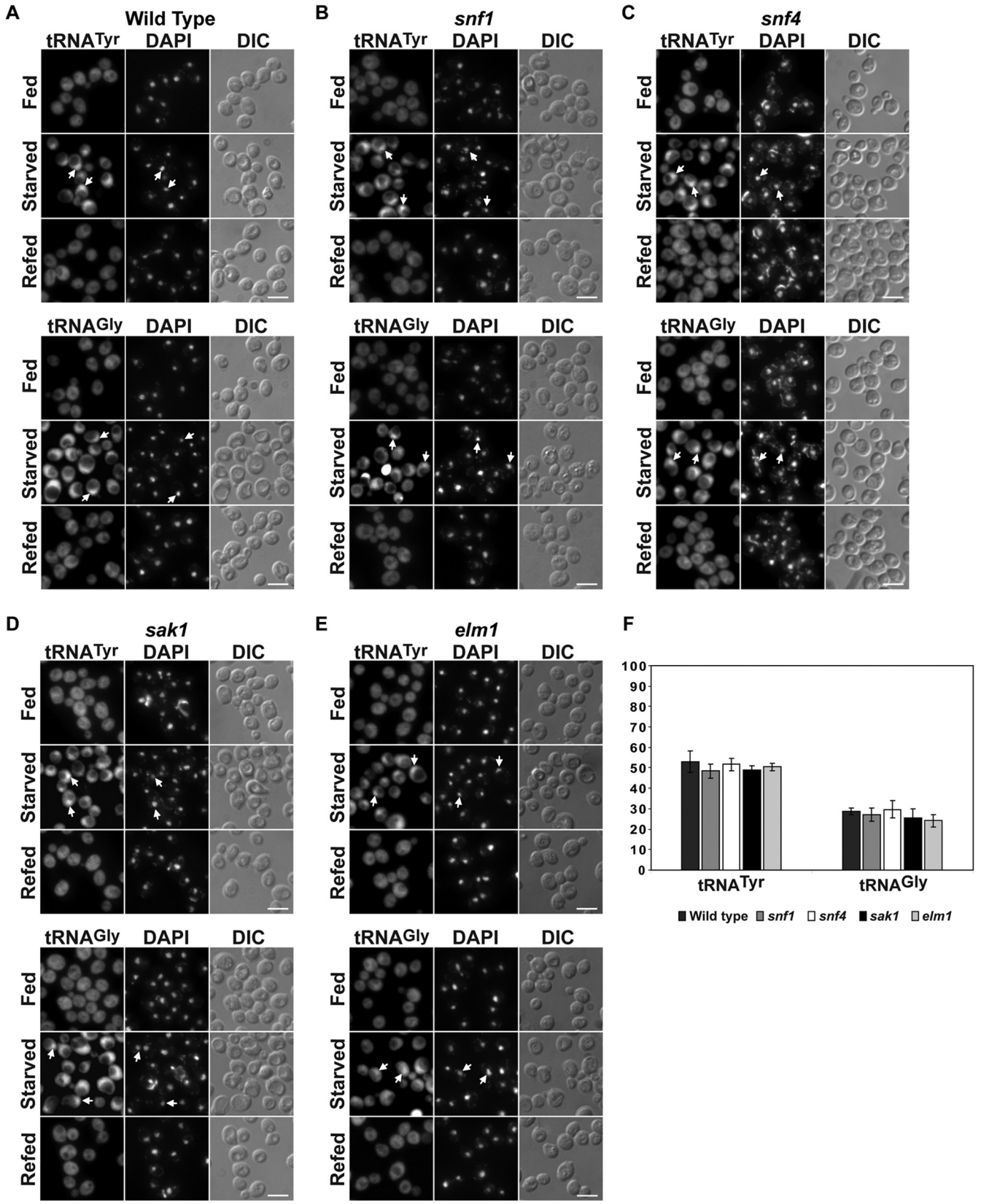

FIG 6 Disruption of the Snf1p signaling pathway does not alleviate nuclear retention of tRNA during glucose deprivation. (A to E) The wild-type strain (A) and the *snf1* (B), *snf4* (C), *sak1* (D), and *elm1* (E) mutant strains were grown in CS medium to mid-logarithmic phase (Fed) and starved of glucose for 60 min in CS lacking glucose (Starved). The starved cells were then provided with glucose at a final concentration of 2% and incubated for 20 min (Refed). The cellular locations of $tRNA^{Tyr}$ and $tRNA^{Gly}$ were monitored by FISH. DNA was visualized by DAPI staining. The arrows indicate cells showing nuclear accumulation of tRNA. Scale bars, 5 μm. (F) The percentage of cells from 3 independent fields of approximately 200 cells displaying nuclear accumulation of $tRNA^{Tyr}$ and $tRNA^{Gly}$ after glucose starvation for 60 min. Statistical analyses were performed using Student's two-tailed *t* test, but no statistically significant differences were found. The error bars indicate standard deviations.

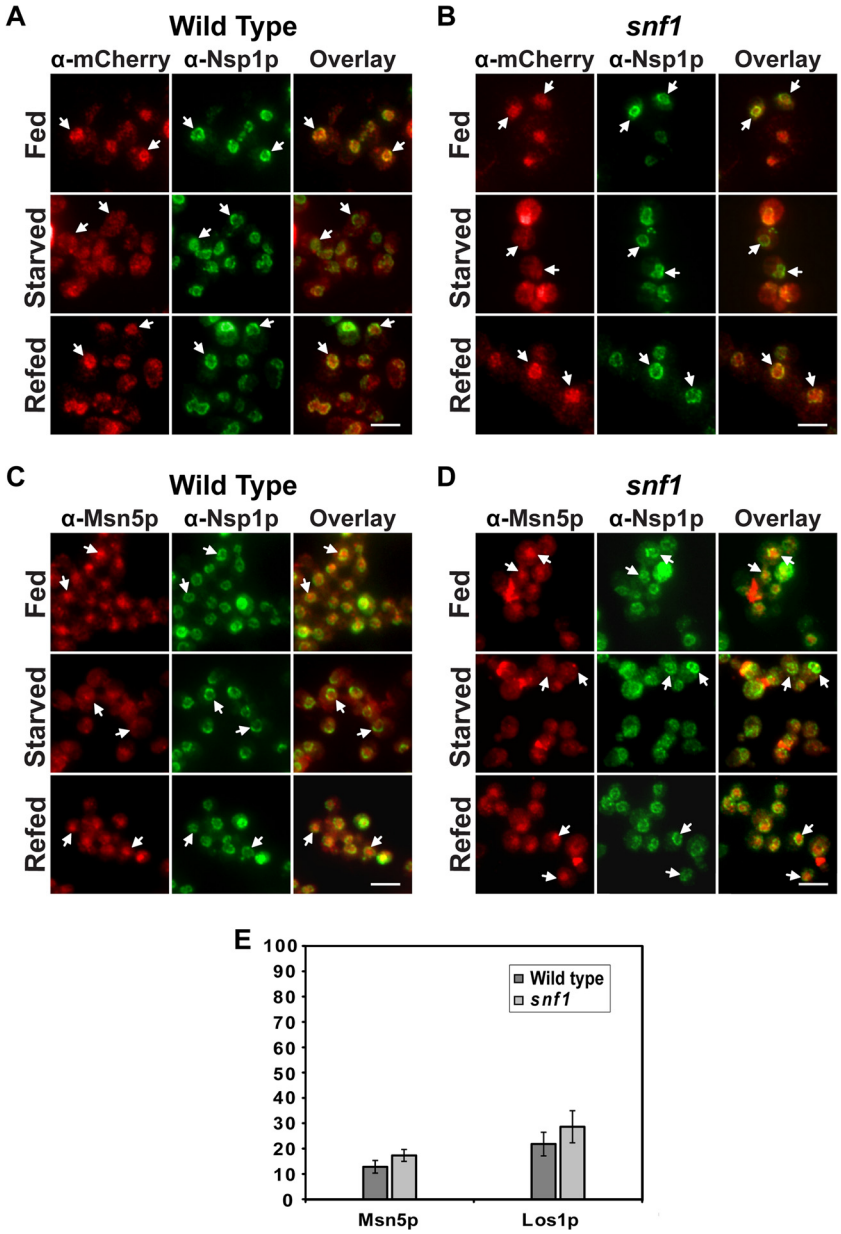

FIG 7 Loss of the function of Snf1p does not restore nuclear localization of the nuclear tRNA export receptors during glucose stress. (A to D) The wild-type and *snf1* strains with and without pCEN-URA-LOS1-mCherry were grown in CS medium lacking uracil to mid-logarithmic phase (Fed) and starved of glucose for 60 min (Starved). The starved cells were provided with glucose at a final concentration of 2% and incubated for 20 min (Refed). The cellular locations of Los1p (A and B), Msn5p (C and D), and Nsp1p (A to D) were monitored by immunofluorescence microscopy. The arrows indicate the locations of Los1p, Msn5p, and Nsp1p. Scale bars, 5 μm. (E) The percentages of cells showing normal localization of the tRNA export receptors after 60 min of glucose starvation were determined from 3 independent fields of ~200 cells. The error bars indicate standard deviations.

pathway that plays a role in the cellular response to glucose availability. In contrast to the Snf1p pathway, the PKA pathway is turned on when glucose is available and turned off when glucose is limiting (3). Thus, it is possible that the return of the nuclear tRNA export receptors is facilitated by a mechanism that involves PKA when glucose is available, which then becomes inactive when glucose is limiting and the PKA pathway is turned off. Ras2p is a G protein that is required in the cascade involved in turning on PKA activity in the presence of glucose. When glucose is available, Ras2p in its GTP-bound state stimulates the activity of the adenylate cyclase Cyr1p, subsequently producing cAMP (56). cAMP binds to the PKA regulatory subunit Bcy1p, causing Bcy1p to release the three partially redundant PKA catalytic subunits, Tpk1p, Tpk2p, and Tpk3p, resulting in activation of PKA activity (11, 12, 57). Previous studies have shown that expression of a constitutively active Ras2p mutant protein, $Ras2p^{Val19}$, in wild-type cells results in constitutive activation of PKA in the absence of glucose (58). Thus, to investigate the involvement of the PKA pathway in controlling nuclear reimport of the receptors and nuclear tRNA export, we first investigated whether nuclear-cytoplasmic traffick-

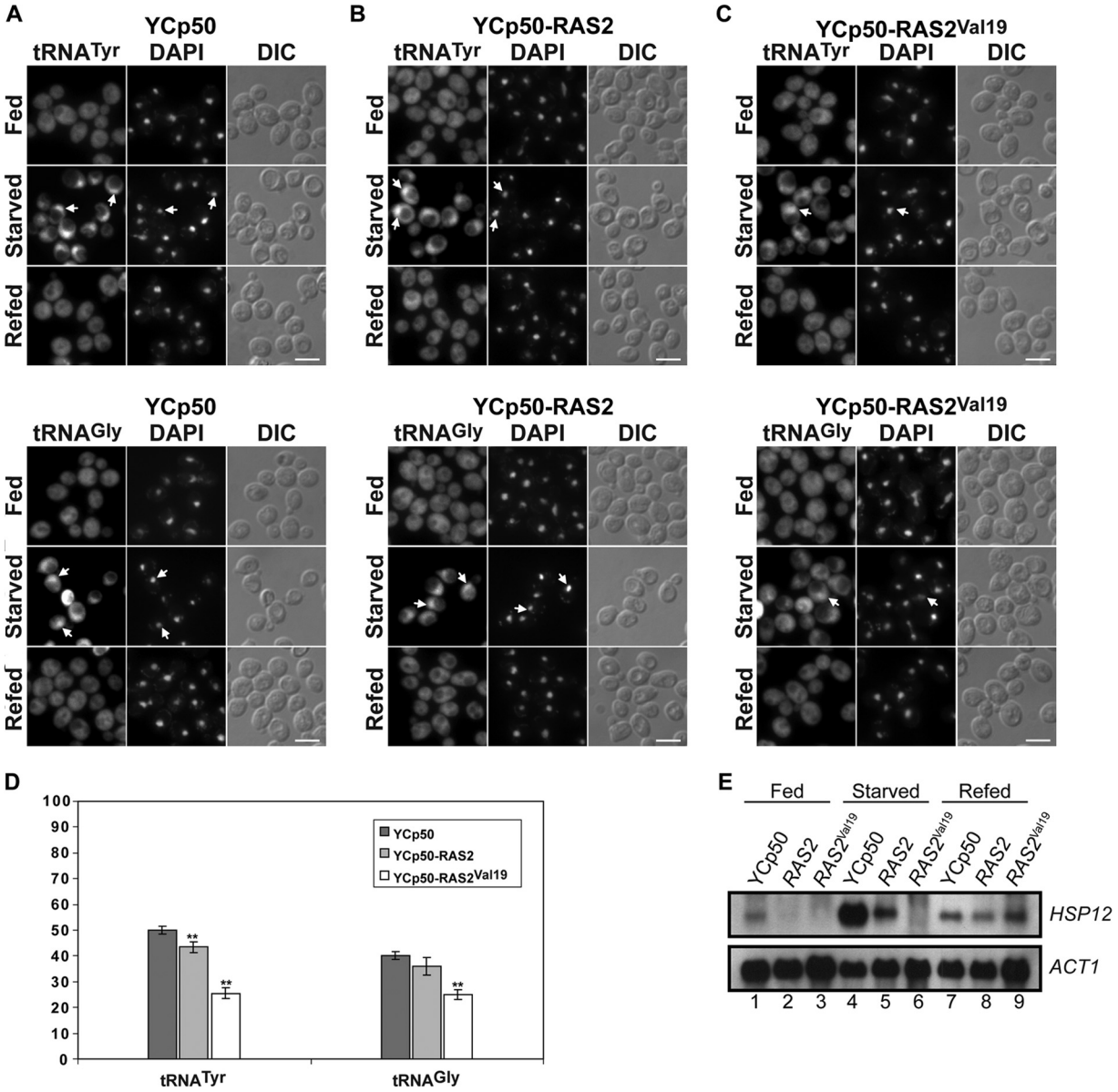

FIG 8 Activation of PKA by expression of constitutively active Rasp2p^{Val19} alleviated nuclear accumulation of tRNA during glucose deprivation. (A to D) Cells transformed with the empty vector YCp50 (A) or YCp50 containing the wild-type *RAS2* gene (B) or the *RAS2*^{Val19} mutant gene (C) were grown in CS medium lacking uracil and containing 2% glucose until mid-logarithmic phase (Fed) before being incubated in medium lacking glucose for 60 min (Starved). The starved cells were resupplemented with glucose at a final concentration of 2% and incubated for 20 min (Refed). The cellular locations of tRNA^{Tyr} and tRNA^{Gly} were monitored by FISH. The arrows indicate cells showing nuclear accumulation of tRNA. Scale bars, 5 μm. (D) The percentages of cells showing nuclear accumulation of tRNA^{Tyr} and tRNA^{Gly} after 60 min of glucose depletion were determined from 3 independent fields of ~200 cells. Results from Student's two-tailed *t* test and standard deviations are shown. **, $P < 0.005$. (E) Northern blot analysis of the expression of *HSP12* was performed on total RNA isolated from samples taken from each strain under the indicated conditions (Fed, Starved, and Refed) to monitor activation of PKA. The actin mRNA level was used to monitor the amount of RNA analyzed.

ing of tRNA is affected when the PKA pathway remained active during glucose deprivation by expression of Ras2p^{Val19} in wild-type cells (Fig. 8A to C).

To confirm that PKA is turned on when Ras2p^{Val19} is present, Northern blot analysis was used to monitor transcription of *HSP12*, which is negatively regulated by PKA (59). Analyses of total RNA (Fig. 8E, top) indicated that transcription of *HSP12* is repressed in glucose-grown wild-type cells harboring the YCp50 plasmid alone (lane 1) or YCp50 carrying the *RAS2* (lane 2) or the *RAS2*^{Val19} (lane 3) gene. In glucose-deprived wild-type cells with the YCp50 plasmid (lane 4) or YCp50 carrying *RAS2* (lane 5), *HSP12* expression was observed, indicating that PKA was turned off. In contrast, transcription of *HSP12* was not detected in glucose-deprived cells expressing the Ras2p^{Val19} mutant protein (lane 6), showing that PKA was turned on in the absence of glucose. The level of transcription of *HSP12* was significantly reduced in starved wild-type cells with YCp50 (lane 7) or YCp50-RAS2 (lane 8) exposed to glucose for 20 min. A low level of *HSP12* mRNA was

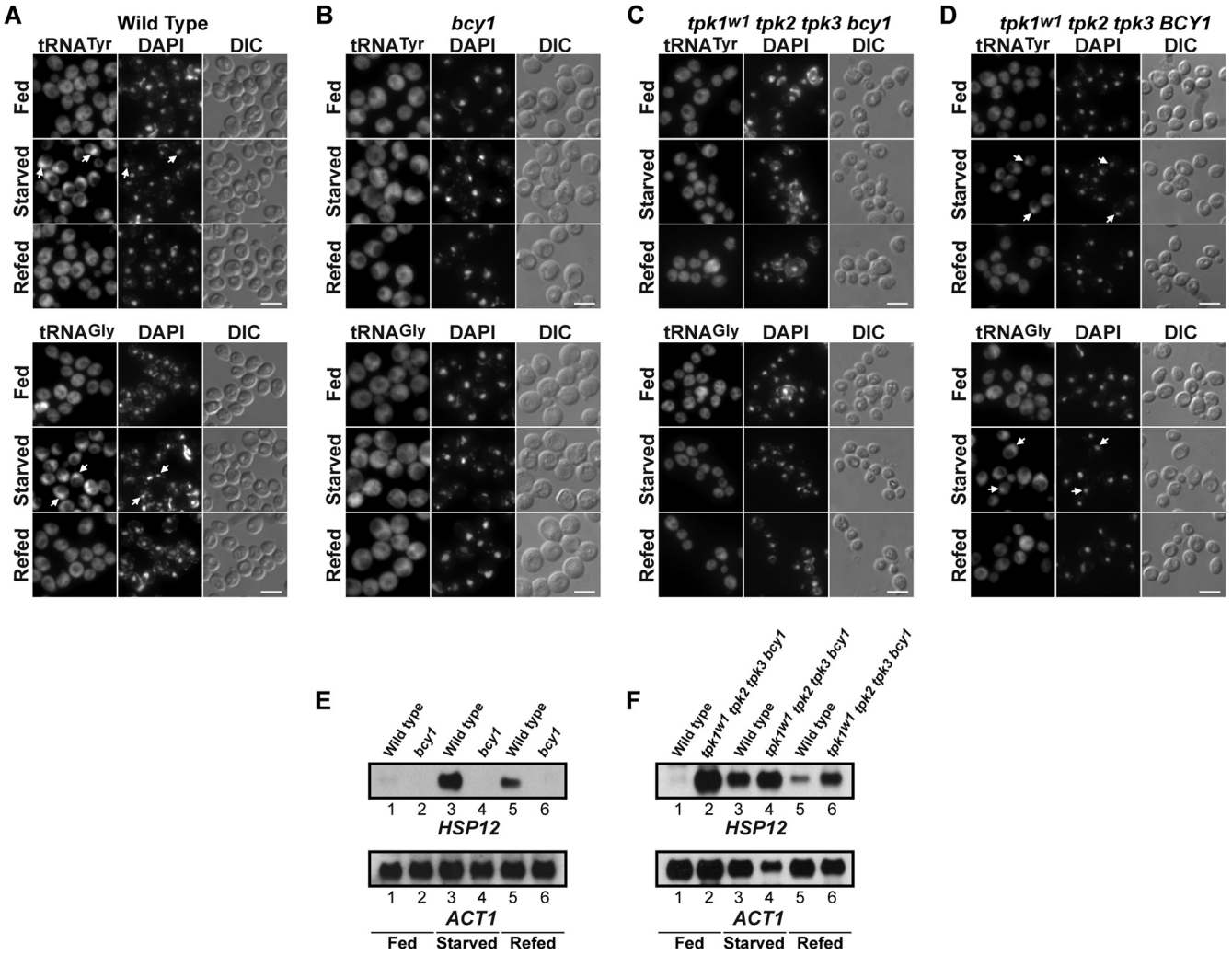

FIG 9 tRNA does not accumulate in the nucleus after glucose starvation when PKA is constitutively active. (A to D) Exponentially growing wild-type (A), *bcy1* (B), *tpk1*^{w1} *tpk2 tpk3 bcy1* (C), and *tpk1*^{w1} *tpk2 tpk3 BCY1* (D) strains were grown in CS medium (Fed) before being starved of glucose for 60 min (Starved). Glucose was added to the starved cells at a final concentration of 2% with incubation for an additional 20 min (Refed). FISH was used to monitor the cellular locations of mature tRNA^{Tyr} and tRNA^{Gly}. Nuclei displaying tRNA accumulation are indicated by arrows. Scale bars, 5 μm. (E and F) Total RNA was isolated from fed, glucose-starved, and refed starved cells, and Northern blot analyses were used to determine the expression of *HSP12* in the *bcy1* (E) and *tpk1*^{w1} *tpk2 tpk3 bcy1* (F) mutant strains and in the wild-type strain (E and F). *ACT1* mRNA was used as a loading control.

detected in starved YCp50-RAS2^{Val19} cells refed with glucose (lane 9). Transcription of *ACT1* indicated that approximately the same amount of total mRNA was used for the analyses (Fig. 8E, bottom).

As observed previously, nuclear export of tRNA^{Tyr} (Fig. 8A to C, top) and tRNA^{Gly} (bottom) was not affected in glucose-fed cells with YCp50 (Fig. 8A) and in cells expressing wild-type Ras2p (Fig. 8B) or the Ras2p^{Val19} mutant (Fig. 8C). However, during glucose deprivation, approximately 50% of cells with YCp50 retained tRNA^{Tyr} and 40% retained tRNA^{Gly} in the nucleus, while 43% of glucose-starved cells expressing wild-type Ras2p accumulated tRNA^{Tyr} and 36% retained tRNA^{Gly} in the nucleus (Fig. 8D). In contrast, only 25% of cells expressing the constitutively active Ras2p^{Val19} mutant protein accumulated a large amount of tRNA^{Tyr} and tRNA^{Gly} in the nucleus during glucose deprivation (Fig. 8D). While in some glucose-starved cells expressing Ras2p^{Val19} the majority of the tRNAs are in the cytoplasm, a small amount of the tRNAs could be detected in the nucleus. These findings suggest that the PKA signaling pathway may be responsible for regulating nuclear tRNA export by facilitating proper relocalization of the nuclear tRNA export receptors to the nucleus after a round of tRNA export to the cytoplasm.

Previous studies have shown that loss of the function of Bcy1p results in PKA being constitutively active in the absence of glucose (59). To test further that the PKA signaling pathway is involved in controlling nuclear tRNA export and the localization of the tRNA export receptors in response to glucose availability, nuclear-cytoplasmic tRNA trafficking (Fig. 9) was monitored in a *bcy1* mutant strain (Fig. 9B), a *bcy1* mutant strain lacking functional Tpk2p and Tpk3p but producing Tpk1p with reduced activity (*tpk1*^{w1} *tpk2 tpk3 bcy1*) (Fig. 9C), and a *tpk1*^{w1} *tpk2 tpk3* mutant strain with wild-type *BCY1* (Fig. 9D). Northern blot analyses indicated that *HSP12* (Fig. 9E, top) is not expressed in the glucose-fed (lane 2), starved (lane 4), and refed (lane 6) *bcy1* mutant strain. *HSP12* is

also not expressed in the isogenic wild-type strain fed with glucose (lane 1) but is expressed when the cells are starved of glucose (lane 3). The level of *HSP12* in starved wild-type cells refed with glucose (lane 5) was significantly reduced compared to that in glucose-starved cells. Consistent with the studies reported, the *tpk1^{w1} tpk2 tpk3 bcy1* strain expressed *HSP12* in the presence (Fig. 9F, lane 2) and absence (lane 4) of glucose, suggesting that the attenuated activity of Tpk1p is unable to repress transcription of *HSP12* in the presence of glucose (59). Nevertheless, these findings indicate that PKA is active in the *bcy1* mutant strain irrespective of whether glucose is present or absent. Deprivation of *BCY1* (Fig. 9A) and *tpk1^{w1} tpk2 tpk3 BCY1* (Fig. 9D) cells of glucose results in accumulation of tRNA^{Tyr} (top) and tRNA^{Gly} (bottom) in the nucleus, and nuclear export of both tRNAs is restored when the starved cells are provided with glucose for 20 min. Like wild-type and *tpk1^{w1} tpk2 tpk3 BCY1* cells, nuclear retention of tRNA^{Tyr} and tRNA^{Gly} was not observed in glucose-fed *bcy1* (Fig. 9B) and *tpk1^{w1} tpk2 tpk3 bcy1* (Fig. 9C) cells. However, in contrast to wild-type and *tpk1^{w1} tpk2 tpk3 BCY1* cells, tRNA^{Tyr} and tRNA^{Gly} were detected primarily in the cytoplasm of *bcy1* (Fig. 9B) and *tpk1^{w1} tpk2 tpk3 bcy1* (Fig. 9C) cells starved of glucose for 60 min, and distribution of the tRNAs remained unchanged when the starved mutant cells were provided with glucose. Thus, despite the inability of the *tpk1^{w1} tpk2 tpk3 bcy1* mutant strain to repress transcription of *HSP12* in the presence of glucose, the low catalytic activity of Tpk1p was able to alleviate nuclear retention of the tRNAs in response to glucose deprivation.

Given that nuclear tRNA export was not affected in the *bcy1* and *tpk1^{w1} tpk2 tpk3 bcy1* mutant strains deprived of glucose, we investigated whether this was due to proper localization of the nuclear tRNA export receptors to the nucleus (Fig. 10). As expected, Los1p is localized to the NPC in the wild-type (Fig. 10A), *bcy1* (Fig. 10B), and *tpk1^{w1} tpk2 tpk3 bcy1* (Fig. 10C) strains fed with glucose, based on colocalization analysis with the nucleoporin Nsp1p. Interestingly, while Msn5p was found to be localized to the nucleus in wild-type (Fig. 10D) and *tpk1^{w1} tpk2 tpk3 bcy1* cells (Fig. 10F), Msn5p was observed at the NPC in the *bcy1* strain (Fig. 10E). Los1p and Msn5p were retained in the cytoplasm in approximately 80% of wild-type cells starved of glucose for 60 min (Fig. 10G), and their localization to the NPC and nucleus, respectively, was restored upon readdition of glucose for 20 min. In contrast, the localization of Los1p and Msn5p did not change significantly in response to glucose deprivation (Fig. 10G) or resupplementation with glucose in both the *bcy1* and *tpk1^{w1} tpk2 tpk3 bcy1* strains, demonstrating that nuclear reimport of the tRNA export receptors is not blocked in the absence of glucose when PKA is constitutively active.

A PKA-independent mechanism plays a role in controlling nuclear tRNA export in response to glucose availability, but not by regulating nuclear reimport of the nuclear tRNA export receptors. Previous studies have shown that inactivation of the three catalytic subunits of PKA severely affects the ability of cells to grow when glucose is available (60). Interestingly, loss of the functions of Msn2p and Msn4p, two transcription factors required for expression of stress response genes when PKA is inactivated during low glucose availability, restored the ability of cells depleted of the three PKA catalytic subunits to grow and divide in the presence of glucose (60). This finding suggests that the efficiency of nuclear tRNA export may not be significantly affected in the quintuple-mutant strain. More importantly, the ability of the mutant strain to grow and divide also suggests that instead of PKA, a PKA-independent mechanism may be involved in regulating nuclear tRNA export by controlling nuclear reimport of the tRNA export receptors. Like the PKA pathway, the function of this mechanism may also be regulated by the cAMP level, which is dictated by glucose availability.

To assess the impact of the loss of the three catalytic subunits on nuclear tRNA export, tRNA localization in the *msn2 msn4* strain was compared to that in a well-characterized *msn2 msn4 tpk1 tpk2 tpk3* mutant strain (Fig. 11) (60). FISH analyses showed that both tRNA^{Tyr} (Fig. 11, top) and tRNA^{Gly} (bottom) were located primarily in the cytoplasm of the *msn2 msn4* (Fig. 11A) and *msn2 msn4 tpk1 tpk2 tpk3* (Fig. 11B) mutant strains fed with glucose. However, in contrast to glucose-fed *msn2 msn4* cells, approximately 9% of *msn2 msn4 tpk1 tpk2 tpk3* cells fed with glucose retained both tRNAs in the nucleus (Fig. 11C). These data show that nuclear tRNA export is not dramatically affected by the loss of PKA.

To test whether this PKA-independent mechanism is regulated by glucose availability, tRNA localization was monitored in the mutant strains deprived of glucose. Like wild-type cells, glucose starvation of *msn2 msn4* (Fig. 11A) and *msn2 msn4 tpk1 tpk2 tpk3* (Fig. 11B) resulted in nuclear accumulation of tRNA^{Tyr} (Fig. 11A and B, top) and tRNA^{Gly} (bottom). Approximately 60% of glucose-starved *msn2 msn4* and *msn2 msn4 tpk1 tpk2 tpk3* cells retained tRNA^{Tyr} in the nucleus, and approximately 32% of the two mutant strains showed nuclear accumulation of tRNA^{Gly} (Fig. 11C). Furthermore, both tRNAs redistributed to the cytoplasm when glucose-starved *msn2 msn4* (Fig. 11A) and *msn2 msn4 tpk1 tpk2 tpk3* (Fig. 11B) cells were refed with glucose. The finding that nuclear tRNA export was blocked in both mutant strains when starved of glucose suggests that Msn2p and Msn4p are most likely not involved in repressing expression of a stress response gene that inhibits nuclear tRNA export when the glucose level is low. In addition, the data suggest that the PKA-independent mechanism is also subjected to regulation by glucose availability, most likely by the cAMP level.

To test whether the PKA-independent mechanism regulates nuclear reimport of the nuclear tRNA export receptors in response to glucose availability, the localization of Msn5p was investigated in *msn2 msn4* (Fig. 12A) and *msn2 msn4 tpk1 tpk2 tpk3* (Fig. 12B) strains by immunofluorescence microscopy. The nuclear envelope was visualized by monitoring the location of the nucleoporin Nsp1p. Msn5p was found in the nuclei of approximately 89% of *msn2 msn4* cells and approximately 69% of *msn2 msn4 tpk1 tpk2 tpk3* cells fed with glucose (Fig. 12C). However, localization of Msn5p in the nucleus was found in only about 24% of *msn2 msn4* cells starved of glucose. A block in nuclear reimport of Los1p was also detected in *msn2 msn4* cells deprived of glucose (data not shown). Proper localization of Msn5p (Fig. 12A) and Los1p (data not shown) to the nucleus was reestablished when starved *msn2 mns4* cells were refed with glucose. In contrast to *msn2 msn4*, Msn5p was detected in the nuclei of approximately 83% of glucose-starved *msn2 msn4 tpk1 tpk2 tpk3* cells (Fig. 12C). In addition, nuclear localization of Los1p was not significantly affected in the quintuple-mutant strain starved of glucose (data not shown). These findings suggest that the PKA-independent mechanism may not be involved in regulating nuclear reimport of the nuclear tRNA export receptors in response to glucose availability. Taken together, the data suggest that PKA is part of a

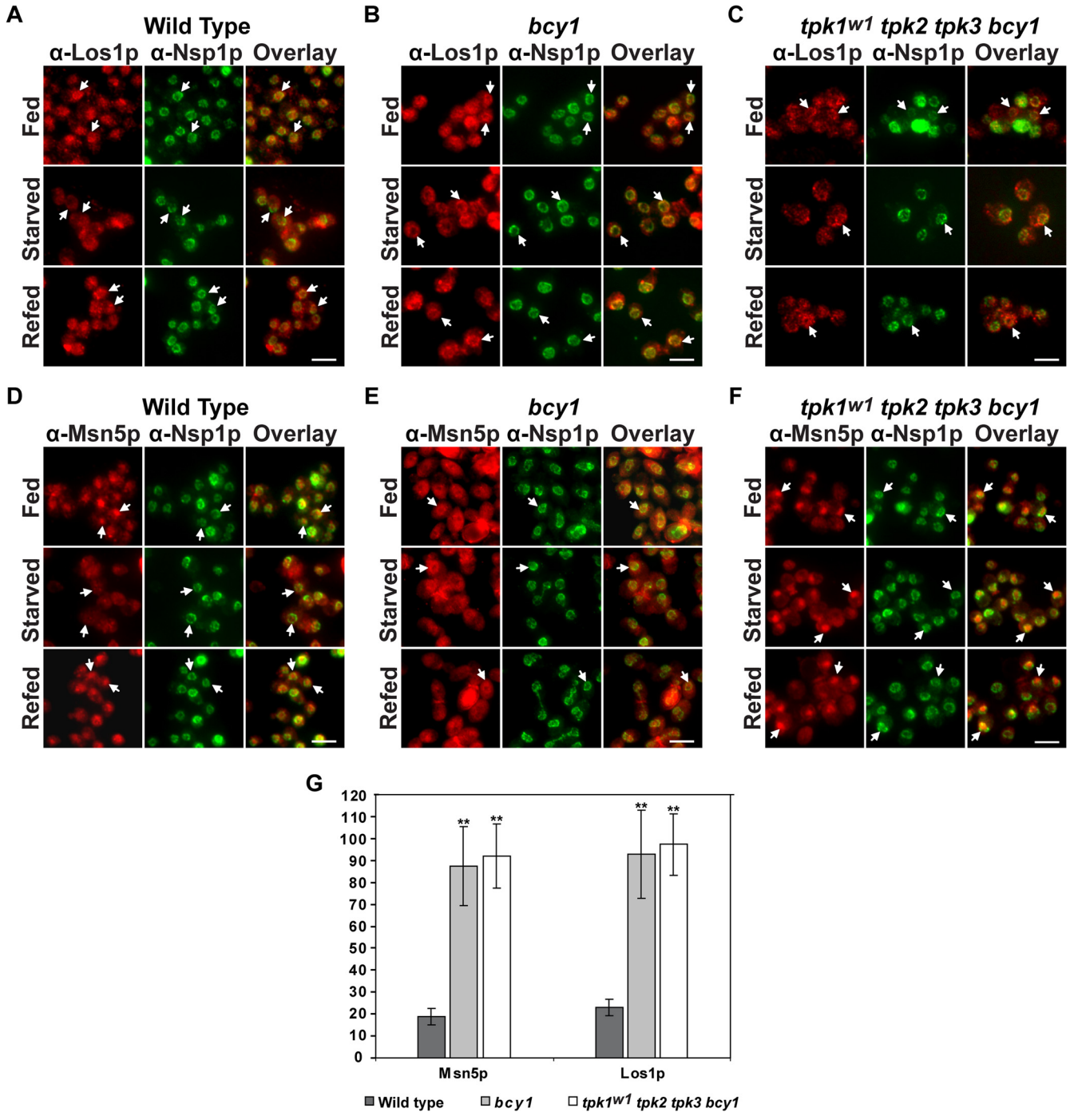

FIG 10 The tRNA export receptors Los1p and Msn5p are retained in the cytoplasm in the wild-type strain during glucose starvation, but not when PKA is constitutively active. (A to C) The wild-type and mutant strains were grown in CS medium (Fed) to an OD_{600} of ~0.6 and incubated in medium lacking glucose for 60 min (Starved). Following starvation, glucose was added at a 2% final concentration, and the cells were incubated for an additional 20 min (Refed). Immunofluorescence microscopy was performed to monitor the location of Los1p in the wild-type strain (A), the *bcy1* strain (B), and the mutant strain containing the catalytic subunit Tpk1p with attenuated activity (*tpk1*^{w1} *tpk2 tpk3 bcy1*) (C). (D to F) The locations of Msn5p in wild-type (D), *bcy1* (E), and *tpk1*^{w1} *tpk2 tpk3 bcy1* (F) cells were determined by immunofluorescence. The nuclear pore complex was visualized using antibodies to the nucleoporin Nsp1p. The arrows indicate the locations of Los1p, Msn5p, and Nsp1p. Scale bars, 5 μm. (G) Percentages of cells showing normal localization of the tRNA export receptors with respect to the percentage of fed cells with normal localization for each strain. The percentages were normalized by dividing the average percentage of cells showing normal localization after 60 min of glucose starvation by the average percentage of fed cells for each strain showing normal localization. Standard deviations were calculated by determining the square root of the sum of the squares of the fractional standard deviations and multiplying by the calculated percentage. Standard deviations and results from Student's two-tailed *t* test are shown; **, $P \leq 0.005$.

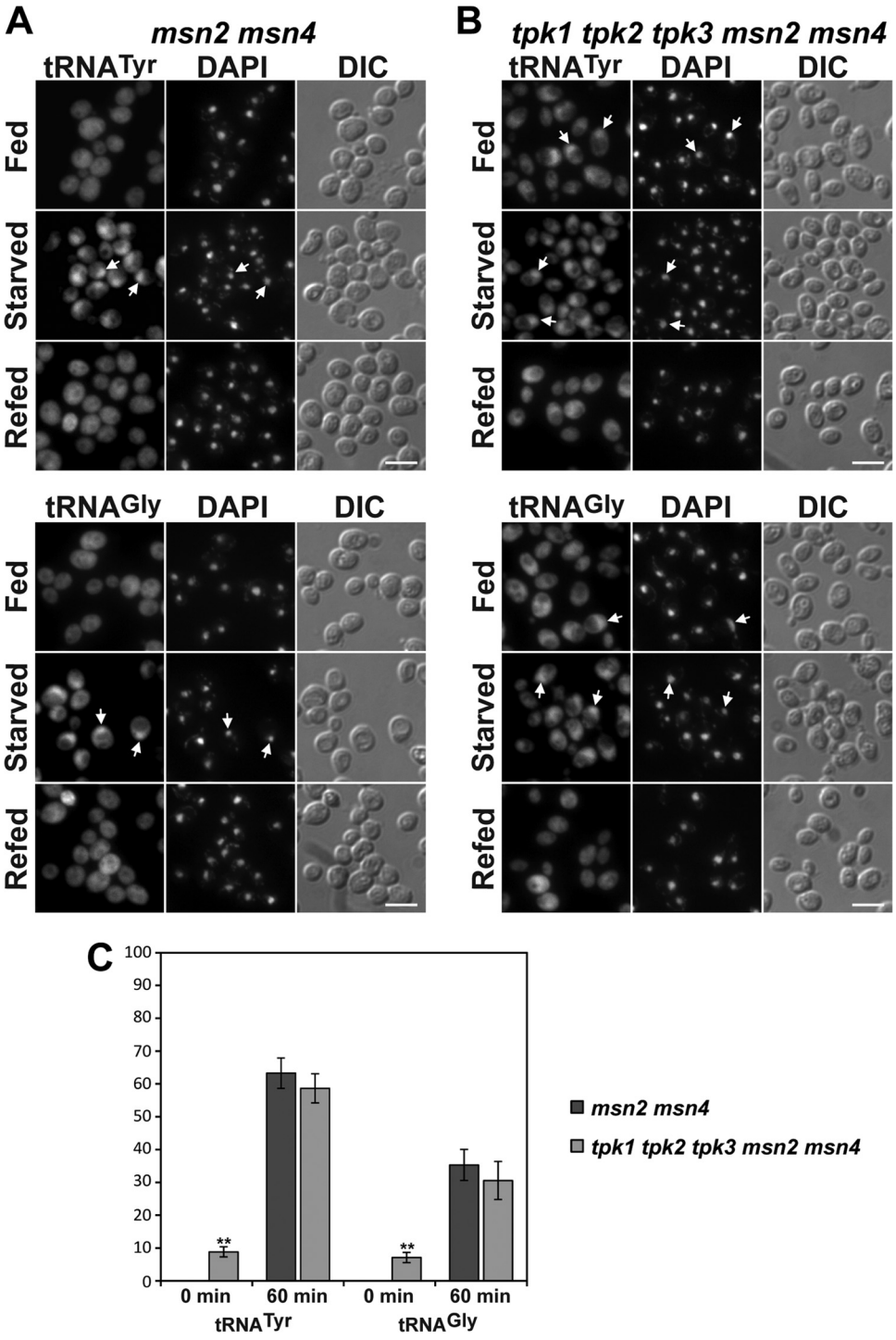

FIG 11 Nuclear tRNA export is regulated by a PKA-independent mechanism. (A and B) Exponentially growing *msn2 msn4* (A) and *tpk1 tpk2 tpk3 msn2 msn4* (B) strains were grown in CS medium (Fed) before being starved of glucose for 60 min (Starved). Glucose was added to the starved cells at a final concentration of 2% with incubation for an additional 20 min (Refed). FISH was used to monitor the cellular locations of mature $tRNA^{Tyr}$ and $tRNA^{Gly}$. Nuclei displaying tRNA accumulation are indicated by arrows. Scale bars, 5 μm. (C) Percentages of cells from 3 independent fields of ~200 cells displaying nuclear accumulation of $tRNA^{Tyr}$ and $tRNA^{Gly}$ before and after glucose starvation. Standard deviations and results from Student's two-tailed *t* test are shown, **, $P < 0.005$.

mechanism that controls nuclear tRNA export in wild-type cells by regulating nuclear reimport of the tRNA export receptors Los1p and Msn5p in response to glucose availability. It is possible that return of the nuclear tRNA export receptors to the nucleus is facilitated by a mechanism that involves PKA when glucose is available, which then becomes inactive when glucose is limiting and the PKA pathway is turned off. Alternatively, when glucose is abundant, PKA could be inhibiting a mechanism that prevents

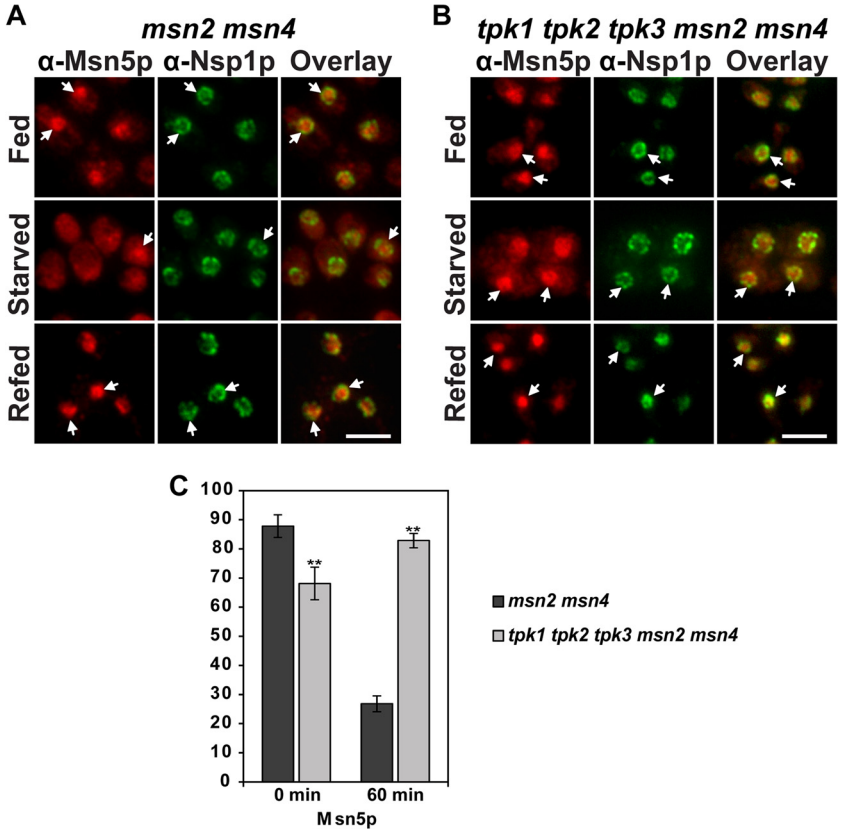

FIG 12 The PKA-independent pathway does not regulate nuclear reimport of the nuclear tRNA export receptors. (A and B) The *msn2 msn4* (A) and *tpk1 tpk2 tpk3 msn2 msn4* (B) yeast strains were grown in CS medium (Fed) to an OD_{600} of ~0.6 before being starved of glucose for 60 min (Starved). The starved cells were provided with glucose at a final concentration of 2% and incubated for an additional 20 min (Refed). Immunofluorescence microscopy was performed to monitor the locations of Msn5p and Nsp1p. The arrows indicate cells displaying nuclear localization of Msn5p. Scale bars, 5 μm. (C) Percentages of cells from 3 independent fields of ~200 cells showing nuclear localization of Msn5p after 60 min of glucose starvation. Standard deviations and results from Student's two-tailed *t* test are shown. **, $P < 0.005$.

nuclear reimport of the nuclear tRNA export receptors when glucose is not available.

Nuclear retention of tRNAs in the absence of glucose is not due to the cells entering the stationary phase, G_0. When glucose is available, PKA phosphorylates the Rim15p protein kinase, which results in Rim15p being sequestered to the cytoplasm by its interaction with the 14-3-3 protein Bmh1/2 (13, 61). Consequently, the cells are able to grow and divide. However, when cells are starved of glucose, Rim15p becomes dephosphorylated, dissociates from the 14-3-3 protein, and translocates to the nucleus. Rim15p in the nucleus activates the transcription factors responsible for expression of stress response genes, and the cells enter the stationary phase (G_0) (13, 61). However, loss of the function of Rim15p prevents the cells from entering G_0 during glucose deprivation. It is therefore possible that the nuclear tRNA export process is inhibited because the cells have entered G_0 during glucose deprivation. To exclude this possibility, we tested whether nuclear tRNA export is affected in a *rim15* mutant strain starved of glucose. For this analysis, wild-type and *rim15* cells were synchronized by treatment with 15 μg/ml nocodazole for 180 min and allowed to grow for an additional 120 min after being released from cell cycle arrest by removal of the nocodazole. The cells were then starved of glucose for 60 min, and the cellular location of tRNA was monitored by FISH (Fig. 13A). Both the wild-type and *rim15* strains synchronized with nocodazole showed uniform distribution of $tRNA^{Tyr}$ (Fig. 13A, top) and $tRNA^{Gly}$ (Fig. 13A, bottom) in the presence of glucose before and after glucose deprivation. Furthermore, nuclear retention of the tRNAs was observed in synchronized and nonsynchronized wild-type and *rim15* cells starved of glucose. The percentages of synchronized and nonsynchronized wild-type and *rim15* cells showing nuclear tRNA retention were about the same (Fig. 13B). As observed previously, the percentages of starved wild-type and *rim15* cells showing nuclear retention of $tRNA^{Gly}$ was lower than that of cells exhibiting nuclear accumulation of $tRNA^{Tyr}$ (Fig. 13B). These data suggest that blocking the cells from entering the stationary phase by depletion of Rim15p did not prevent inhibition of nuclear tRNA export. Thus, it is unlikely that inhibition of nuclear tRNA export and cytoplasmic retention of the nuclear tRNA export receptors during glucose deprivation are caused by the cells entering the stationary phase.

PKA does not appear to regulate nuclear reimport of the nuclear tRNA export receptors by direct phosphorylation of the receptors. A mechanism by which PKA could facilitate localization of the tRNA export receptors to the nucleus is by phosphorylation of the proteins directly when glucose is available. Alternatively, loss of PKA function when glucose is absent could lead to activation of a kinase that phosphorylates the receptors to block

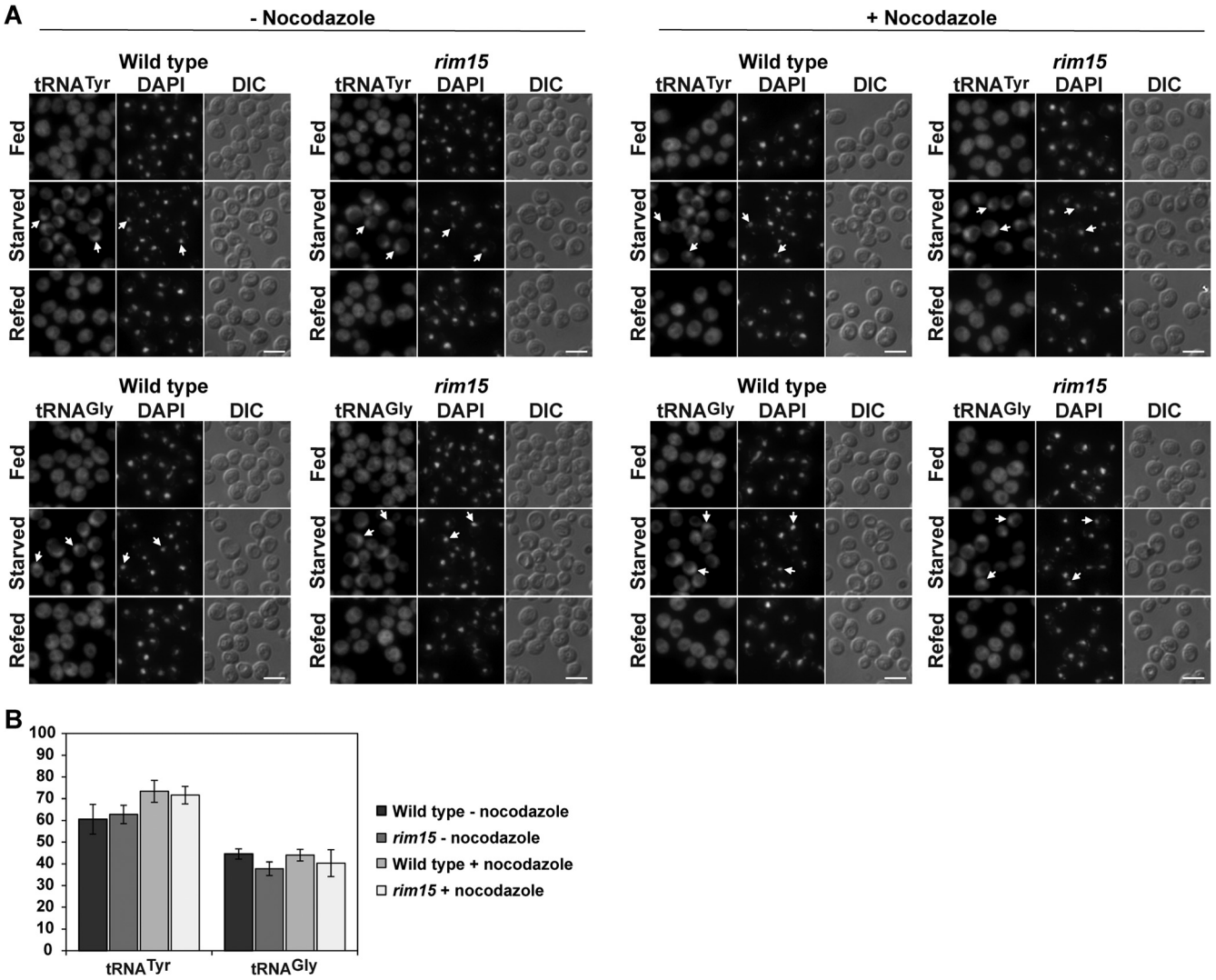

FIG 13 Nuclear accumulation of tRNA during glucose deprivation is not caused by cells entering the stationary phase. (A) The wild-type and *rim15* strains were synchronized by addition of 15 μg/ml of nocodazole for 180 min and then released and allowed to grow for an additional 120 min or treated with the drug vehicle dimethyl sulfoxide (DMSO) (Fed). Following synchronization, the cells were cultured in CS medium lacking glucose for 60 min (Starved) and resupplemented with glucose at a final concentration of 2% and incubated for 20 min (Refed). The nuclear-cytoplasmic distribution of mature tRNA in wild-type and *rim15* strains was monitored by FISH. The arrows indicate nuclei showing nuclear tRNA accumulation. DNA was visualized using DAPI staining. Scale bars, 5 μm. (B) The percentages of cells showing nuclear accumulation of $tRNA^{Tyr}$ and $tRNA^{Gly}$ after 60 min of glucose depletion were determined from 3 independent fields of ~200 cells. Statistical analyses were performed using Student's two-tailed *t* test, and no statistically significant differences were found. The error bars indicate standard deviations.

translocation of the receptors to the nucleus. Recent large-scale studies aimed at identifying phosphorylated proteins in *S. cerevisiae* suggest that Los1p and Msn5p are not subjected to phosphorylation (62). Nevertheless, we investigated whether Los1p and Msn5p are phosphorylated in response to glucose availability by an alternative strategy. This approach involves isolation of phosphorylated proteins using Phos-tag-Agarose (Wako) chromatography, followed by Western blot analyses to detect whether Los1p and Msn5p are among the isolated phosphoproteins. Phos-tag binds to phosphates attached to serine, threonine, and tyrosine residues of proteins and to phosphates linked to other macromolecules and has been used extensively to detect phosphorylation of proteins from eukaryotic cells, including yeast (63). We established that this strategy isolated phosphorylated proteins from total cell extract prepared from glucose-grown cells, but not from the total cell extract treated with λ-phosphatase to dephosphorylate phosphoproteins. The vast majority of Los1p and Msn5p was detected in the fraction containing proteins that were not retained on the column. However, a small amount of both proteins was found to interact with Phos-tag-Agarose incubated with λ-phosphatase-treated and untreated cell lysates prepared from glucose-fed and -starved cells (data not shown). These findings suggest that Msn5p and Los1p retained on the Phos-tag-Agarose matrix may be due to the proteins interacting nonspecifically with the resin and that they may not be phosphorylated when cells are fed with glucose or starved of glucose. Furthermore, it was not possible to ascertain whether the two receptors were phosphorylated by colocalization Western blot analyses of total cell extract prepared

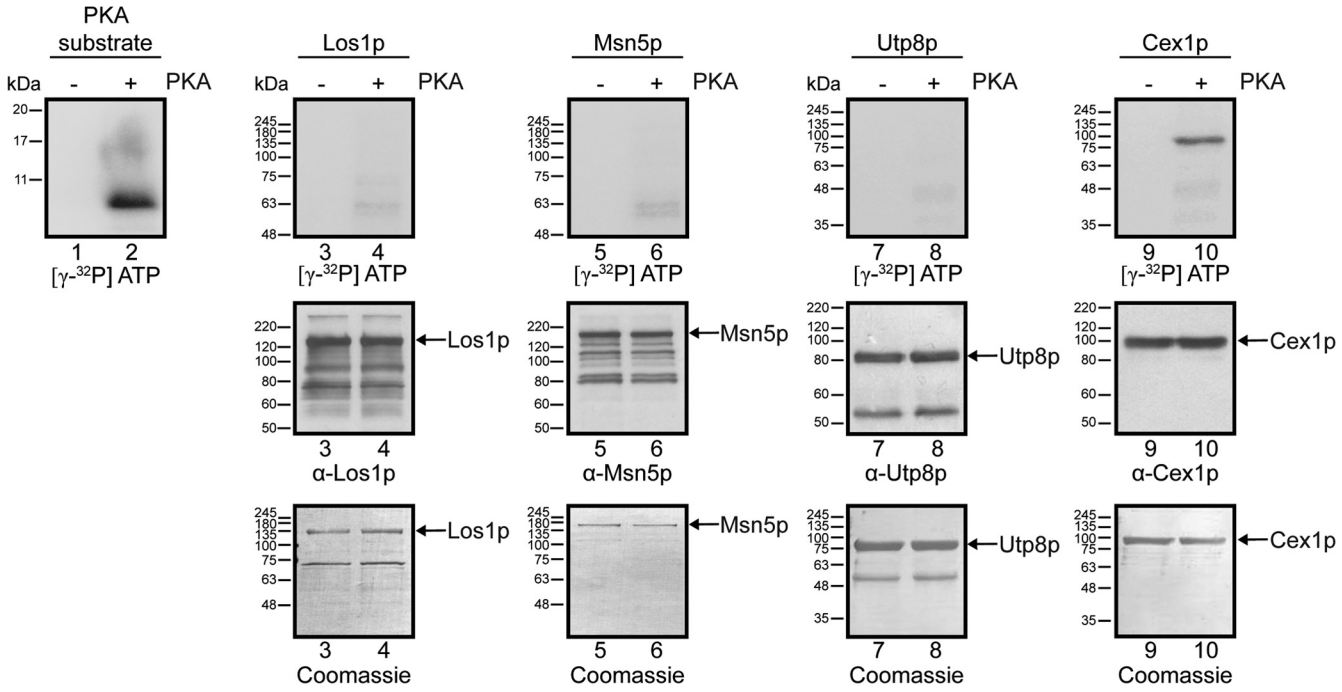

FIG 14 PKA does not phosphorylate the tRNA export receptors *in vitro*. Purified His-tagged recombinant Los1p, Msn5p, Utp8p, or Cex1p was incubated without (−) or with (+) 1 U of bovine PKA and 5 μCi of [γ-^{32}P]ATP at 25°C for 30 min in PKA phosphorylation buffer. The reaction mixtures were separated by SDS-PAGE and imaged using a phosphorimager. A peptide containing a PKA phosphorylation site was used as a positive control for the *in vitro* phosphorylation. Western blot analysis and Coomassie staining were used to verify that the same amount of each protein was used for the analysis.

from cells grown in the presence and absence of glucose using a combination of α-Los1p and commercially available α-phosphoserine or α-phosphothreonine, or α-Msn5p and α-phosphoserine or α-phosphothreonine. This was primarily due to the lack of specificity of the α-phospho-amino acid antibodies.

To test directly whether Msn5p and Los1p are substrates for PKA, we investigated whether bovine PKA phosphorylates recombinant Msn5p and Los1p *in vitro* (Fig. 14). This enzyme has been shown previously to phosphorylate recombinant yeast PKA protein substrates (64, 65). As a control, phosphorylation of Cex1p, which plays a cytoplasmic role in the nuclear tRNA export process, *in vitro* by the bovine PKA was monitored, as Cex1p was shown to be phosphorylated *in vivo* and to contain PKA phosphorylation motifs. Phosphorylation of a peptide containing a PKA phosphorylation site (PKA substrate) was monitored to confirm that the enzyme is active. Phosphorylation of Utp8p was also tested, as nuclear tRNA export was restored in glucose-starved cells overproducing the protein and it has been demonstrated to be phosphorylated *in vivo*. Phosphorylation of the PKA substrate was observed in the presence of PKA (Fig. 14, lane 2), but not when PKA was absent (lane 1), indicating that the assay can specifically detect PKA phosphorylation of a substrate. Los1p (Fig. 14, lanes 3 and 4), Msn5p (lanes 5 and 6), and Utp8p (lanes 7 and 8) phosphorylation was not detected in the presence or absence of PKA. In contrast, Cex1p (lanes 9 and 10) was found to be phosphorylated only in the presence of the PKA catalytic subunit. Western blot analysis and Coomassie blue staining showed that equal amounts of each recombinant protein were incubated in the presence or absence of PKA. These findings suggest that PKA may not regulate localization of the tRNA export receptors Los1p and Msn5p or the function of Utp8p by direct phosphorylation.

DISCUSSION

Recent studies have established that glucose deprivation causes inhibition of the nuclear tRNA export process in *S. cerevisiae* (30, 46). While the mechanism responsible for regulating nuclear tRNA export in response to the glucose level is not understood, findings from this study strongly suggest that it is most likely due to the function of the nuclear tRNA export receptors and the intranuclear tRNA chaperone Utp8p being controlled by glucose availability (Fig. 4). How the glucose level influences Utp8p function in nuclear tRNA export is not known, but evidence obtained suggests that the ability of the tRNA export receptors to function in nuclear tRNA export in response to glucose availability is most likely related to regulation of nuclear reimport of the tRNA export receptors after a round of tRNA export to the cytoplasm (Fig. 3). This conclusion is in accordance with previous studies showing cytoplasmic accumulation of several nuclear export receptors, including the nuclear tRNA export receptors, when the glucose level is low (47). Inhibition of nuclear reimport of the tRNA export receptors is not due to the cells entering stationary phase, as nuclear accumulation of tRNA was observed in glucose-deprived *rim15*, a mutant strain that is unable to enter the stationary phase when PKA is turned off under low glucose levels (Fig. 13). Furthermore, it is also unlikely that accumulation of the tRNA export receptors in the cytoplasm during glucose deprivation is due to expression of a stress response gene controlled by the transcription factors Msn2p and Msn4p, since nuclear retention of mature tRNAs and cytoplasmic accumulation of the tRNA export receptors were observed in *msn2 msn4* cells during glucose deprivation (Fig. 11). Why the tRNA export process is directly regulated when protein translation and transcription of genes, including those for

tRNAs and components of the ribosome, are downregulated during nutrient stress is not understood (1–3). A possible explanation is that the nuclear tRNA export process is regulated to conserve energy, as ATP is utilized during the tRNA aminoacylation quality assurance step in the nucleolus and GTP is consumed to facilitate unloading of the nuclear tRNA export receptors of the tRNA cargo at the cytoplasmic face of the NPC. Additionally, regulation of nuclear tRNA export may provide an additional mechanism to control the rate of protein synthesis in response to nutrient availability.

One of the causes of inhibition of nuclear tRNA export in wild-type *S. cerevisiae* cells deprived of glucose is a block in nuclear reimport of the nuclear tRNA export receptors (Fig. 3). Localization analyses of tRNA and the tRNA export receptors in mutant strains lacking an active Snf1p suggest that Snf1p is not involved in the regulatory mechanism, as both nuclear tRNA export and relocalization of the receptors to the nucleus are inhibited when the mutant strains are deprived of glucose (Fig. 6 and 7). In contrast, nuclear tRNA export and relocalization of the tRNA export receptors to the nucleus were not affected in mutant strains with constitutively active PKA during glucose deprivation (Fig. 8, 9, and 10). Taken together, these results suggest that PKA activity is required for proper relocalization of the tRNA export receptors to the nucleus and that inhibition of PKA activity during glucose deprivation leads to a block in nuclear reimport of the nuclear tRNA export receptors. Collectively, the data suggest that PKA is associated with a mechanism responsible for regulating relocalization of the receptors to the nucleus in response to glucose availability. However, it is not understood how inhibition of PKA activity during glucose deprivation leads to a block in nuclear reimport of the nuclear tRNA export receptors. Furthermore, while it is also not known why nuclear reimport of the tRNA export receptors is regulated in response to glucose availability, it is possible that controlling the amount of tRNA export receptors available for nuclear tRNA export may, in part, conserve GTP for use in metabolic processes required to survive during nutrient stress.

Interestingly, investigation of nuclear tRNA export in *msn2 msn4 tpk1 tpk2 tpk3* cells uncovered the involvement of a PKA-independent mechanism in regulating nuclear tRNA export in response to glucose availability (Fig. 11). This mechanism, however, does not play a role in regulating nuclear reimport of the nuclear tRNA export receptors, as nuclear import of the receptors was not affected when the quintuple mutant was deprived of glucose (Fig. 12). How the PKA-independent mechanism regulates nuclear tRNA export is not understood. Furthermore, while it is not known whether both PKA and the PKA-independent mechanism act together to regulate nuclear tRNA export, the finding that the major defect observed during glucose deprivation of wild-type cells is inhibition of nuclear import of the nuclear tRNA export receptors strongly suggests that the PKA-related mechanism may be primarily responsible for regulating nuclear tRNA export in response to glucose availability. However, further studies are required to understand the significance and contribution of the PKA-independent regulatory mechanism.

During the process of exporting tRNAs from the nucleus to the cytoplasm, the tRNA export receptors interact directly with a number of nucleolar and cytoplasmic components. Cex1p is a cytoplasmic component of the nuclear tRNA export process that interacts with the nuclear tRNA export receptors at the cytoplasmic face of the NPC to assist with dissociation of the receptor-tRNA-Gsp1p-GTP export complex (41, 42). Cex1p has been shown to have a PKA phosphorylation site and to be phosphorylated by PKA *in vitro* (Fig. 14). Furthermore, Cex1p has been shown by phosphoproteomic studies to be phosphorylated *in vivo* (62). Thus, it is possible that PKA inhibition results in activation of a mechanism that dephosphorylates Cex1p, leading to inhibition of the function of Cex1p in dissociation of the tRNA-receptor-Gsp1p-GTP complex. The lack of dissociation of the export complex would prevent reimport of the tRNA export receptors back to the nucleus, as it is generally thought that only nuclear export receptors free of their cargoes are allowed to reenter the nucleus. However, this is unlikely to be the case, as overexpression of Cex1p did not restore nuclear export of tRNA during glucose deprivation, whereas overexpression of the tRNA export receptors or Utp8p alleviated nuclear accumulation of tRNAs (Fig. 4).

PKA does not appear to facilitate nuclear reimport of the nuclear tRNA export receptors by phosphorylation of the receptors, as PKA did not phosphorylate Msn5p and Los1p *in vitro* (Fig. 14) and phosphoproteomic studies established that the two proteins are not phosphorylated *in vivo* (62). Thus, it is unlikely that phosphorylation/dephosphorylation of the receptors plays a role in regulation of nuclear reimport of the nuclear tRNA export receptors. It is possible, therefore, that when glucose is abundant, PKA inhibits by phosphorylation a mechanism that is responsible for cytoplasmic retention of the tRNA export receptors when the glucose level is low. For instance, phosphorylation of a cytoplasmic component prevents the component from interacting with the tRNA export receptors, allowing the receptors to traffic between the nucleus and cytoplasm, or phosphorylation of a kinase inhibits activation of a cytoplasmic component that participates in the mechanism responsible for cytoplasmic retention of the receptors. However, further studies are required to understand the mechanism by which PKA facilitates the regulation of nuclear reimport of the tRNA export receptors in response to glucose availability.

Nuclear export of tRNAs in *S. cerevisiae* involves multiple pathways and the nucleolar components Utp8p, Utp9p, and Utp22p (26, 33, 35). However, of the three Utp proteins, Utp8p is the only component of the intranuclear phase of the tRNA export process that participates in all the nuclear tRNA export pathways. Interestingly, like the nuclear tRNA export receptors, overproduction of Utp8p also improved the efficiency of nuclear export of functional tRNAs made from both intronless and intron-containing pre-tRNAs during glucose deprivation (Fig. 4). This finding suggests that Utp8p function may also be subjected to regulation in response to glucose availability. However, unlike regulation of the nuclear tRNA export receptors, regulation of the function of Utp8p is not caused by a block in nuclear import and nucleolar localization of the protein (data not shown). Furthermore, the function of Utp8p does not appear to be regulated by PKA-catalyzed phosphorylation, as Utp8p was not phosphorylated *in vitro* by PKA (Fig. 14). In addition, regulation of Utp8p function during glucose deprivation is most likely not involved in the mechanism that causes cytoplasmic accumulation of the tRNA export receptors, as depletion of Utp8p does not affect localization of the tRNA export receptors to the nucleus (data not shown). Thus, it is possible that the function of Utp8p in either translocating aminoacyl-tRNAs from the nucleolus to the nuclear tRNA export receptor or facilitating formation of the receptor-tRNA-Gsp1p com-

plex is subjected to regulation in response to glucose availability. Regulation of Utp8p function may provide an important feedback mechanism to control the steady-state rate of ATP consumption in the nuclear tRNA aminoacylation quality assurance step during glucose deprivation.

S. cerevisiae is a highly adaptable organism that uses complex mechanisms to control nuclear-cytoplasmic tRNA trafficking in response to nutrient availability. The mechanism used by *S. cerevisiae* to regulate nuclear tRNA export is also dependent on the type of nutrient stress. Previous studies have shown that amino acid or nitrogen deprivation affects nuclear export of functional tRNAs made from intron-containing precursors, but not nuclear export of functional tRNAs made from intronless pre-tRNAs (24–26). Inhibition of nuclear export of tRNAs from intron-containing pre-tRNAs during nitrogen deprivation is due to regulation of the function of the nuclear tRNA export receptors (25). The function of the tRNA export receptors is, in part, regulated by the TORC1 signaling pathway (25). However, TORC1 does not influence the function of the tRNA export receptors by controlling nuclear reimport of the receptors (25). In contrast, glucose deprivation causes inhibition of nuclear export of tRNAs made from both intronless and intron-containing precursors. Like amino acid or nitrogen deprivation, glucose depletion also affects the function of the nuclear tRNA export receptors, but the function of the tRNA export receptors in nuclear tRNA export is controlled by regulation of nuclear reimport of the receptors by a mechanism involving PKA. Moreover, in contrast to amino acid and nitrogen deprivation, glucose deprivation also results in regulation of the function of Utp8p. Thus, it is possible that coordinated regulation of the intranuclear function of Utp8p and the NPC translocation step when glucose availability is limiting facilitates rapid downregulation of the nuclear tRNA export process to conserve energy. In addition to PKA, a PKA-independent mechanism is also involved in regulating nuclear tRNA export in response to glucose availability. However, this mechanism does not regulate nuclear reimport of the tRNA export receptors. Finally, this study provides a strong foundation for future studies directed at delineating the mechanism by which PKA regulates nuclear reimport of the nuclear tRNA export receptors in response to glucose availability.

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