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Functional Analysis of the RNA Polymerase II C-terminal Domain Kinase Ctk1 in the Yeast *Saccharomyces cerevisiae*

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Erklärung

Diese Dissertation wurde im Sinne von §13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Herrn Professor Ralf-Peter Jansen betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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During this thesis, the following articles were published:

Hurt E, Luo MJ, **Röther S**, Reed R, Sträßer K. "Cotranscriptional recruitment of the serine-arginine-rich (SR)-like proteins Gbp2 and Hrb1 to nascent mRNA via the TREX complex." **PNAS** U S A. 2004 Feb 17;101(7):1858-62.

Lariviere L, Geiger S, Hoeppner S, **Röther S**, Sträßer K, Cramer P. "Structure and TBP binding of the Mediator head subcomplex Med8-Med18-Med20." **Nature Structural and Molecular Biology** 2006 Oct;13(10):895-901.

Röther S, Clausing E, Kieser A, Sträßer K. "Swt1, a novel yeast protein, functions in transcription." **Journal of Biological Chemistry** 2006 Dec 1;281(48):36518-25.

Krebs S, Medugorac I, **Röther S**, Sträßer K, Forster M. "A missense mutation in the 3-ketodihydrosphingosine reductase FVT1 as candidate causal mutation for bovine spinal muscular atrophy." **PNAS** U S A. 2007 Apr 17;104(16):6746-51.

Röther, S and Sträßer K. "The RNA polymerase II CTD kinase Ctk1 functions in translation elongation." **Genes and Development**, 2007; Jun 1; 21 (11).

This article is highlighted in the same issue of Genes and Development by a perspective written by Michael Hampsey and Terry Goss Kinzy: "Synchronicity: policing multiple aspects of gene expression by Ctk1." **Genes and Development**, 2007; Jun 1; 21 (11).

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Summary

Gene expression encompasses a multitude of different steps, starting with transcription in the nucleus, co-transcriptional processing and packaging of the mRNA into a mature mRNP, export of the mRNP through the nuclear pore and finally the translation of the message in the cytoplasm. The central coordinator for coupling of the nuclear events is the differentially phosphorylated C-terminal domain (CTD) of RNA polymerase II (RNAP II). The phosphorylation pattern of the CTD not only dictates the progression through the transcription cycle but also the recruitment of mRNA processing machineries. Coupling of transcription to mRNA export is achieved by the TREX complex, which consists in the yeast *S. cerevisiae* of the heterotetrameric THO complex important for transcription elongation, the SR-like proteins Gbp2 and Hrb1, and Tex1 and the mRNA export factors Sub2 and Yra1. By direct interaction with Yra1, the mRNA export receptor Mex67-Mtr2 is then recruited to the mRNP and transports the mRNP through the nuclear pore complex to the cytoplasm.

In a genetic screen for factors that are crucial for TREX complex function in the living cell, Ctk1, a cyclin dependent kinase (CDK) that phosphorylates the C-terminal domain (CTD) of RNAP II during transcription elongation, was identified (Hurt et al. 2004).

Surprisingly, besides the TREX components Gbp2 and Hrb1, Ctk1 co-purified ribosomal proteins and translation factors. Using sucrose density centrifugation, it could be shown that Ctk1 indeed associates with translating ribosomes *in vivo*, suggesting a novel function of this protein in translation. This assumption was confirmed by *in vitro* translation assays: loss of Ctk1 function leads to a reduction in translational activity.

More specifically, Ctk1 is important for efficient translation elongation and contributes to the accurate decoding of the message. Cells depleted for Ctk1 are more sensitive towards drugs that impair translational accuracy and show an increase in the frequency of miscoding *in vivo*. The function of Ctk1 during decoding of the message is most likely direct, as in extracts of cells depleted for Ctk1 the defect in correct decoding of the message can be restored to wild type levels by addition of purified CTDK-I complex.

An explanation for the molecular mechanism of Ctk1's function is provided by the identification of Rps2 as a novel substrate of Ctk1. Rps2 is a protein of the small ribosomal subunit, located at the mRNA entry tunnel and known to be essential for translational accuracy. Importantly, Rps2 is phosphorylated on serine 238 by Ctk1, and cells containing an *rps2-S238A* mutation show an increased sensitivity towards drugs that affect translational accuracy and an increase in miscoding as determined by *in vitro* translation extracts. The role

of Ctk1 in translation is probably conserved as CDK9, the mammalian homologue of Ctk1, also associates with polysomes.

Since Ctk1 interacts with the TREX complex, which functions at the interface of transcription and mRNA export, Ctk1 might bind to the mRNP during transcription and accompany the mRNP to the ribosomes, where Ctk1 enhances efficient and accurate translation of the mRNA. This study could be an example of a novel layer of control in gene expression: the composition of the mRNP determines its translational fate, including efficiency and accuracy of translation.

1 Introduction

Studies from many groups over recent years have revealed that several steps in gene expression previously thought to be discrete are part of a sophisticated network with a high degree of coordination, interconnection and interdependence. The tight coupling within this network encompasses chromatin remodelling and transcription, co-transcriptional maturation of the synthesized mRNA, including 5' capping, splicing, cleavage and polyadenylation, packaging of the mRNA in a mature mRNP, the export of the mRNP to the cytoplasm and finally translation of the message. Only a highly regulated interplay of these processes ensures efficient and accurate expression of the genetic information (for review see Maniatis and Reed 2002; Proudfoot et al. 2002; Reed and Hurt 2002; Cole and Scarcelli 2006).

1.1 The mRNA transcription cycle

Eukaryotic RNA polymerase II (RNAP II) is a multisubunit protein complex that transcribes the genomic information stored within the DNA into messenger RNA (mRNA). The process of transcription can be divided into initiation, transcript elongation, termination and recycling of RNAP II (for review see Prelich 2002; Svejstrup 2004; Meinhart et al. 2005). A key element in guiding the polymerase through the transcription cycle and in coupling the transcription process to mRNA processing events is the C-terminal domain (CTD) of Rpb1, the largest subunit of RNAP II. The tail-like CTD protrudes from the catalytic core of RNAP II and is flexibly located at the exit groove of the pre-mRNA. The CTD is composed of heptapeptide repeats with the consensus sequence Y_1-S_2-P_3-T_4-S_5-P_6-S_7. The number of the repeats varies species-dependent, *e.g.* 26 in the yeast *Saccharomyces cerevisiae* and 52 in human. These repeats can be heavily modified by phosphorylation on serine 2 (S2) and serine 5 (S5), allowing four possible phosphorylation states: unphosphorylated, phosphorylated at S2, phosphorylated at S5, and phosphorylated at both sites, S2 and S5.

The phosphorylation state of the CTD is a determinant for progression through the transcription cycle. The balanced action of CTD cyclin dependent kinases (CDK) and phosphatases is reflected by an initially high S5 phosphorylation level at the promoter region, which then decreases when RNAP II approaches the 3' end of the gene. To the contrary, S2 phosphorylation is low in the 5' region of the gene but increases when RNAP II progresses towards the 3' end (Komarnitsky et al. 2000; Cho et al. 2001,

Figure **1**).

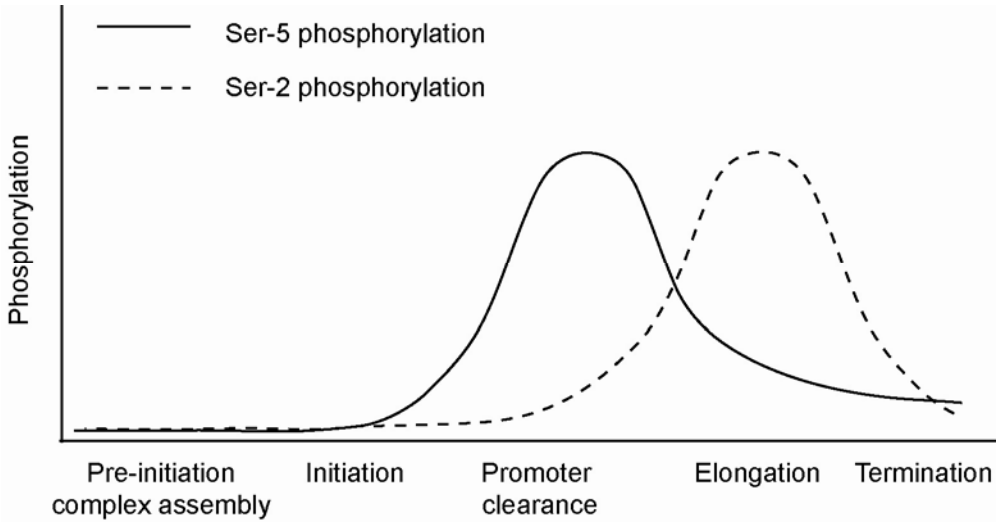

Figure 1: Phosphorylation during the transcription cycle (Svejstrup 2004).
The graph shows the level of phosphorylation at serine 2 and serine 5 of the CTD heptapeptide repeat during the transcription cycle.

For transcription initiation, hypophosphorylated RNAP II is recruited together with general transcription factors (TF) and the mediator complex, which transmits signals from activators and repressors to RNAP II, to the promoter, leading to the assembly of the preinitiation complex (PIC, for an overview see Figure 2). PIC assembly can be prevented by early phosphorylation of the hypophosphorylated CTD on S5 by the CDK subunit of the mediator, Srb10/Srb11 (CDK8/cyclin C in *H. sapiens*, Hengartner et al. 1998). However, phosphorylation of the CTD after PIC formation on S5 by the TFIIH subunit Kin28/Ccl1 (CDK7/CyclinH in *H. sapiens*) results in the transition from PIC to promoter clearance and initiation (Hengartner et al. 1998). S5 phosphorylation by Kin28 is regulated by Srb10 as this kinase can also phosphorylate the cyclin of Kin28 upon repressory signals, thus inhibiting initiation at the stage of the PIC (Akoulitchev et al. 2000). After PIC formation and phosphorylation on S5 by Kin28, mediator and general transcription factors remain partially in a scaffold at the promoter and thus allow for a rapid entry of new polymerases to transcribe the gene (Zawel et al. 1995; Svejstrup et al. 1997).

Transition from initiation to productive elongation is characterized in mammals by phosphorylation of S2 of the CTD and Spt5, a subunit of the negative transcription elongation factor DSIF. Both phosphorylation events are carried out by CDK9/cyclin T and are necessary for productive transcription elongation (Marshall and Price 1995; Marshall et al. 1996; Wada et al. 1998; Yamaguchi et al. 1998). In yeast, CDK9 has two potential homologues: Ctk1 and Bur1, which are both kinases essential for efficient transcription elongation. Bur1 forms a

CDK/cyclin pair with Bur2 (Yao et al. 2000; Keogh et al. 2003) and was shown to be important for transcriptional activation by phosphorylation of Rad6, which in turn is responsible for the H3K4 methylation by the COMPASS complex (Wood et al. 2005). Ctk1 forms together with the cyclin Ctk2 and a third protein Ctk3 (Sterner et al. 1995) the heterotrimeric CTDK-I complex, which phosphorylates S2 of the CTD of Rpb1 (Cho et al. 2001), necessary for efficient transcription elongation (Figure 2).

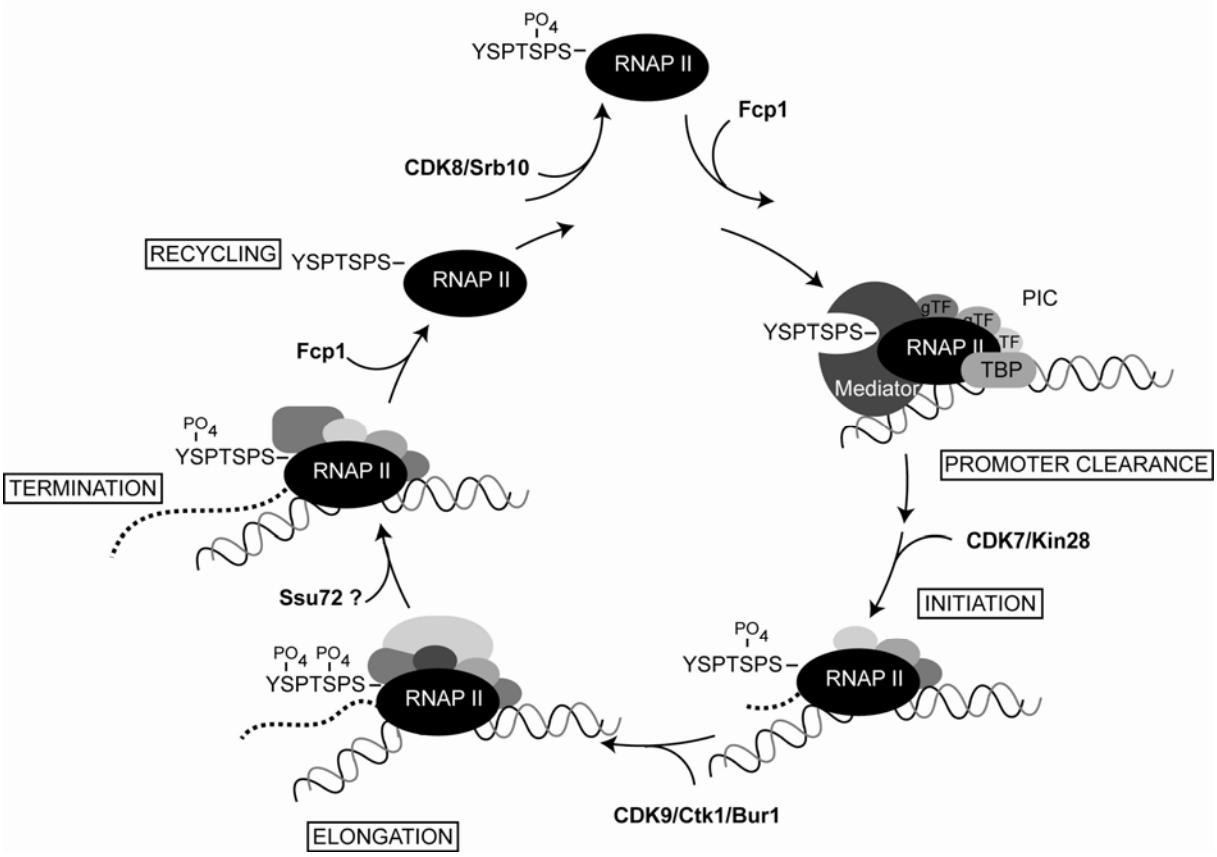

Figure 2: Simplified model of the mRNA transcription cycle (based on Prelich 2002; Svejstrup 2004).
Hypophosphorylated RNAP II is assembled in a PIC together with TATA-box binding protein (TBP), general transcription factors (gTFs) and mediator. Shortly after initiation, the CTD is phosphorylated on S5 by Cdk7/Kin28, which stimulates promoter escape. Phosphorylation on S2 by CDK9/Ctk1 results in productive elongation. The phosphatases Ssu72 and Fcp1 dephosphorylate the CTD, thereby recycling RNAP II in its hypophosphorylated form. Cdk8/Srb10 can repress transcription by phosphorylating the CTD on S5 prior to preinitiation complex formation.

After transcription is terminated, RNAP II is displaced from the DNA template and has to be recycled in order to participate in a new round of transcription. Recycling necessitates not only the removal of elongation specific co-factors but also the dephosphorylation of the CTD by CTD phosphatases. In yeast, Fcp1 was shown to dephosphorylate both S2 and S5 *in vitro*

(Lin et al. 2002), and is thought to play a major role in recycling of RNAP II by dephosphorylating S2 (Hausmann and Shuman 2002). The second known phosphatase, Ssu72, seems to be involved in initiation, elongation and termination of transcription and preferably dephosphorylates S5 (Krishnamurthy et al. 2004). *In vitro* experiments have shown that Ssu72 dephosphorylates a CTD substrate previously phosphorylated by Kin28, and thus it was speculated that Ssu72 might be involved in controlling the transition from initiation to processive elongation (Krishnamurthy et al. 2004). To date, the knowledge about CTD phosphatases is much less in comparison to CTD kinases, and the exact mechanism of regulation is still unclear and under investigation.

1.2 The transcription cycle is directly linked to mRNA processing

The phosphorylation state of the CTD not only dictates the progress through the transcription cycle but also influences the recruitment of the mRNA processing machinery for capping, splicing and polyadenylation of the message (Figure 3). Although these events occur sequentially, they are tightly interconnected and coordinated by the CTD, which serves as a docking site for the enzymes.

After synthesis of about 20-30 nt, the 5'-triphosphate end of the pre-mRNA is capped by 7-methylguanosine and bound by the cap binding complex. Efficient capping requires promoter-proximal pausing of RNAP II, and the recruitment of capping enzymes is stimulated by the phosphorylation of S5 of the CTD (Coppola et al. 1983), which provides a docking site for the capping apparatus (Komarnitsky et al. 2000; Pei et al. 2001). As soon as capping is completed, the polymerase is reactivated. This mechanism is most likely a "checkpoint" that ensures that only capped transcripts are further elongated (Pei et al. 2003). Spliceosome components bind directly to hyperphosphorylated (S2 and S5) form of the CTD and introns are removed co-transcriptionally (Du and Warren 1997; Kim et al. 1997; Hirose et al. 1999). After RNAP II transcribes past the polyadenylation site, the mRNA is cleaved and polyadenylated by the 3' processing machinery, which binds to S2 phosphorylated CTD (Hirose and Manley 1998; Barilla et al. 2001; Ahn et al. 2004).

The interdependence and interconnection of all these co-transcriptional processes becomes apparent in several aspects. The cap binding complex enhances not only splicing as it directly interacts with splicing factors and promotes the recognition of the cap-proximal 5' splice site (Colot et al. 1996; Lewis et al. 1996), but also has a stimulatory effect on the cleavage of the pre-mRNA (Flaherty et al. 1997). Crosstalk between splicing and polyadenylation has also

been shown, *e.g.* for the IgM gene, where selection of the polyadenylation site is dependent on splicing and determines whether the protein is soluble or a membrane protein (Takagaki et al. 1996; Takagaki and Manley 1998).

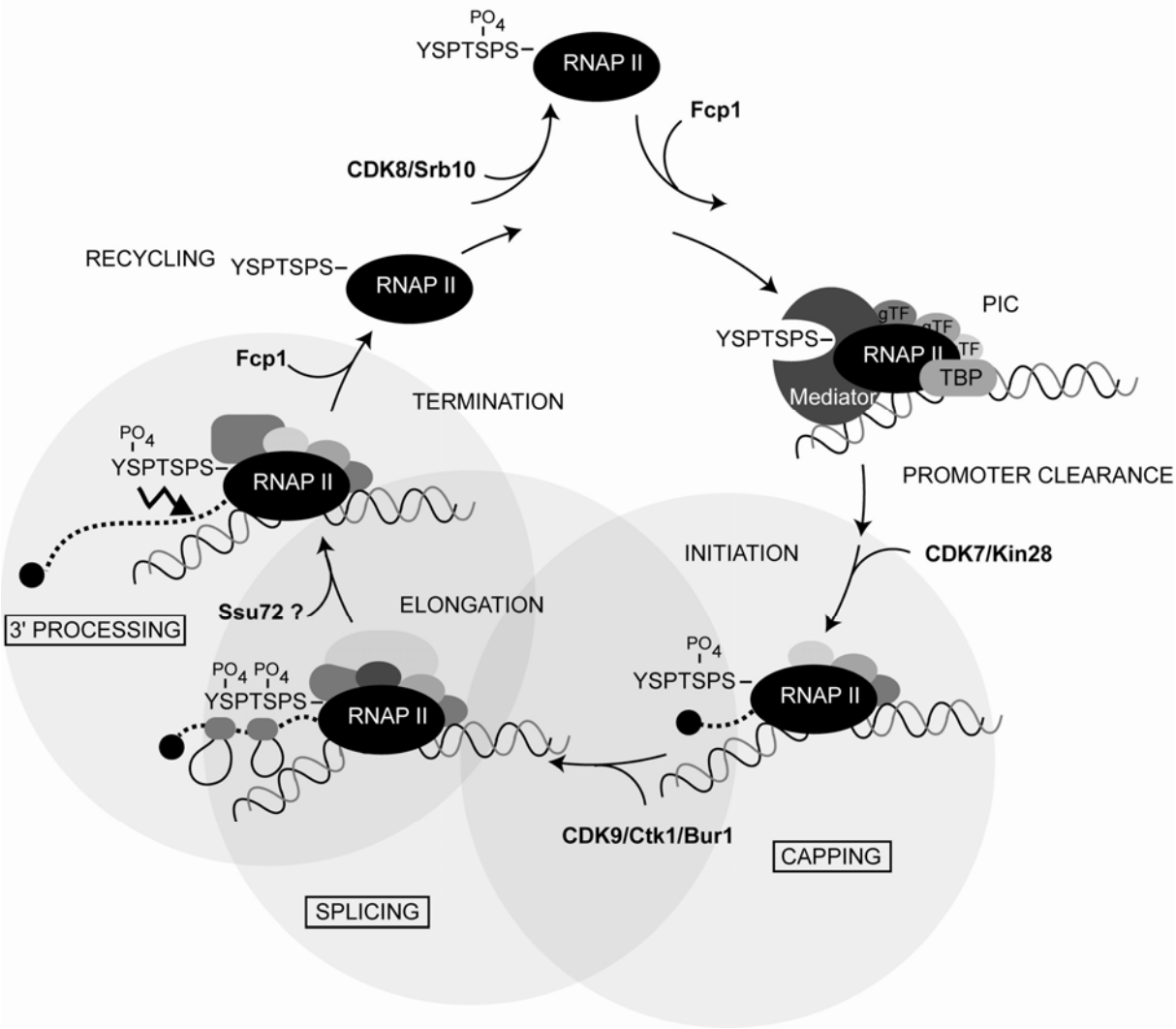

Figure 3: Capping, splicing and polyadenylation of the message occur co-transcriptionally and are regulated by the phosphorylation state of the CTD.
Phosphorylation of S5 by Cdk7/Kin28 results in recruitment of the capping machinery. Elongating polymerase, phosphorylated on S2 and S5 recruits proteins involved in the splicing process. S2 phosphorylation at the 3' end of the gene is essential for recruitment of the 3' processing machinery, ensuring proper maturation of the nascent transcript.

1.3 Transcription, quality control and export of the mRNA

The transcription cycle is not only defined by transcription, chromatin remodelling and pre-mRNA processing, but also by the recruitment of proteins to the nascent transcript that ensure RNA integrity and direct the RNA for export to the cytoplasm (Figure 4).

The key player in coupling transcription to mRNA export is the highly conserved TREX complex (Strasser et al. 2002). In the yeast *S. cerevisiae*, TREX is composed of the heterotetrameric THO complex, of the mRNA export proteins Sub2 and Yra1, the two SR-like proteins Gbp2 and Hrb1 and Tex1, a protein of so far unknown function. THO, which consists of Tho2, Thp2, Mft1 and Hpr1 is associated with actively transcribed genes over the entire length of the open reading frame (ORF) and is needed for efficient transcription elongation (Chavez and Aguilera 1997; Piruat and Aguilera 1998; Chavez et al. 2000; Strasser et al. 2002). Even though THO components are not essential in *S. cerevisiae*, THO is apparently able to prevent DNA/RNA hybrid formation, which would otherwise result in an elongation block for the next polymerase (Huertas et al. 2006). THO has also been implicated in linking transcription to RNA metabolism and genome stability (Jimeno et al. 2002) as mutants of THO display high levels of transcription dependent hyper-recombination. This phenotype might be a secondary effect of an elongation defect which might result in exposition of transcription units to recombination machineries (Reed 2003). Importantly, for the subsequent export of the mRNA into the cytoplasm, THO is necessary to load the mRNA export proteins Sub2 and Yra1 co-transcriptionally onto the nascent transcript (Strasser et al. 2002; Abruzzi et al. 2004). Sub2 is a DECD box helicase involved in splicing and mRNA export. As Sub2 binds directly to Yra1 it was speculated that it exhibits its function by recruiting the mRNA export factor Yra1, which has RNA-RNA annealing activity (Portman et al. 1997), to the nascent transcript (Strasser and Hurt 2001). Gbp2 and Hrb1 associate stably with TREX and belong to the family of SR-like proteins. In metazoans, SR proteins are splicing factors that bind to exon sequences in pre-mRNA and recruit the spliceosome to the flanking 5' and 3' splice sites (Fu 1995; Graveley 2000). Although a direct involvement of Gbp2 and Hrb1 in mRNA export has not been shown, they associate with actively transcribed genes, bind to the nascent transcript, are exported into the cytoplasm in dependence of the THO complex components and remain bound to the mRNP during translation (Hacker and Krebber 2004; Hurt et al. 2004).

TREX is also linked to the exosome, a 3'-5' exonuclease complex that degrades faulty mRNAs and is important for rRNA processing (for review see Houseley et al. 2006). Apparently, proper mRNP formation is monitored by the exosome, resulting in retention and destruction of aberrant mRNPs at the site of transcription. The tight coupling between TREX and the exosome ensures that any transcripts lacking TREX (and thereby the mRNA export transport machinery) are targeted for destruction (Andrulis et al. 2002; Libri et al. 2002; Zenklusen et al. 2002).

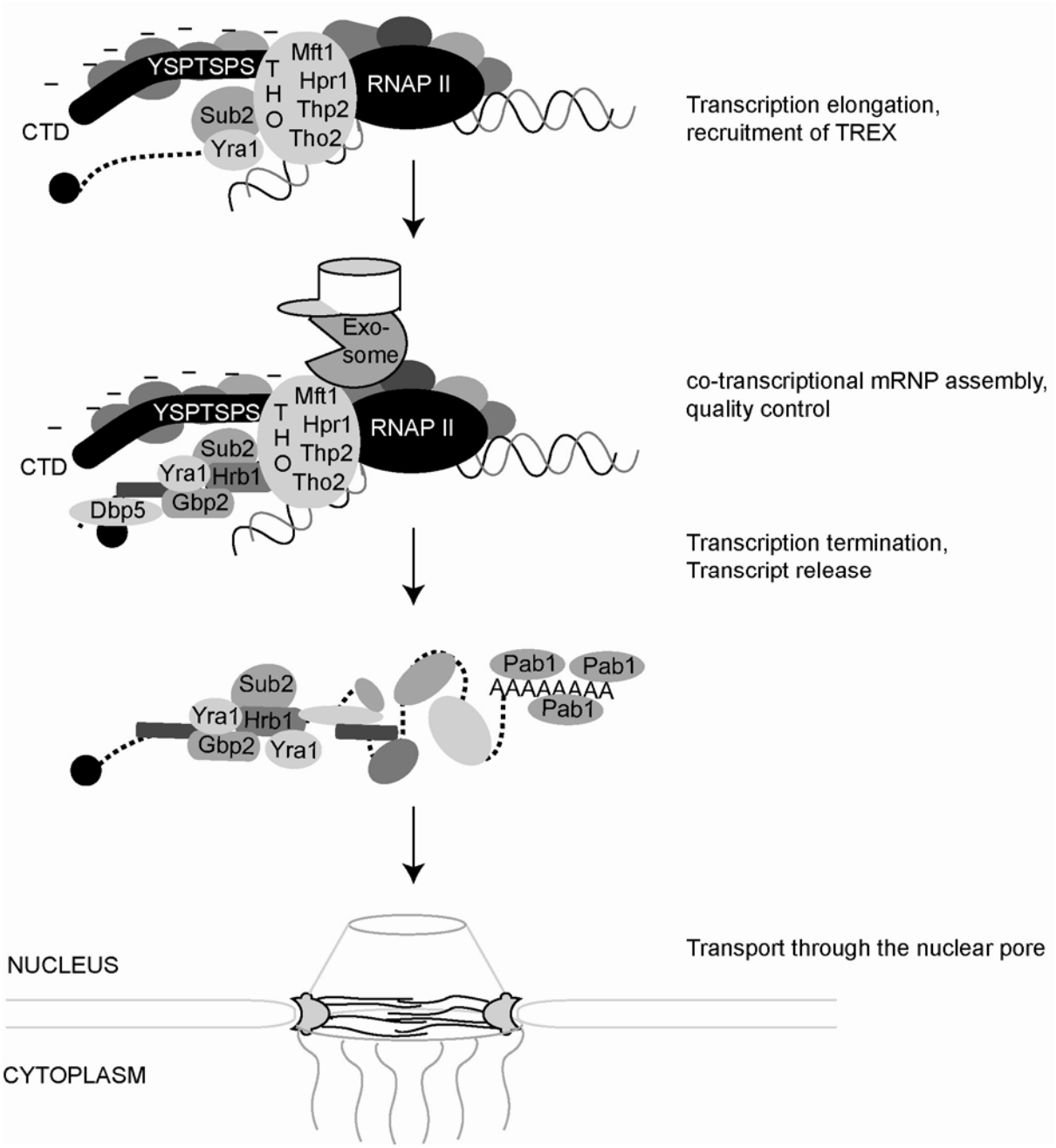

Figure 4: Model of the co-transcriptional recruitment of mRNA export factors (adopted from Aguilera 2005).
THO is co-transcriptionally bound to nascent mRNAs through interactions with factors implicated in transcription elongation. THO subsequently recruits then Sub2 and Yra1. Correct mRNP assembly and 3' processing are subject to quality control by the exosome. After transcription termination and transcript release, the mRNP is exported into the cytoplasm.

In summary, TREX couples transcription to mRNA export by ensuring co-transcriptional mRNP assembly and packaging of the mRNA into an exportable mRNP complex. THO recruits Sub2 and Yra1 to the nascent transcripts and the RNA annealing activity of Yra1 and

the putative ATPase activity of Sub2 might facilitate the correct folding of mRNP complexes and the arrangement of the mRNA into an export competent mRNP. Importantly, most of the proteins described above for *S. cerevisiae* are conserved in evolution and an equivalent function has been shown in higher eukaryotes (Reed and Cheng 2005).

Physical contact of Yra1 to the also highly conserved mRNA export receptor Mex67-Mtr2 mediates the binding of Mex67-Mtr2 to the mRNP, and the mRNA is exported to the cytoplasm by interaction of Mex67-Mtr2 with the phenylalanine-glycine (FG) repeats of the nuclear pore proteins (Strasser et al. 2000) that are components of the nuclear pore complexes (NPC). FG repeats are thought to function as permeability barrier and present binding sites for cargo-receptor complexes moving through the pore (Ryan and Wente 2000; Tran and Wente 2006). How exactly the mRNP passes through the channel is still under discussion, and it is speculated that the export of the mRNP is facilitated by pulling the message outward, *e.g.* by displacing the molecules from the emerging message that facilitate the passage through the NPC, such as Mex67-Mtr2 (Lund and Guthrie 2005) or by ribosomes that initiate translation on the emerging mRNA (Cole and Scarcelli 2006).

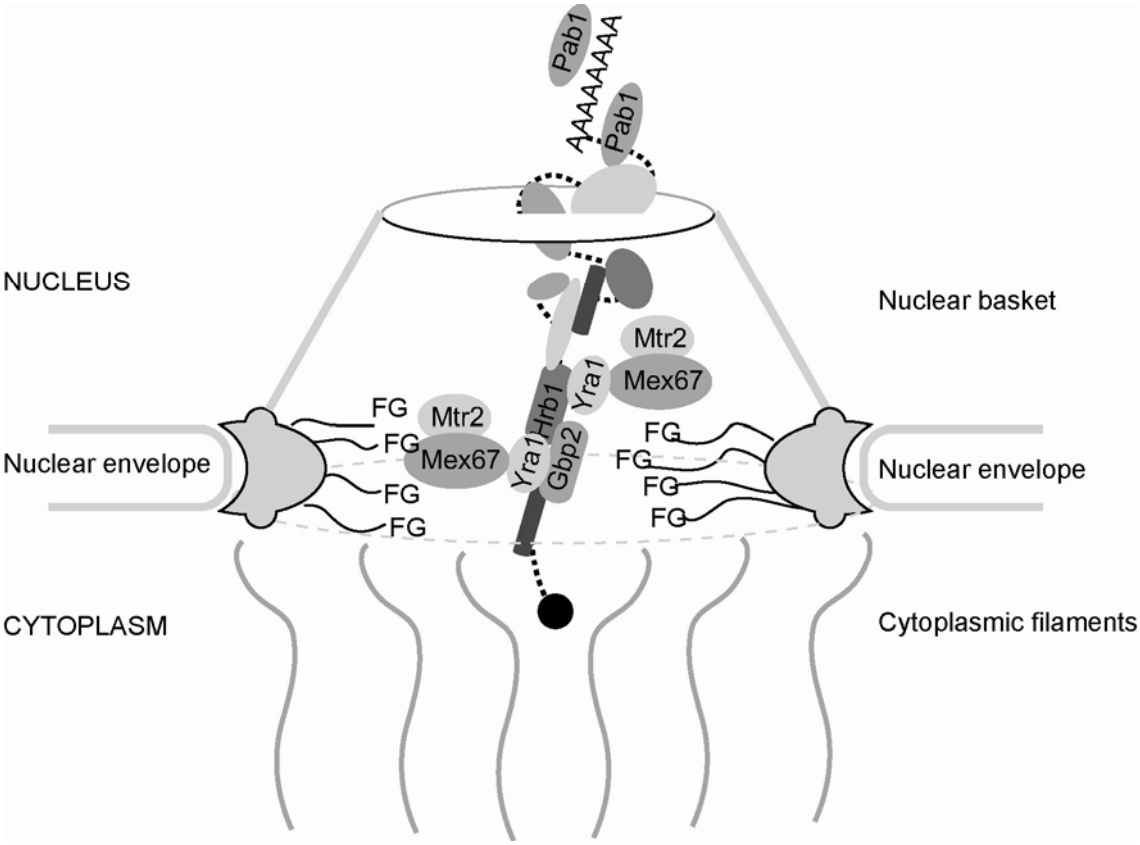

Figure 5: Transport of the mRNP through the nuclear pore.
Transport of the mRNP through the nuclear pore channel is achieved by interaction of Mex67-Mtr2 with the FG repeats of the nuclear pore proteins.

1.4 The translation of the message

The translation of the message is accomplished by large ribonucleoprotein machineries in the cytoplasm, the ribosomes. In eukaryotes, ribosomes consist of a large 60S and a small 40S ribosomal subunit. The 60S subunit is composed of the 25S, 5.8S and 5S rRNA and about 45 ribosomal proteins, while the 40S subunit is composed of the 18S rRNA and about 32 ribosomal proteins.

Translation initiation is a complex process involving multiple steps (Figure 6, for review see Preiss and Hentze 2003; Kapp and Lorsch 2004; Holcik and Sonenberg 2005). A ternary complex consisting of the GTP-bound GTPase eukaryotic translation initiation factor 2 (eIF2) and methionyl tRNA (^{met}tRNA) associates together with eIF3, eIF1 and eIF5 to a multifactor complex (MFC). eIF1A promotes the binding of the MFC to the 40S subunit, resulting in a 43S preinitiation complex. The 43S subunit is then recruited to eIF4F bound mRNA, in which secondary structures in the 5' untranslated region (UTR) are already partially removed by the action of eIF4F and eIF4B. eIF4F is a multisubunit complex consisting of the cap-binding protein eIF4E, the DEAD-box helicase eIF4A, important for the unwinding of secondary structures in the 5' UTR, and eIF4G, which provides a scaffold for the interaction with the polyA binding protein bound to the poly(A) tail. This interaction is important for the circularization of the mRNA, enhances the efficiency of translation and mediates regulatory signals. The bound 43S complex starts to scan the mRNA for the start codon. As soon as the AUG is bound to the peptidyl (P) site of the ribosome, eIF5B in its GTP-bound form acts as a GTPase activating protein (GAP) for eIF2 on the 48S preinitiation complex. As soon as the GTP of eIF2 is hydrolyzed, the initiation factors leave the ribosome. In a second GTP hydrolysis event, which is stimulated by the ribosome, eIF5B is released and the 60S subunit joins the 40S subunit to form an 80S monosome (Figure 6).

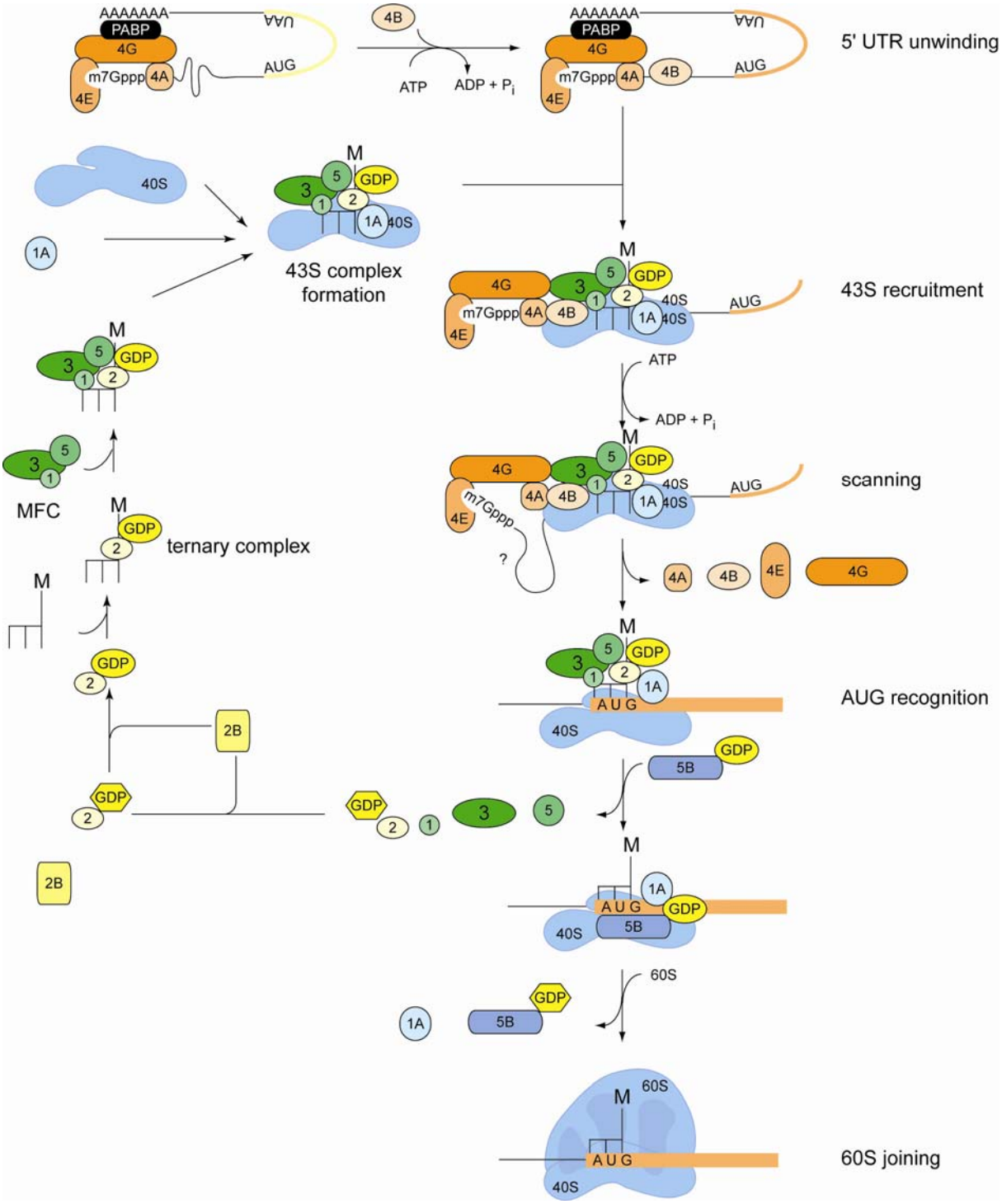

Figure 6: Initiation of translation (adopted from Preiss and Hentze 2003).
Initiation involves the recognition and the recruitment of mRNAs by the translation machinery and results in the assembly of the start codon in the peptidyl-site of the 80S ribosome. For details see text.

During translation elongation (for review see Kapp and Lorsch 2004, Figure 7), eukaryotic elongation factor 1A (eEF1A) in the GTP-bound form binds to aminoacyl-tRNA (aa-tRNA) and recruits the aa-tRNA to the acceptor (A) site of the ribosome. The correct codon-

anticodon basepairing leads to a stimulation of GTP hydrolysis by the ribosome. The ribosome is also responsible for peptide-bond catalysis, resulting in an empty tRNA in the P-site and a new peptidyl-tRNA in the A-site. eEF2, a GTP-driven translocase moves the mRNA and peptidyl-tRNA after peptide-bond formation to the P-site and the empty tRNA to the exit (E) site of the ribosome. Uniquely in fungi, a third factor, eEF3 facilitates the exit of deacetylated tRNA from the E-site and simultaneously stimulates the binding of new aatRNA to the ribosomal A-site (Figure 7).

As soon as the ribosome encounters a stop codon, a ternary complex consisting of eukaryotic release factor 1 (eRF1), eRF3 and GTP promotes the hydrolysis of the peptidyl-tRNA bond by the ribosome, which results in the release of the protein (for review see Mugnier and Tuite 1999; Bertram et al. 2001; Nakamura and Ito 2003).

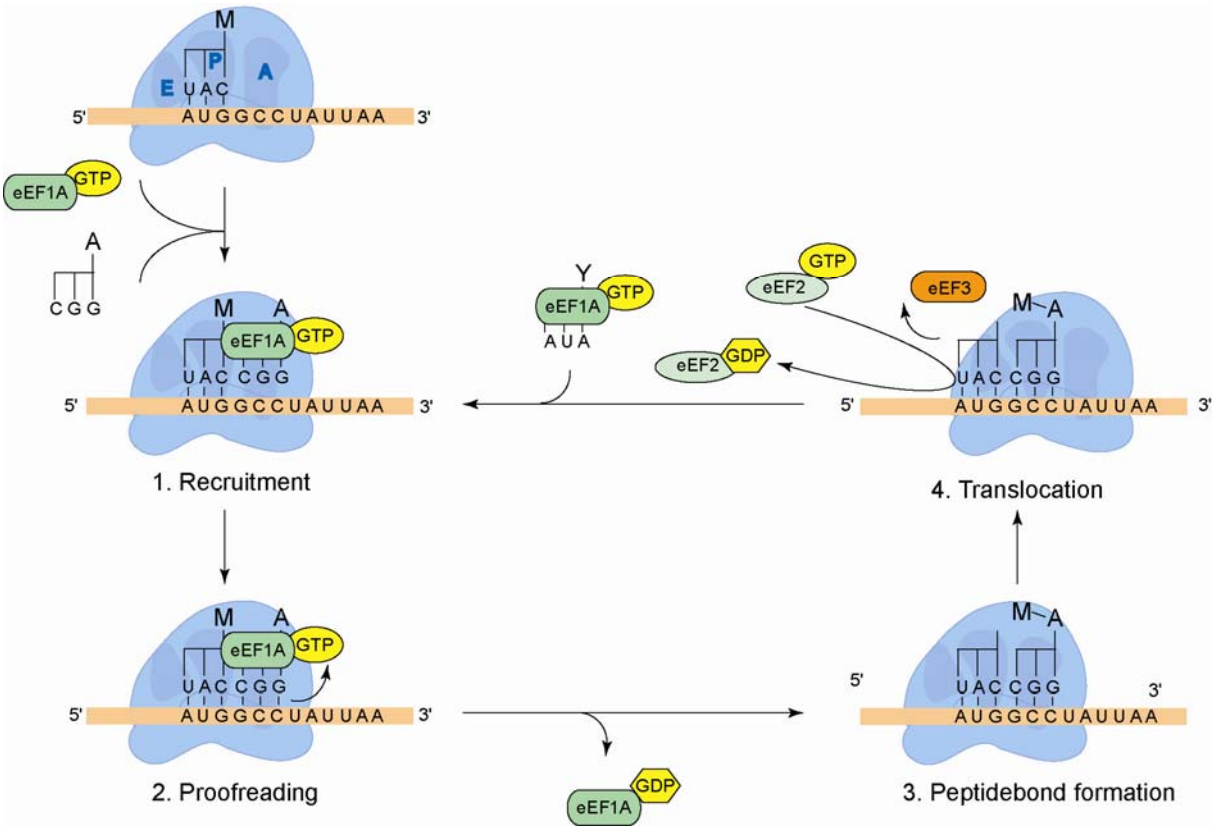

Figure 7: Elongation of translation (adopted from Kapp and Lorsch 2004).
A ternary complex consisting of eEF1A, aatRNA and GTP is recruited to a vacant A-site. Codon recognition stimulates GTP hydrolysis and peptide bond formation. Translocation of the A- and P-site tRNAs results in a free A-site and an empty tRNA in the E-site and tRNA-bound to the nascent peptide chain in the P-site. For details see text.

1.5 Aim of this work

Gene expression starts with the transcription of the message in the nucleus and ends with translation of the encoded protein in the cytoplasm. In recent years, it has been shown that the single steps during gene expression are highly interconnected. The conserved TREX complex, which couples transcription to mRNA export, is one example for a protein complex coupling intranuclear processes. In a genetic screen for factors that are crucial for TREX complex function in the living cell, Ctk1, the kinase subunit of the CTDK-I complex, was identified (Hurt et al. 2004). As CTDK-I phosphorylates the CTD of the largest subunit of RNA polymerase II, this observed genetic interaction suggested a link between transcription and TREX recruitment. Thus, initially, a potential role of Ctk1 in coupling transcription to TREX and mRNA export should be analyzed using genetic and biochemical methods.

Surprisingly, the biochemical characterization indicated that Ctk1 is not only involved – as expected – in nuclear events, but also seemed to have an unexpected cytoplasmic function in translation.

Consequently, the aim of this work became the analysis of the function of Ctk1 in translation. First, this finding should be confirmed using a combination of different independent approaches. Second, the translational process, *i.e.* translation initiation, elongation or termination, Ctk1 is involved in should be identified. This should be accomplished by using *in vitro* approaches, such as different kinds of *in vitro* translation assays. Upon identification of the process, the impact of Ctk1's function in translation in the living cell should be investigated. As Ctk1 is a kinase, a potential substrate and the phosphorylation site should be identified. With the help of the substrate and the site phosphorylated by Ctk1, it should be possible to give a molecular explanation of Ctk1's function in translation.

In addition, to analyze whether the process is conserved throughout evolution, initial experiments should be performed with cultured mammalian cells.

The results of this study should allow speculations about a potential coupling of transcription and translation by Ctk1, which could be an example for the function of a protein in a novel layer of gene expression control.

2 Results

2.1 The deletion of *CTK1* leads to a severe growth defect

In order to identify factors that are crucial for the function of TREX in the living cell, a genetic screen was performed. One of the synthetic lethal mutants identified in this screen was complemented by the *CTK1* gene (Hurt et al. 2004). As Ctk1 phosphorylates the CTD of the largest subunit of RNAP II during transcription elongation, the observed genetic interaction between TREX, which acts at the interface of transcription and mRNA export, and Ctk1 suggested a functional link. For the analysis of the function of *CTK1* in the living cell, the *CTK1* gene was knocked out. Whereas wild type (wt) cells took about 2-3 days to form colonies, colony formation in the *Δctk1* strain took about 4 days. In addition, the colonies showed non-uniform colony morphology (small, medium and big colonies). Thus, although *CTK1* is not essential in *S. cerevisiae*, its deletion leads to a severe growth defect and a cold-sensitive phenotype (Figure 8 and Lee and Greenleaf 1991; Sterner et al. 1995).

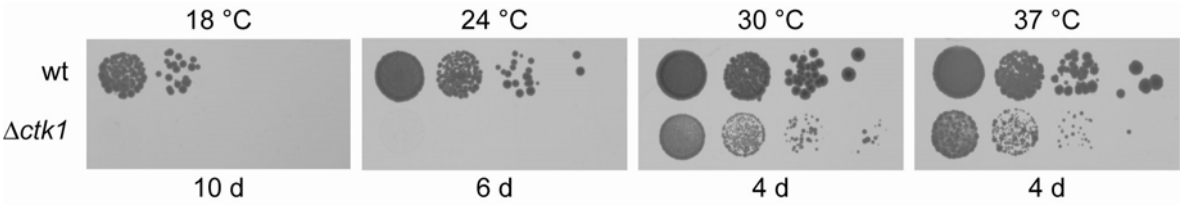

Figure 8: Deletion of *CTK1* leads to a severe growth defect and a cold-sensitive phenotype.
10-fold serial dilutions of wild type (wt) and *Δctk1* cells were spotted onto YPD plates and incubated at 18°C, 24°C, 30°C and 37°C.

2.2 The kinase activity of Ctk1 is essential for cell viability

The severe growth phenotype observed in *Δctk1* cells could be due to the missing catalytic activity, or the lack of Ctk1 as an interaction partner, or due to a combination thereof. In order to analyze this, mutants of *CTK1* were generated that should affect either the binding of ATP or the binding of the substrate, but not the overall folding of the protein. As Ctk1 is a member of the family of well characterized Cdc2/CDC28-related cell cycle cyclin dependent kinases (CDK, Lee and Greenleaf 1991), the sites of the mutations were chosen based on sequence homology of Ctk1 with CDK2, a mammalian CDK involved in G1 and S-phase events (Pagano et al. 1993). Although Ctk1 shares only about 40% overall amino acid homology with human CDK2 (http://www.ebi.ac.uk/clustalw), some stretches responsible for the kinase

activity are nearly identical (Figure 9a). Crystallographic studies in the early 1990s with human CDK2 revealed the molecular mechanism by which these kinases transfer the γ-phosphate of ATP to the substrate (De Bondt et al. 1993). In CDK2, the ATP and the essential Mg^{2+} ion are held in position by ionic and hydrogen-bonding interactions with several amino acid residues, especially Lys33 and Asp145. Based on the positioning of the ATP molecule, the authors speculated that the substrate binds in a cleft between the two lobes affiliated to Lys33 and Asp145, thereby positioning the Ser-hydroxyl group of the substrate near the γ-phosphate of ATP so that the phosphotransferase reaction is catalyzed.

Based on these data, two mutations were introduced into the *CTK1* gene, affecting either the binding of ATP or the binding of the substrate (Figure 9b). In order to decrease the binding of ATP, Lys212, the amino acid corresponding to CDK2 Lys33, was substituted by alanine, resulting in the "kinase-dead" or ATP-binding mutant. Although the substrate can bind to this mutant initially, it will dissociate from the kinase immediately as phosphate transposition is not possible. Substitution of Asp306 (corresponding to Asp145 in CDK2) for alanine disturbs the coordination of the Mg^{2+} ion and consequently the positioning of the ATP molecule as the charges are destabilized. Thus, the substrate will bind, but as the nucleophilic attack of the Ser-hydroxyl group is extremely prolonged, the substrate is trapped and will dissociate from the kinase with very low kinetics, resulting in the "trapping mutant" (Gibbs and Zoller 1991; Iyer et al. 2005). As shown in Figure 9c, these mutations in Ctk1 caused a similar phenotype as the complete knockout of the gene. When either the ATP-binding was disturbed (K212A) or the dissociation of the substrate inhibited (D306A), Ctk1 was no longer able to fulfill its function as indicated by the small size of the colonies, which were comparable to *Δctk1* colonies in contrast to those containing *CTK1*. Thus, the severe growth defect observed in *Δctk1* cells is most likely caused by the missing kinase function.

(a)

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HsCDK2       1 ------------------------------------------------------------
ScCtk1       1 MSYNNGNTYSKSYSRNNKRPLFGKRSPNPQSLARPPPPKRIRTDSGYQSNMDNISSHRVN

HsCDK2       1 ------------------------------------------------------------
ScCtk1      61 SNDQPGHTKSRGNNNLSRYNDTSFQTSSRYQGSRYNNNNTSYENRPKSIKRDETKAEFLS

HsCDK2       1 ----------------------MEN---------------------------------
ScCtk1     121 HLPKGPKSVEKSRYNNSSNTSNDIKNGYHASKYYNHKGQEGRSVIAKKVPVSVLTQQRST

                                      K33
HsCDK2       4 --FQKVEKIGEGTYGVVYKARNKLTG---EVVALKKIRLDTETEGVPSTAIREISLLKEL
ScCtk1     181 SVYLRIMQVGEGTYGKVYKAKNTNTE---KLVALKKLRLQGEREGFPITSIREIKLLQSF
                                      K212
HsCDK2      59 NHPNIVKLLDVIHTEN-KLYLVFEFLHQDLKKFMDASALTG-IPLPLIKSYLFQLLQGLA
ScCtk1     238 DHPNVSTIKEIMVESQKTVYMIFEYADNDLSGLLLNKEVQI--SHSQCKHLFKQLLLGME
                    D145
HsCDK2     117 FCHSHRVLHRDLKPQNLLINTEGAIKLADFGLARAFGVPVRTYTHEVVTLWYRAPEILLG
ScCtk1     296 YLHDNKILHRDVKGSNILIDNQGNLKITDFGLARKMNS-RADYTNRVITLWYRPPELLLG
                    D306
HsCDK2     177 CKYYSTAVDIWSLGCIFAEMVTRRALFPGDSEIDQLFRIFRTLGTPDEVVWPGVTSMPDY
ScCtk1     355 TTNYGTEVDMWGCGCILVELFNKTAIFQGSNELEQIESIFKIMGTPTINSWPTLYDMPWF

HsCDK2     237 KPSFPKWARQDFSKVVPPLD-----EDGRSLLSQMLHYDPNKRISAKAALAHPFFQDVTK
ScCtk1     415 FMIMPQQQTTKYVNNFSEKFKSVLPSSKCLQLAINLLCYDQTKRFSATEALQSDYFKEEPK

HsCDK2     292 PVPHLRL-----------------------------------------------
ScCtk1     475 PEPLVLDGLVSCHEYEVKLARKQKRPNILSTNTNNKGNGNSNNNNNNNDDDDK
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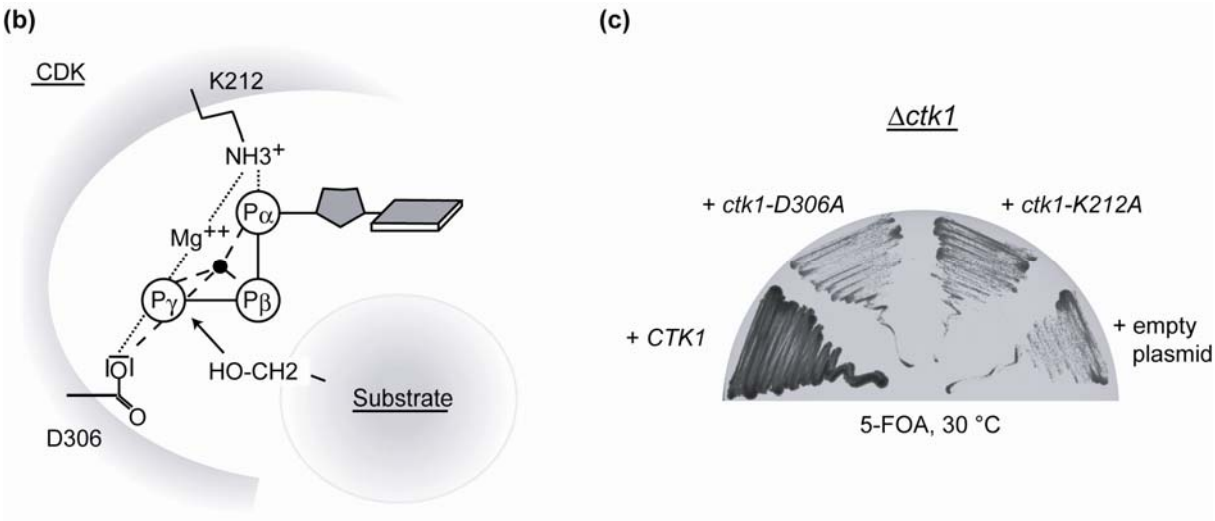


Figure 9: Mutations in the active center of Ctk1 impair growth.
(a) Alignment of CDK2 (*H. sapiens*), Cdc28 (*S. cerevisiae*), and Ctk1 (*S. cerevisiae*). The alignment was done using ClustalW (http://www.ebi.ac.uk/clustalw) and BoxShade http://www.ch.embnet.org/software/BOX_form.html). Conserved, identical residues are black boxed; semi-conserved residues are grey-boxed. Ctk1 shares about 40% homology with CDK2. **(b)** Schematic illustration of the ATP binding pocket in Ctk1, according to DeBondt et al. (1993). For the kinase-dead mutant, Lys212 was substituted for alanine. ATP cannot bind anymore and the substrate is not phosphorylated. For the trapping mutant, Asp306 was substituted for alanine. The substrate can bind but is not phosphorylated as the ATP is not correctly positioned. The substrate dissociates from the kinase with very low kinetics and is therefore trapped. Potential hydrogen bonds are shown as thin dashed lines and coordination bonds as thick dashed lines. **(c)** Mutations in the active center of Ctk1 impair growth. The *CTK1* shuffle strain was transformed with plasmids encoding for *CTK1*, *ctk1-D306A* or *ctk1-K212A* and an empty plasmid. Growth was assessed after restreaking transformants onto a plate containing 5-FOA and incubation at 30°C for 5 d.

2.3 *CTK1* interacts genetically with TREX

CTK1 was originally identified in a synthetic lethality (sl) screen with a deletion of *MFT1* (Hurt et al. 2004). Therefore, it was interesting to see whether *CTK1* is only synthetically lethal with genes encoding the THO complex (*HPR1*, *MFT1*, *THO2*, *THP*2) involved in transcription elongation (Chavez and Aguilera 1997; Piruat and Aguilera 1998; Chavez et al. 2000) or with the entire TREX complex, including also *SUB2*, *YRA1*, *GBP2* and *HRB1* (Strasser et al. 2002; Hurt et al. 2004), which could indicate a novel function of Ctk1 during mRNA export. Furthermore, synthetic lethality between *CTK1* and the mRNA export receptor *MEX67* was assessed to see, whether *CTK1* is also genetically linked to downstream events of mRNA export, *i.e.* the export of the mRNP to the cytoplasm.

2.3.1 The principle of synthetic lethality

In general, two alleles are synthetically lethal if the combination of otherwise viable mutations in these genes leads to cell death. One approach to address synthetic lethality is the construction of a double knockout strain of the genes of interest, carrying the respective wt copies on a *URA3* plasmid (shuffle strain), which can then be lost by restreaking the cells onto plates containing 5-FOA (*X*shuffle *Y*shuffle). This strain is transformed with different combinations of plasmids carrying either the intact gene *X* or *Y*, or mutated (mut) *x* or *y*. If the strain carrying both mut *x* and mut *y* is inviable on 5-FOA, the mutant alleles are synthetically lethal (Figure 10a). On a more molecular level, synthetic lethality can hint for a physical or a functional interaction between the two gene products. In the case of physical interaction, cells survive when mutations in one of the two interacting proteins do not disrupt the interaction interface, whereas the combination of both mutations impairs the interaction and leads to cell death (Figure 10b, I). Synthetic lethality can also reflect a functional involvement of the two proteins in the same (Figure 10b, II) or in redundant pathways (Figure 10b, III), *i.e.* cells can survive if one of the two proteins is inactive, whereas the cell will die if both proteins are inactive.

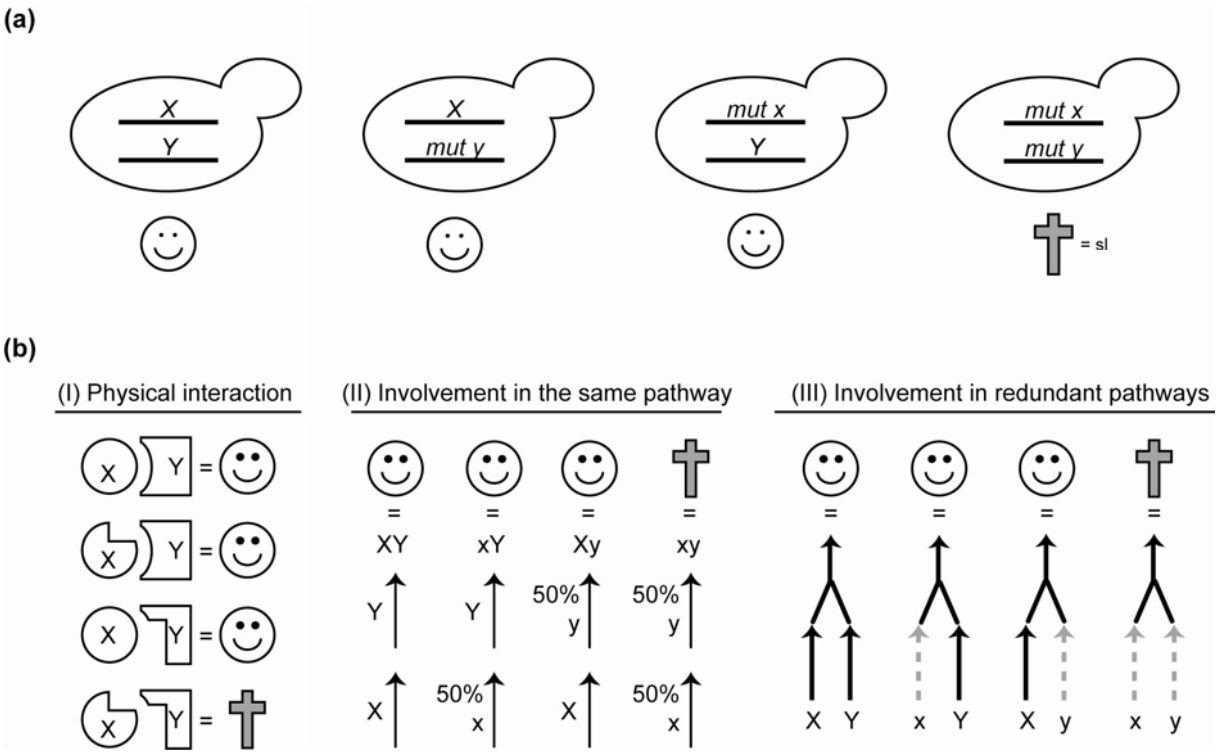


Figure 10: Principles of synthetic lethality.
(a) Single mutations in the genes of interest do not affect cell growth (*X* muty or mut*x* *Y*). If however the combination of two mutations (mut*x* muty) confers cell death, the two genes are synthetically lethal. **(b)** Synthetic lethality can be based on either physical interactions (I), or the involvement of two proteins in the same (II) or in alternative (III) pathways.

2.3.2 *CTK1* is synthetically lethal with THO

In order to test whether the genetic interaction of *CTK1* with *MFT* can be extended to the entire THO complex double deletion strains of *CTK1* and *MFT1*, *HPR1* or *THO2*, respectively, were created. On plates containing 5-FOA (Figure 11) the cells were viable if one of the wt genes was present. However, the presence of only the kinase-dead or the trapping mutant of Ctk1 or no plasmid (=*Δctk1*) resulted in cell death, showing that the observed synthetic lethality between *CTK1* and *MFT1* is not restricted to only *MFT1* but can also be extended to other components of THO. It is noteworthy that the addition of *MFT1* or *HPR1* or *THO2* on a plasmid did not rescue the growth phenotype to the same extent as *CTK1* did. This was expected since *e.g. Δctk1Δhpr1* plus a plasmid encoding for *HPR1* reflects a *Δctk1* strain, which shows a severe growth defect (Figure 8). Even though the creation of a *Δctk1Δthp2* shuffle strain was not successful, the observed synthetic lethality between *CTK1* and *MFT1*, *HPR1* and *THO2* suggests that *CTK1* is also synthetically lethal with *THP2*.

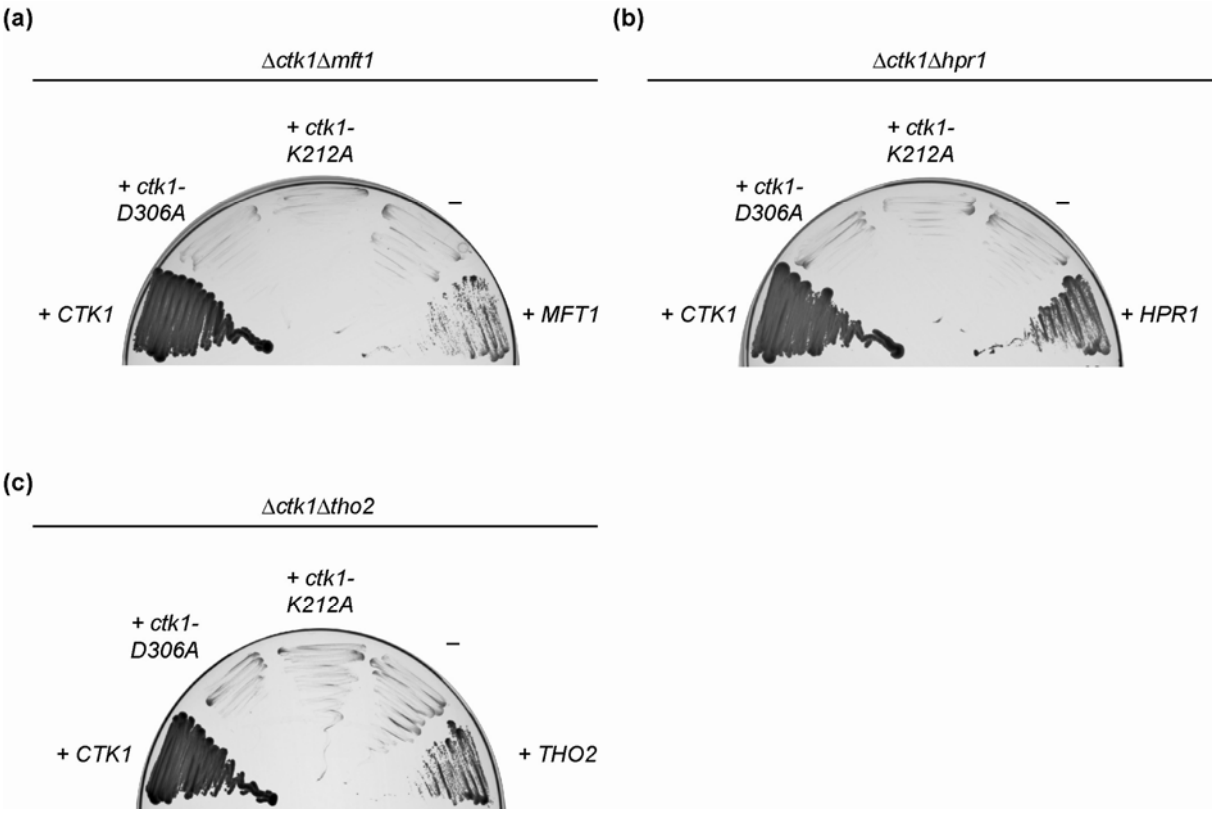


Figure 11: *CTK1* interacts genetically with *MFT1, HPR1* and *THO2,* components of the THO complex.
The strains *CTK1Δmft1* **(a)**, *CTK1Δhpr1* **(b)** and *CTK1Δtho2* **(c)** were transformed with plasmids encoding *CTK1*, *ctk1-D306A* (trapping mutant), *ctk1-K212A* (kinase-dead mutant) and *MFT1*, *HPR1*, *THO2*, or an empty plasmid. Transformants were restreaked onto plates containing 5-FOA and growth was analyzed after incubation at 30°C for 5 days. No growth indicates a synthetic lethal relationship between *CTK1* and *MFT1*, *HPR1* or *THO2*, respectively.

2.3.3 *CTK1* is synthetic lethal with *YRA1* and *MEX67*, but not *SUB2*

When the double knockout strains of *CTK1* with the mRNA export factors *SUB2*, *YRA1* or the mRNA export receptor *MEX67* were tested for synthetic lethality, it turned out that *CTK1* was synthetically lethal with temperature sensitive mutants of *YRA1* and *MEX67*, but not *SUB2* (Figure 12).

Yra1, the major yeast RNA-RNA annealing protein, which is most likely responsible for the targeting of RNAP II transcripts to Mex67-Mtr2, is a 25 kDa multidomain protein composed of an N-terminal domain, an arginine and glycine (RGG) rich domain, containing a putative nuclear localization signal (NLS), an RNA recognition motif (RRM), an additional NLS-domain and a C-terminal domain (Strasser and Hurt 2000, Figure 12a). Analysis of a temperature sensitive mutant of *YRA1* showed that especially the C-terminal domain might be

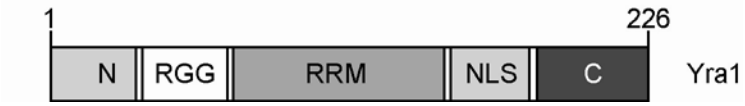
important for mRNA export, and domain analysis revealed that the NLS+C as well as the N+RGG domain of Yra1 directly bind to the Mex67-Mtr2 heterodimer (Strasser and Hurt 2000). Interestingly, *Δctk1* was found to be sl with mutant *yra1* in cases where the NLS or the C-terminal domain of Yra1 was deleted, allowing the speculation that Ctk1 might be a part of the exported mRNP and the interaction of both proteins before or during transport or subsequent export might be necessary for the survival of the cell.

In contrast, *CTK1* was not sl with alleles of the DECD-box helicase *SUB2* (data not shown). However, the observed sl between *CTK1* and THO and *YRA1*, respectively, still might indicate that *CTK1* interacts genetically with other alleles of *SUB2* than the tested ones.

Mex67, which constitutes together with Mtr2 the mRNA export receptor, is composed of an N-terminal, a leucine rich region (LRR), middle and a C-terminal domain (Figure 12b). The LRR domain is most likely involved in protein-protein interactions (Kobe and Deisenhofer 1994) and interestingly, *CTK1* is synthetically lethal with two ts mutants in the N+LRR domain of *MEX67*, suggesting that protein-protein interactions could be affected when the deletion of *CTK1* is combined with these mutant *mex67* alleles. Further biochemical and sequence analysis of these mutant *mex67* alleles might help to provide information why *CTK1* is sl with these mutations, *i.e.* the deletion of *CTK1* could affect binding of Mex67 to the nuclear pore or to another protein of the mRNP. The middle domain of Mex67 binds to Mtr2, which is a prerequisite for the *in vivo* interaction of Mex67 with the nuclear pore (Segref et al. 1997; Santos-Rosa et al. 1998; Strasser et al. 2000). The C-terminal domain of Mex67 is not essential for cell viability. However, it was speculated that this domain may modulate efficient nuclear export of the shuttling Mex-Mtr2 by interacting with additional export factors such as Xpo1 (Strasser et al. 2000). Δ*ctk1* was sl with temperature sensitive mutants of *MEX67* in which the C-terminal domain is truncated, suggesting that Ctk1 is implicated in the process of the export of the mRNP through the nuclear pore, which is also supported by the observed genetic interaction between *CTK1* and mutations in *YRA1* that are essential for either efficient transport through the pore or the interaction with Mex67-Mtr2.

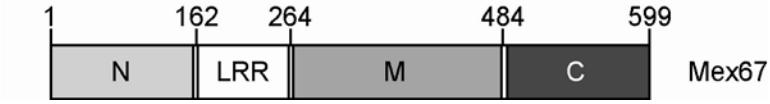
The observed allele-specific synthetic lethality between *CTK1* and *YRA1* or *MEX67*, respectively shows that Ctk1 not only interacts genetically with proteins involved in transcription elongation and early mRNA export, but also with proteins that are implicated in the transport process of the mRNP through the nuclear pore.

(a)



YRA1 allele	deleted domain	*CTK1*	empty plasmid
YRA1	-	+	+
yra1-ΔN	N	+	+
yra1-ΔRGG	RGG	+	+
yra1-ΔRBD	RBD	+	+
yra1-ΔNLS	NLS	+	-
yra1-ΔC	C	+	-

(b)



MEX67 allele	affected domain	*CTK1*	empty plasmid
MEX67	-	+	+
mex67-5	M	+	+
mex67-Cts4	C	+	-
mex67-Cts19	C	+	-
mex67-N+LRRts1	N+LRR	+	+
mex67-N+LRRts2	N+LRR	+	+
mex67-N+LRRts3	N+LRR	+	-
mex67-N+LRRts4	N+LRR	+	-
mex67-N+LRRts5	N+LRR	+	+
mex67-N+LRRts6	N+LRR	+	+

Figure 12: *Δctk1* is synthetic lethal with alleles of *YRA1* and *MEX67*.
CTK1 YRA1 and *CTK1 MEX67* were transformed with either *CTK1* or an empty plasmid and ts alleles of *YRA1 or MEX67*. Transformants were restreaked onto plates containing 5-FOA and growth was analyzed after incubation at 30°C for 5 days. No growth annotated by a "-" indicates synthetic lethality between *Δctk1* and the respective allele. **(a)** Yra1 is composed of an N-terminal, an arginine and glycine rich (RGG), an RNA recognition motif (RRM), a nuclear localization (NLS) and a C-terminal domain. *Δctk1* is sl with *YRA1* bearing mutations in the NLS or C-terminal domain. **(b)** Mex67 is a multidomain protein, containing an N-terminal, LRR, middle and a C-terminal domain. *Δctk1* is sl with mutations in *MEX67* in the N+LRR and the C-terminal domain.

2.3.4 *CTK1* is not synthetic lethal with *GBP2* and *HRB1*

Gbp2 and Hrb1 are highly homologous mRNA binding proteins that are recruited co-transcriptionally to the nascent mRNA in dependence of THO and are stably associated with TREX (Hacker and Krebber 2004; Hurt et al. 2004). Together with the mRNA, they travel to the cytoplasm and are part of the translating ribosome (Windgassen and Krebber 2003; Windgassen et al. 2004). In order to test, whether *Δctk1* also interacts genetically with these two TREX components, synthetic lethality between *CTK1* and *GBP2* or *HRB1*was assessed (Figure 13a). *CTK1* and *HRB1* did not interact genetically as indicated by the growth on plates containing 5-FOA when either the *ctk1* kinase mutants or *Δctk1* was combined with *Δhrb1*. In contrast, the double deletion of *CTK1* and *GBP2*, as well as the combination of the *ctk1* kinase mutants with the deletion of *GBP2* grew slower than *Δctk1* or *Δgbp2* cells. The high homology of Gbp2 and Hrb1 (Hacker and Krebber 2004) suggested that these proteins fulfill redundant functions in the cell. However, the observed difference in growth between the *Δctk1Δhrb1* and *Δctk1Δgbp2* strain might be a first hint of an involvement of these proteins in different functions or pathways, maybe correlated to a so far unknown function of Ctk1.

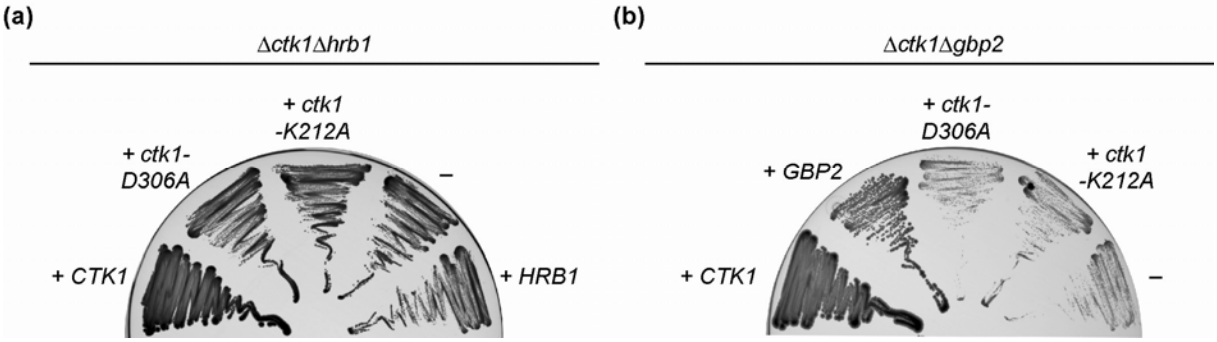


Figure 13: *CTK1* is not synthetic lethal with *HRB1* but interacts genetically with *GBP2*. *CTK1Δgbp2* and *CTK1Δhrb1* strains were transformed with *CTK1*, *ctk1-D306A*, *ctk1-K212A*, an empty plasmid and either *GBP2* or *HRB1*. Transformants were restreaked onto plates containing 5-FOA and incubated for 5 days at 30°C. **(a)** *CTK1* and *HRB1* are not synthetically lethal as the double deletion transformed with either the inactive kinase mutants (*ctk1-D306A*, c*tk1-K212A*) or an empty plasmid is viable. **(b)** *CTK1* is synthetic sick with *GBP2* as shown by slower growth when mutant *ctk1* (deletion or inactive kinase mutants) was combined with *Δgbp2*.

2.3.5 Deletion of *CTK1* does not lead to an mRNA export defect

The interaction between *CTK1* and TREX suggested a function of Ctk1 in mRNA export. Therefore, a potential mRNA export defect in *Δctk1* cells was assessed. As a positive control for nuclear accumulation of mRNA, a temperature-sensitive mutant allele of the mRNA export receptor *MEX67*, *mex67-5*, was used. This mutant leads to a complete block of mRNA-export if the temperature is raised to 37°C (Segref et al. 1997).

Figure **14** shows that in contrast to the nuclear accumulation of poly(A)$^+$ RNA in the *mex67-5* mutant at 37°C, the deletion of *CTK1* does not lead to a mRNA export defect, as the mRNA is evenly distributed over the cytoplasm. This finding suggests that although Ctk1 interacts genetically with TREX, it is not necessary for the transport of the mRNP to the cytoplasm.

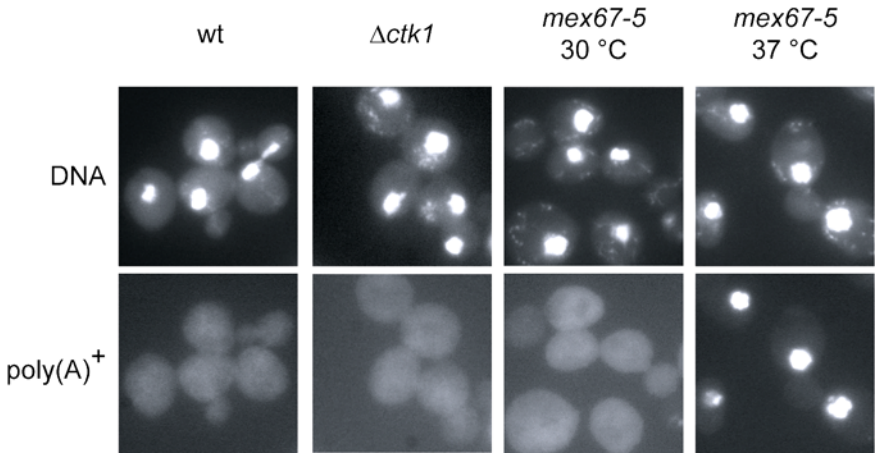


Figure 14: *Δctk1* cells do not show an mRNA export defect.
Nuclei of the cells were stained with DAPI and the poly(A)-tail of bulk mRNA was visualized using a Cy3-labelled oligo(dT) probe. In wt, *Δctk1* and *mex67-5* at 30°C, poly(A)$^+$ mRNA is equally distributed over the cell. After shifting *mex67-5* cells to 37°C, mRNA export is blocked and the mRNA accumulates in the nucleus.

2.4 Ctk1 interacts with ribosomal proteins and proteins involved in translation

Previously, it was shown that Ctk1 physically interacts with TREX components (Hurt et al. 2004). In order to get further insights into the mechanism how Ctk1 could function with regard to a putative connection between transcription and mRNA export, novel interacting partners of Ctk1 were identified using tandem affinity purification (Figure 15a, Figure 15b). Mass spectrometry of proteins co-purifying with Ctk1-TAP yielded the CTDK-I complex (Ctk1, Ctk2 and Ctk3) and the TREX proteins Gbp2 and Hrb1. Surprisingly, besides these

"expected" proteins, mainly proteins with a function in translation as well as ribosomal proteins were found. To test the specificity of this observed co-purification, a non-tagged wt and a Ceg1-TAP strain were purified. Ceg1 was chosen as a control because it is a subunit of the nuclear capping enzyme complex associated with the nascent transcript with a similar expression level as Ctk1 (http://www.yeastgenome.org; Shibagaki et al. 1992; Kim et al. 2004). As shown in Figure 15c, neither Ceg1-TAP (lane 2) nor the non-tagged control strain (lane 3) showed such a high level of co-purifying ribosomal proteins and proteins involved in translation as did Ctk1-TAP (lane 1), indicating that the observed interaction of ribosomal proteins and proteins involved in translation with Ctk1 is indeed specific.

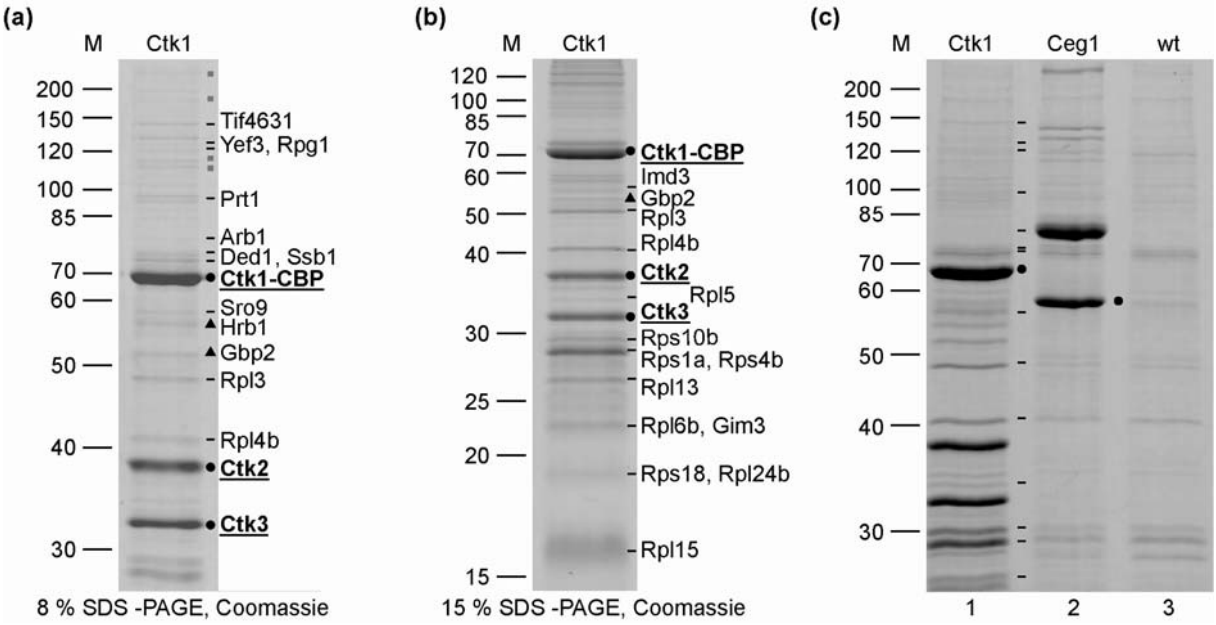


Figure 15: Ctk1 interacts with ribosomal proteins *in vivo*.
(a), **(b)** TAP-tagged Ctk1 was purified from *S. cerevisiae*. Co-purifying proteins were separated by an 8% (a) or a 15% (b) SDS-PAGE and identified by mass spectrometry (C. Turck, MPI for Psychiatry, Munich). Bands indicated by a full circle are components of the CTDK-I complex, triangles represent Gbp2 and Hrb1, horizontal lines mark ribosomal proteins or proteins involved in translation and grey squares correspond to the following proteins (from top to bottom): Bem2, Kem1, Ubp3 and Syp1. Other bands present in the purification could not be identified unambiguously. **(c)** The co-purification of ribosomal proteins with Ctk1 seems to be specific as control purifications of Ceg1 (lane 2) or of a non-tagged wt control (lane 3) show markedly less co-purification of ribosomal proteins when compared to Ctk1 (lane 1). Tagged proteins are indicated by a full circle and straight lines represent ribosomal proteins or proteins involved in translation.

However, the analysis of the co-purifying proteins did not reveal specificity for a distinct translational process. Ribosomal proteins belonged to the 40S small ribosomal subunit as well as the 60S large ribosomal subunit, and the identified translation factors were involved in

either translation initiation (Ded1, Prt1, Rpg1, and Tif4631), translation elongation (Yef3), or proteins associated with translating ribosomes or ribosome biogenesis (Sro9, Ssb1, Arb1). As the translation factors are present in only very low amounts compared to Ctk1 and ribosomal proteins the observed interaction between Ctk1 and *e.g.* Prt1 is most likely mediated by ribosomal proteins.

The obtained results were astonishing as almost no proteins related to the described nuclear function of Ctk1 were found, but instead mainly proteins involved in translation were identified. Assuming that the observed co-purification is specific and considering the genetic interaction between *CTK1* and *TREX*, it might be that Ctk1 exits the nucleus bound to the mRNP. Thus, it was interesting to investigate the composition of Ctk1 co-purifying proteins when mRNA export was blocked. To achieve accumulation of mRNA inside the nucleus, a ts allele of *MEX67*, *mex67-5*, was used (Segref et al. 1997, see also

Figure **14**). When Ctk1 was purified from mRNA export blocked cells, the pattern of co-purifying proteins changed slightly. At about 30 kDa, one band appeared that was identified as Yra1, a component of TREX. This interaction, which only becomes more prominent when mRNA export is blocked, suggests that Ctk1 might indeed be a component of the exported mRNP and might thereby be transported to the cytoplasm to interact with ribosomes.

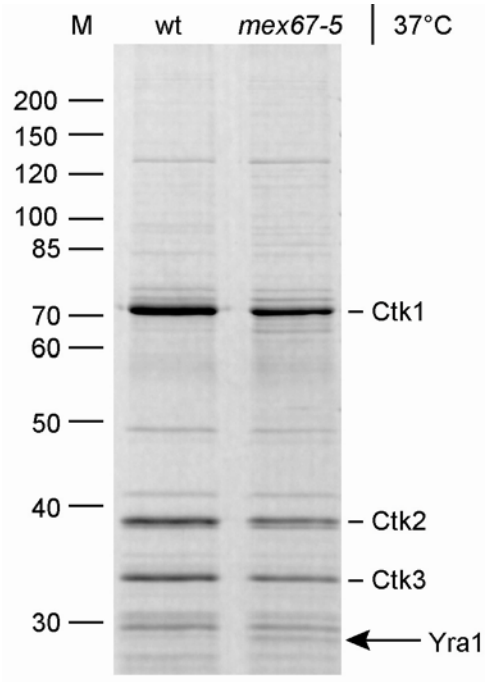


Figure 16: Ctk1 interacts with Yra1 if the mRNA export is inhibited.
Ctk1 was purified from wt and mRNA export blocked (*mex67-5*) cells after shift of the cultures to 37°C. The arrow indicates the newly appearing Yra1 band in the Ctk1 purification from *mex67-5* cells at 37°C.

2.5 Ctk1 associates with translating ribosomes

The biochemical association of Ctk1 with ribosomes and translation factors suggests a role for Ctk1 in translation. However, it is known that TAP purifications tend to contain ribosomes as contaminants (Gavin et al. 2002). Therefore, the association of Ctk1 with ribosomes was verified using polysome density gradient centrifugation, where lysates are separated into polysomes, 80S monosomes, 60S subunits, 40S subunits and soluble proteins and the migration of a protein in the gradient can be assessed by Western blotting. To arrest translating ribosomes, cells were treated with cycloheximide, which inhibits the translocation of the aminoacyl-tRNA from the A- to the P-site (Obrig et al. 1971). Each fraction of the gradient was then assessed for either the presence of TAP-tagged proteins, *e.g.* Ctk1, or Rpl6 as a protein of the large ribosomal subunit and Rps8 as a protein of the small ribosomal subunit. Compared to ribosomal proteins, Ctk1 was present in fractions containing ribosomal subunits, monosomes and polysomes (Figure 17), indicating that Ctk1 associates with translating ribosomes.

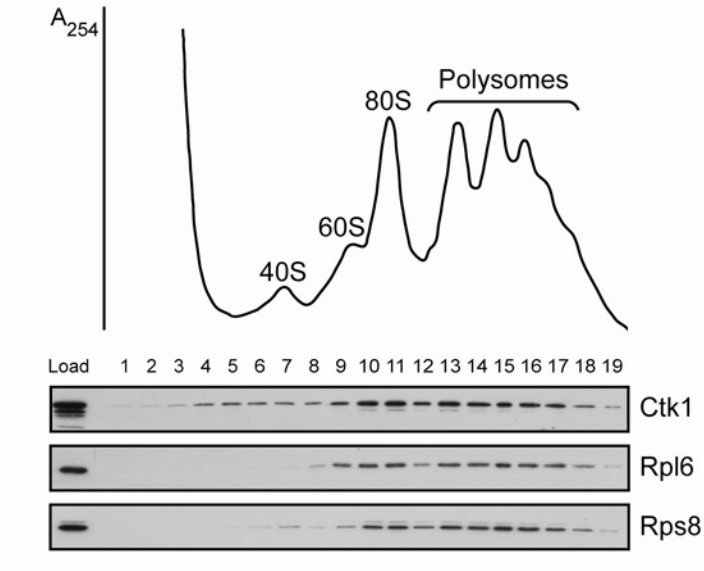


Figure 17: Ctk1 associates with translating ribosomes.
Sucrose density gradients were performed and ribosomal fractions (40S, 60S, 80S and polysomes) were determined by A_{254nm} measurement of the gradient fractions. Each fraction was analyzed by Western blotting using an anti-protein A antibody in order to assess the presence of TAP-tagged Ctk1. Anti-Rpl6 and Rps8 antibodies were used to show the distribution of the large and the small ribosomal subunit, respectively. Ctk1's migration pattern is similar to that of ribosomal proteins and can be found at 40S, 60S, 80S and polysomes.

Even though this finding confirmed the results of the TAP (Figure 15), it could be that the observed migration behavior is due to a high molecular weight, *e.g.* nuclear transcription complex. Therefore, the sedimentation pattern of proteins involved in diverse cellular processes was analyzed after sucrose density gradient centrifugation. Rbp1, the largest subunit of RNAP II, which is phosphorylated by Ctk1, and Paf1, a subunit of the Paf complex involved in transcription elongation and poly A length control (Squazzo et al. 2002; Mueller

et al. 2004), are present exclusively in the fractions with the lowest molecular weight of a sucrose density gradient (Figure 18a, b). This result shows that the sedimentation behaviour of Ctk1 apparently does not correlate with the presence of transcription complexes.

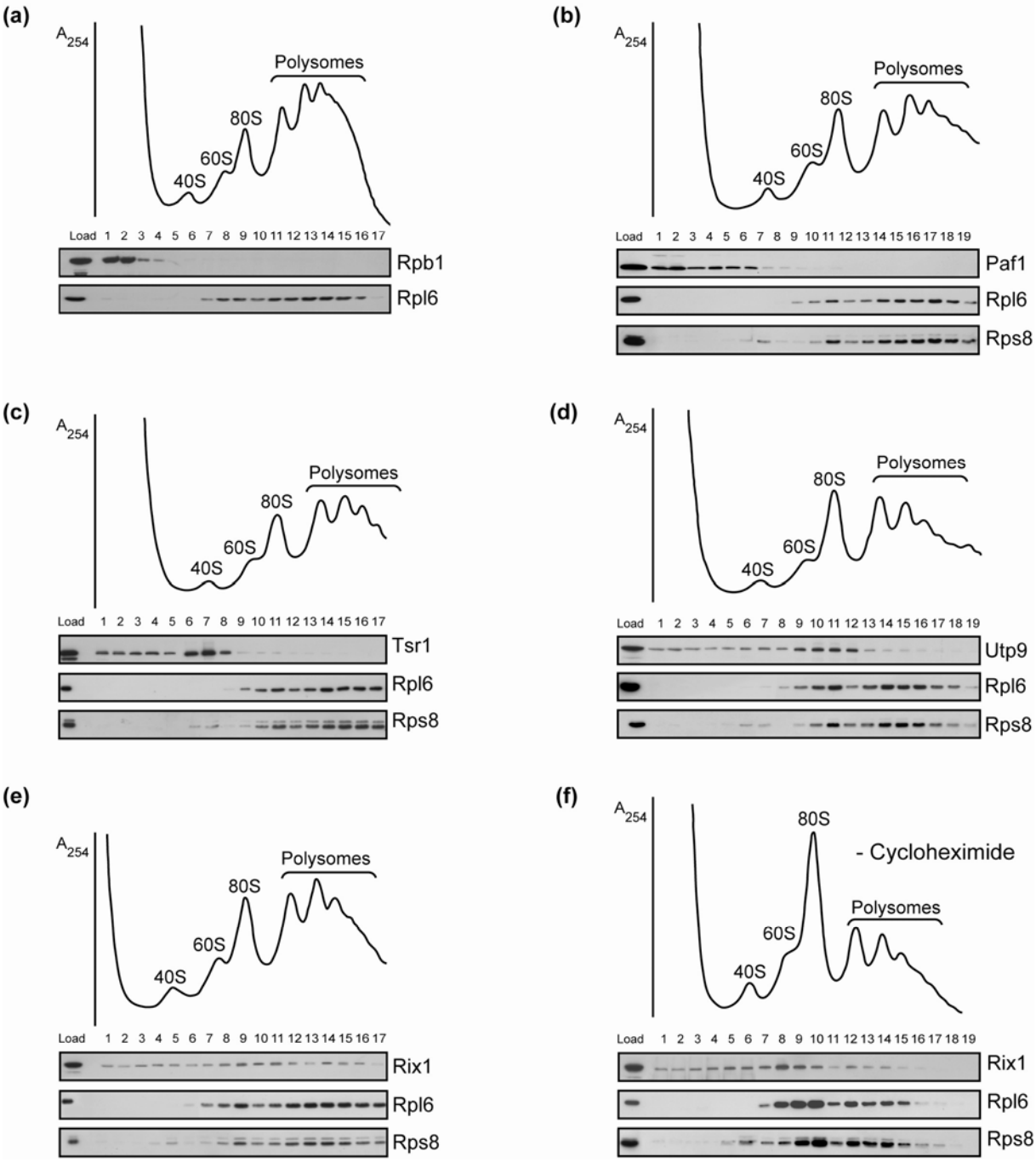


Figure 18: The sedimentation behaviour of Ctk1 does not correspond to the sedimentation behaviour of transcription or pre-ribosomal complexes.
TAP-tagged proteins were visualized using anti-protein A antibodies. As controls, antibodies against Rpl6 (large ribosomal subunit) and Rps8 (small ribosomal subunit) were used. **(a)** Rpb1, the largest subunit of RNAP II is part of the soluble fraction. **(b)** Paf1, a protein involved in transcription elongation and poly(A) length control is present in the soluble fractions. **(c)** Tsr1, a component of the 40S pre-ribosomal subunit co-migrates with the 40S subunit. **(d)** Utp9, a component of the 90S pre-ribosome sediments in a peak between 80S

monosomes and the polysome fractions. **(e)** Rix 1, a component of the 60S pre-ribosomal subunit, migrates similar to Ctk1. **(f)** In absence of cycloheximide, the migration pattern of Rix1 shifts towards the 60S subunit.

Besides the described function in transcription elongation, Ctk1 has been implicated in RNA polymerase I transcription and the maintenance of the rDNA locus (Bouchoux et al. 2004; Grenetier et al. 2006), suggesting that Ctk1 could be involved in ribosome biogenesis and could therefore be associated with ribosomal precursor particles. Thus, the migration of exemplary proteins of 90S, pre-40S and pre-60S particles was analyzed in sucrose density gradients. Tsr1, a protein of the pre-40S particle, involved in 18S rRNA processing and 40S maturation (Gelperin et al. 2001), showed the expected predominant 43S association (Figure 18c). Utp9, a component of the rRNA processing machinery of the 90S pre-ribosomal particle showed a typical distribution for 90S components, which is characterized by a main peak between 80S and the first polysomal peak (Dragon et al. 2002, Figure 18d). In contrast, Rix1, a component of the pre-60S particle, important for 60S biogenesis and its export into the cytoplasm (Bassler et al. 2001; Nissan et al. 2002), showed a similar distribution as Ctk1 (Figure 18e). Whereas the examples of pre-40S and 90S particles, Tsr1 and Utp9, clearly did not show a sedimentation pattern like Ctk1, the migration behaviour of the pre-60S protein, Rix1 was similar to that of Ctk1, suggesting that Ctk1 might be associated with pre-60S particles.

To distinguish between a general involvement of Ctk1 in translation or in pre-60S biogenesis, polysomes were disrupted by either omission of cycloheximide, which leads to ribosomal run-off from the message, or by the addition of the elongation terminator puromycin. Both methods should not affect ribosome biogenesis and should therefore allow a differentiation between general translation and ribosome biogenesis.
Gradients without cycloheximide are characterized by an increase in 80S monosomes and a concomitant loss of polysomes, reflecting the ribosomal run-off. When cycloheximide was omitted, Ctk1 as well as the ribosomal proteins shifted to the fractions corresponding to the 80S monosomes (Figure 19a), whereas Rix1, the component of the pre-60S particle, shifted to the 60S fractions in absence of cycloheximide (Figure 18f). Although the migration patterns of Rix1 and Ctk1 were similar under standard conditions, the processes these proteins are involved in are most likely different. As Ctk1 shows a similar migration pattern as ribosomal proteins, Ctk1 could be specifically associated with ribosomes engaged in translation. This assumption was further confirmed by the addition of puromycin prior to preparation of the extracts. Puromycin is a structural analogue of the 3’ end of an aminoacyl-adenosin-5’-

phosphate-tRNA and substitutes for an incoming aminoacyl tRNA at the ribosomal A-site. The amino group of puromycin reacts with the carboxyl group of the peptidyl tRNA thereby forming peptidyl-puromycin, which results in premature release of the polypeptide (Nathans 1964). This release is reflected by a complete loss of polysomes and an increase in the 80S monosome peak. In puromycin treated cells, Ctk1 as well as Rpl6 and Rps8 shifted from the high molecular weight fractions to fractions corresponding to the 80S peak (Figure 19b), proving that Ctk1 is specifically associated with translating ribosomes.

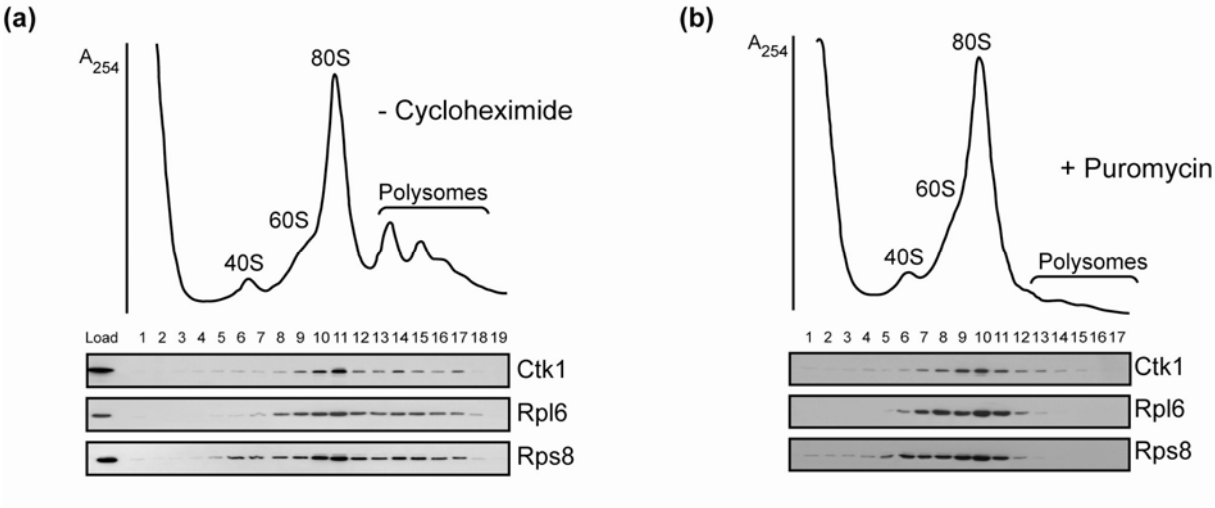


Figure 19: The association of Ctk1 with translating ribosomes is specific.
Omission of cycloheximide or addition of puromycin leads to an increased 80S monosomal peak and a concomitant reduction of polysomes. TAP-tagged proteins were visualized using anti-protein A antibodies. As controls, antibodies against Rpl6 (large ribosomal subunit) and Rps8 (small ribosomal subunit) were used. **(a)** In absence of cycloheximide, Ctk1's distribution within the gradient shifted accordingly to that of ribosomal proteins, resulting in a peak corresponding to 80S monosomes. **(b)** When puromycin is added, Ctk1 and the ribosomal proteins Rpl6 and Rps8 shift to the fractions corresponding to the 80S peak.

2.6 Ctk1 is associated with ribosomes *in vivo*

The results obtained by TAP and with the sucrose density gradients showed that Ctk1 is associated with ribosomes. This was not only in contrast to the described nuclear functions of Ctk1 but also to its predominant nuclear localization (Lee and Greenleaf 1991). It could be that the observed association is due to a specific but artificial binding of Ctk1 to ribosomes, *i.e.* that Ctk1 binds to a distinct domain that is also present in translating ribosomes. To show that Ctk1 is also associated with ribosomes *in vivo*, cells were crosslinked with formaldehyde *in vivo* (Nielsen et al. 2004) and the extracts were prepared under high-salt (500 mM) conditions to dissociate any protein (including Ctk1) from the ribosome that was not present

at the ribosomes during the crosslinking process. Under these conditions, a small amount of Ctk1 was clearly associated with polysomes, monosomes and ribosomal subunits, compared to the sedimentation behavior of Rpl6 (lanes 8, 9, 10, 14, 15, 16) and Rps8 (lanes 6, 10, 15) (Figure 20). That Ctk1 is only partially associated with ribosomes *in vivo*, is expected as Ctk1 is a predominantly nuclear protein (Lee and Greenleaf 1991) and the small amount of Ctk1 present at ribosomes would be consistent with a catalytic function of this protein during translation.

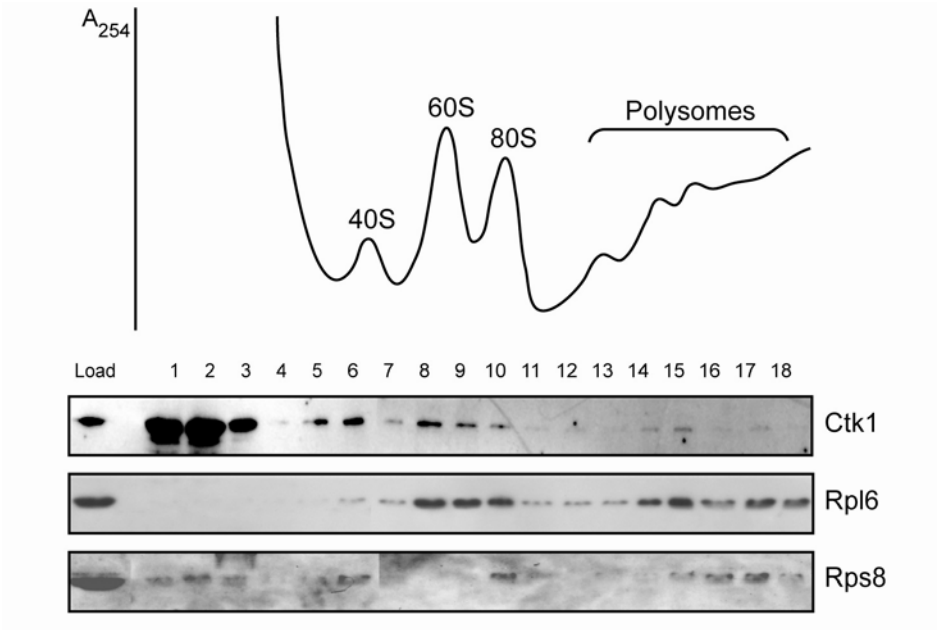


Figure 20: Ctk1 is associated with ribosomes *in vivo*.
Cells were crosslinked using formaldehyde and the extract was prepared in presence of 500-mM KCl to dissociate any protein from ribosomes that was not present during the cross-linking process. Ctk1, Rpl6 and Rps8 were visualized by Western blotting of each fraction of the gradient.

2.7 Ctk2 and Ctk3 also associate with translating ribosomes

Ctk1 was described to constitute together with the cyclin Ctk2 and a third protein Ctk3 the active CTDK-I complex (Sterner et al. 1995). Since it was possible that Ctk1 exhibits its cytoplasmic function independently of Ctk2 and Ctk3, or that *e.g.* only Ctk2 or only Ctk3 are present in a cytoplasmic complex together with Ctk1, it was interesting to see if the other two subunits of the CTDK-I complex are also associated with ribosomes. As shown in Figure 21 the migration pattern of Ctk1 and Ctk3 resembled that of Ctk1. Both proteins were associated with ribosomal subunits, monosomes and polysomes. When ribosomes were disrupted into

40S and 60S subunits by EDTA, Ctk2 (Figure 21b) and Ctk3 (Figure 21d) shifted concomitantly to the fractions containing either the ribosomal subunits or the soluble proteins. Thus, Ctk2 and Ctk3 also associate with polysomes, indicating that Ctk1, Ctk2 and Ctk3 function together in a cytoplasmic CTDK-I complex. This finding supports the hypothesis of a catalytic function of Ctk1 in translation as it was shown that deletion of either of the subunits renders the kinase inactive (Hautbergue and Goguel 1999; Hautbergue and Goguel 2001).

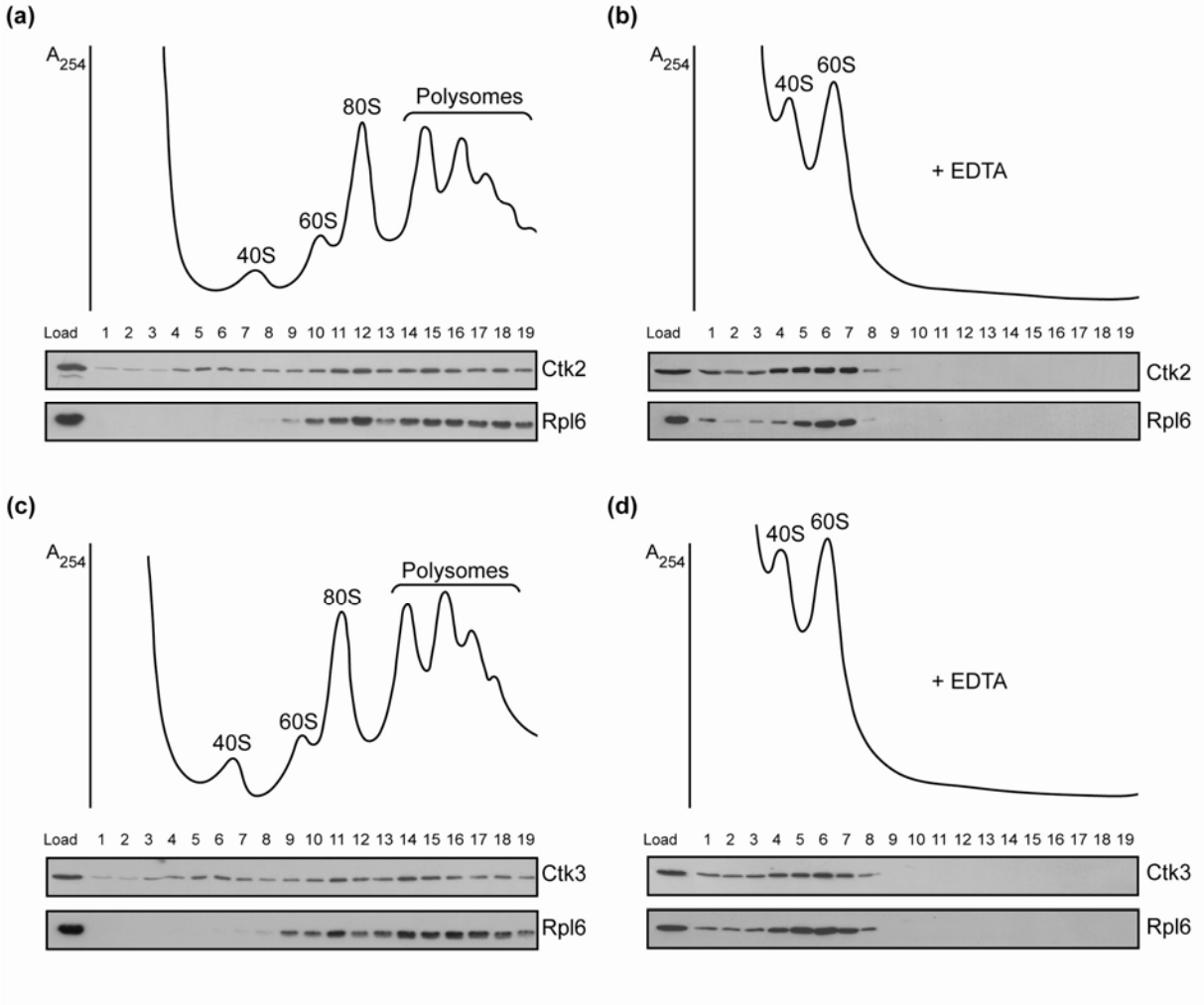


Figure 21: Ctk2 and Ctk3 also associate with translating ribosomes.
The migration pattern of TAP-tagged Ctk2 and Ctk3 was analyzed by Western blotting using an anti-protein A antibody. The sedimentation behavior of large ribosomal protein Rpl6 was assessed by an antibody directed against Rpl6 and served as a reference. Ctk2 **(a)** and Ctk3 **(c)** are present at translating ribosomes. Upon addition of EDTA the ribosome is disrupted and Ctk2 **(b)** and Ctk3 **(d)** shift to the subunits and soluble fractions.

2.8 Loss of Ctk1 function causes a decrease in translational activity in *in vitro* translation extracts

The association of Ctk1, Ctk2 and Ctk3 with translating polysomes suggested an involvement of the CTDK-I complex in translation. Usually, in order to elucidate a possible function of a protein in translation, a factor-free (such as amino acids, ions) translation competent extract is prepared and the influence of the protein can be investigated *in vitro* by measuring the incorporation of a radioactively labelled amino acid into peptides. The comparison of the translational activity of *Δctk1* extracts to wt extracts showed that deletion of *CTK1* resulted in a decrease in translation to about 10% (Figure 22a) of wt activity, indicating that Ctk1 seems to be necessary for efficient translation.

Ctk1 is known to be important for RNA polymerase II transcription (Cho et al. 2001), and the recruitment of 3'-mRNA processing factors to the nascent mRNA during transcription (Ahn et al. 2004). Therefore it could be that the lack of translational activity with endogenous mRNA in extracts prepared from *Δctk1* cells is due to less protein or that the mRNA is not as well translatable as RNA from wt cells, for example because the poly(A)-tail is truncated. To address this issue, mRNA was prepared from wt cells and tested in *in vitro* translation assays after destruction of endogenous mRNA with nuclease. Figure 22b shows that the translational activity of *Δctk1* cells was also decreased to about 20% when wt RNA was added in the translation reaction. As an additional template, *in vitro* transcribed luciferase mRNA was included in the analysis. In extracts prepared from *Δctk1* cells luciferase activity is reduced to about 10% in comparison to wt (Figure 22c).

Importantly, mRNA prepared from *Δctk1* cells was translated in wt extracts as efficiently as mRNA from wt cells (Figure 22d), indicating that the mRNA of *Δctk1* cells is fully translatable and can therefore not account for the observed defects in translation. Furthermore, the extracts of wt and *Δctk1* cells used in the in vitro translation assays contained the same amount of ribosomal proteins as assessed by Western blotting against Rps8 and Rpl6 (Figure 22e). Taken together, neither the quality of the mRNA nor the amount of ribosomal proteins can account for the observed decrease in translation of *Δctk1* cells, implicating a novel function for Ctk1 in efficient translation.

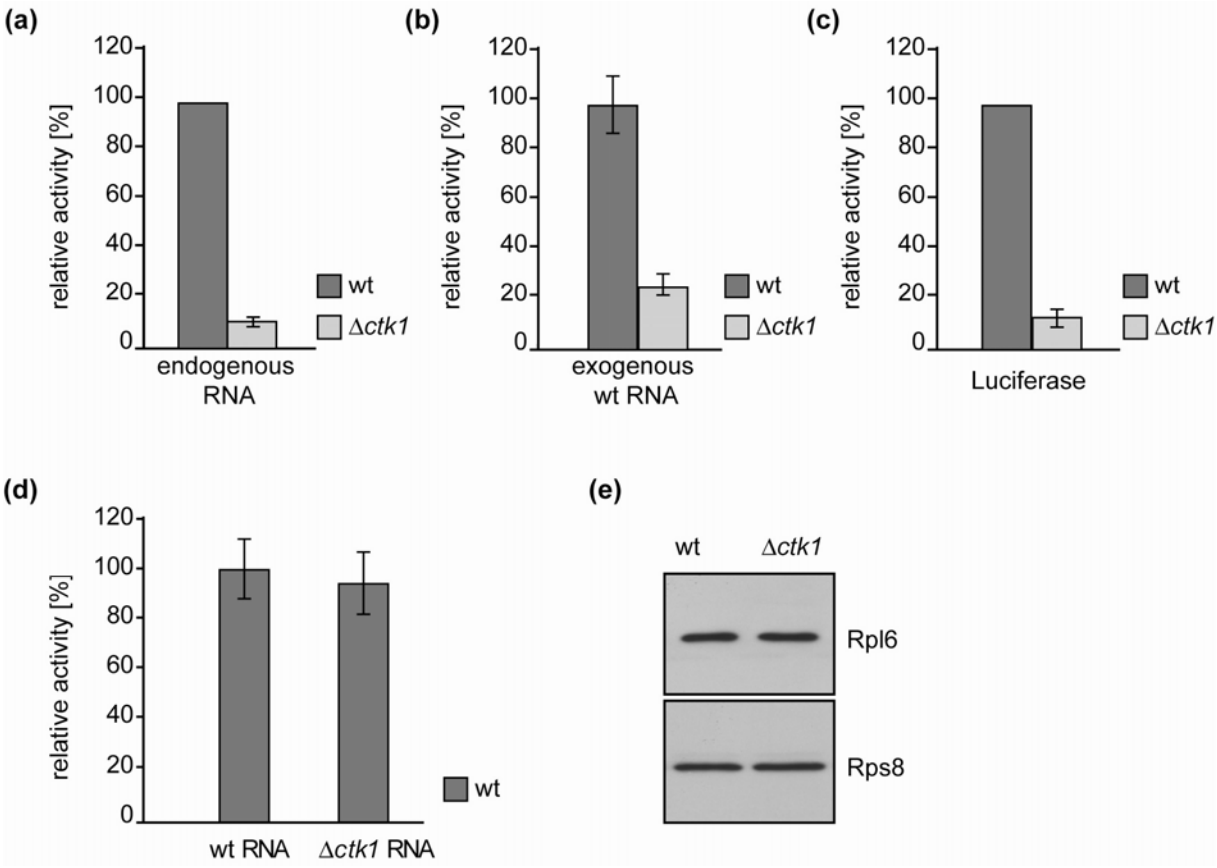


Figure 22: Ctk1 is needed for efficient translation.
Translation competent extracts were prepared from wt cells (dark grey) and *Δctk1* cells (light grey) and equal amounts were tested in the translation reaction. The translational activity of extracts prepared from *Δctk1* cells with endogenous mRNA **(a)**, exogenous wt mRNA **(b)** and *in vitro* transcribed luciferase mRNA **(c)** is reduced in comparison to extracts prepared from wt cells. **(d)** mRNA prepared from *Δctk1* cells is fully translatable in extracts from wt cells. **(e)** Extracts prepared from wt and *Δctk1* cells contain equal amounts of ribosomal proteins as assessed by Western blotting using antibodies specific for a protein of the large (Rpl6) and the small (Rps8) ribosomal subunit. Translational activity of wt and *Δctk1* extracts using wild type or *Δctk1* mRNA as a template was determined after destruction of endogenous mRNA with micrococcal nuclease and incubation with L-[^{35}S]-methionine. Luciferase activity of wt and *Δctk1* extracts was determined by measuring the luminescence of the luciferase protein after the translation reaction. For all experiments wild type activity was set to 100% and the decrease in activity of extracts lacking Ctk1 was calculated accordingly. Values and error bars represent the results of two *in vitro* translation measurements for three independent extracts each.

As the deletion of *CTK1* leads to a severe growth phenotype (Figure 8, Figure 23b and c), the observed decrease in translation might be a secondary effect, caused by an accumulation of various defects. To exclude this possibility, a genomic depletion system for *CTK1* was designed based on a strain carrying a C-terminal TAP-tagged version of Ctk1 (to be able to determine protein levels using the anti-protein A antibody) driven by the *GAL1* promoter (*GAL1::CTK1-TAP*). Consequently, in galactose-containing media (YPG) Ctk1 will be

expressed, whereas in glucose-containing media (YPD) the expression will be repressed (Figure 23a).

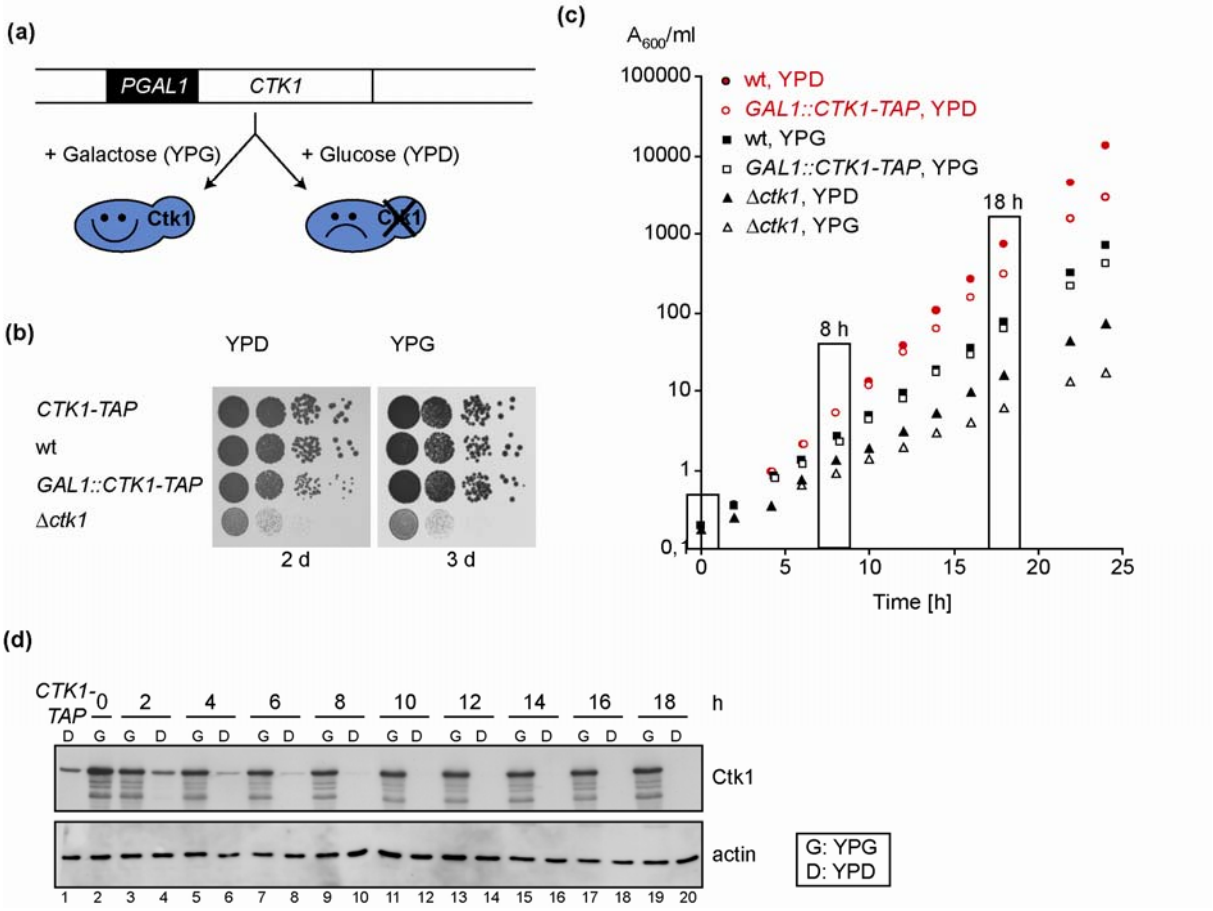


Figure 23: Depletion system for Ctk1.
(a) Schematic illustration of the employed depletion system. In galactose-containing medium (YPG), Ctk1 is expressed whereas in glucose-containing medium (YPD) the expression of Ctk1 is repressed. **(b)** Depletion of Ctk1 leads to slower growth on plates containing glucose. 10-fold serial dilutions of *CTK1-TAP*, wt, *GAL1::CTK1-TAP* and *Δctk1* cells spotted onto YPD and YPG plates after incubation at 30°C for 2 and 3 days, respectively. **(c)** Growth curves of wt, *GAL1::CTK1-TAP*, or Δ*ctk1* cells in galactose containing medium (YPG, open circles, squares and triangles) and after shift to glucose containing medium (YPD, filled circles, squares and triangles) by measuring optical density at 600 nm. **(d)** Ctk1 is not detectable anymore on a Western blot after 8 h in glucose-containing medium. The same amount of protein extracted from cells of the *GAL1::CTK1-TAP* strain grown in galactose or glucose-containing medium at the indicated time points was subjected to SDS-PAGE and Western blotting. As loading control the amount of actin was assessed.

When growth of wt, *GAL1::CTK1-TAP* and *Δctk1* cells in YPG and YPD was measured, it turned out that the expression of Ctk1 from the *GAL1* promoter leads to a slight reduction in growth (Figure 23b and c, compare wt and *GAL1::CTK1-TAP* in YPG) and as a expected to overexpression of Ctk1 (Figure 23d; compare first two lanes). The growth rate of a wt and the *GAL1::CTK1-TAP* strain was identical for the first 8 hours after shift from YPG to YPD,

whereas after 8 hours *GAL1::CTK1-TAP* started to grow at a lower rate than the wt (Figure 23c). Corresponding to the measured growth rates, the expression of Ctk1 was detectable for the first 8 h after shift to glucose-containing medium (Figure 23d, lanes 4, 6, 8, and 10). After 18 h in YPD, the Ctk1-depleted cells (*GAL1::CTK1-TAP*, YPD) grew more slowly than the wild type strain (wt, YPD), but still significantly faster than the *Δctk1* strain (*Δctk1*, YPD, Figure 23c), which was also evident after two days of growth on plates (Figure 23b; YPD).

To test for a direct effect of Ctk1 in translation, extracts were prepared from cells grown in i) YPG, where the translational activity should be similar to wt cells, ii) after a shift to YPD for 8 h, when the cells grow still as wt, but Ctk1 is not detectable anymore as assessed by Western blotting and iii) after growth for 18 h in YPD, where the cells grow significantly slower than wt.

Wt and *GAL1::CTK1-TAP* cells grown in galactose, *i.e.* Ctk1 is expressed, show the same translational activity with endogenous mRNA present in *in vitro* translation extracts, exogenous wt mRNA, and *in vitro* transcribed luciferase mRNA (Figure 24a). When *GAL1::CTK1-TAP* cells were grown for 8 h in YPD, *i.e.* Ctk1 is repressed, the translational activity of these extracts dropped to about 70% compared to extracts from a wt strain (Figure 24b). Depleting the extracts for 18 hours of Ctk1 by growth in YPD resulted in a drop of translational activity to about 30% of the activity of wt cells (Figure 24c), comparable to the effect observed in extracts prepared from *Δctk1* cells (Figure 22). The observed decrease in translation activity is (as in the case of *Δctk1* extracts) not due to the presence of less ribosomal proteins, because the amount of ribosomal proteins was identical when equal amounts of the translation extracts were tested (Figure 24d). Accordingly, the translatability of the mRNA of Ctk1-depleted cells was tested. The mRNA from either strain (wt in YPG and YPD and *GAL1::CTK1-TAP* in YPG and YPD) was equally well translatable in wt extracts. Thus, the above experiments suggested that loss of Ctk1 function leads to a decrease in translational activity, most likely in a direct manner as the decrease in translation occurs prior to an impairment of growth. However, when the CTDK-I complex was purified and added to *in vitro* translation extracts prepared from wt or *GAL1::CTK1-TAP* cells after growth for 18 hours in YPD, the activity of the depleted extract could be rescued from 20 to only about 25% (data now shown). Even though this result argues against a direct involvement of Ctk1 in translation, it might be that Ctk1 recruits another component important for efficient translation to the ribosome. As such a component would be absent from extracts prepared from cells depleted of Ctk1, the sole addition of the CTDK-I complex could not restore translational activity.

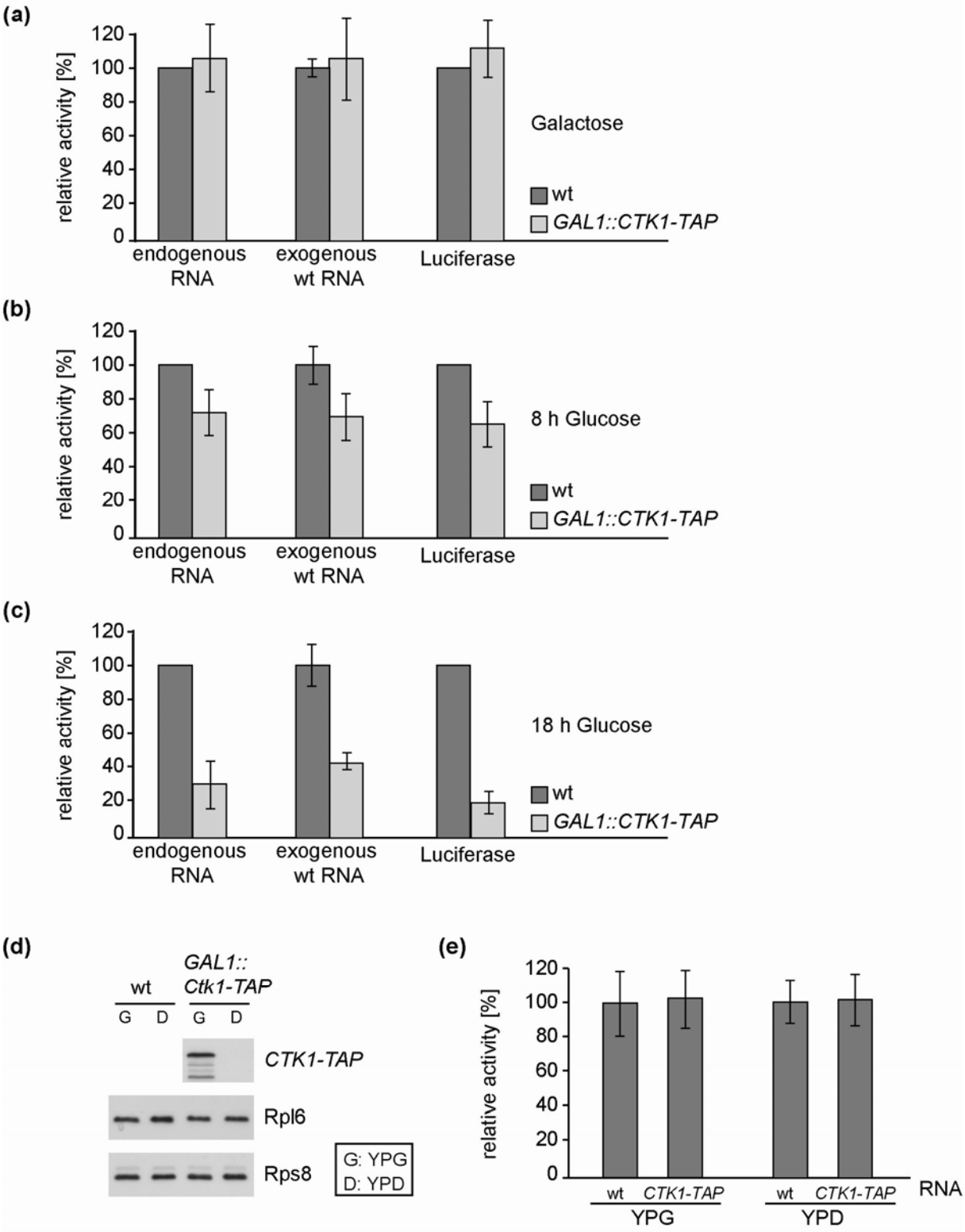


Figure 24: Depletion of Ctk1 causes a translation defect prior to impairment of growth.
(a) Translation extracts were prepared from wt and *GAL1::CTK1-TAP* cells grown in galactose-containing medium. Both extracts have the same translational activity when tested with endogenous mRNA (left panel), exogenous wt RNA (middle panel) or *in vitro* transcribed luciferase mRNA. **(b)** Depletion of Ctk1 for 8 h leads to a reduced level in translation. When wt and *GAL1::CTK1-TAP* cells are shifted for 8 h to medium containing

glucose prior to preparation of extracts, the translational activity drops to about 70% with either mRNA template, in comparison to wt extracts. **(c)** When Ctk1 was depleted for 18 h by growth in glucose-containing medium, the translational activity of the extracts was reduced to 30%. Values and error bars represent the results of two *in vitro* translation measurements obtained for three independent extracts each. **(d)** After 18 h depletion in glucose-containing medium, the extracts analyzed in (c) contain equal amounts of ribosomal proteins as assessed by Western blotting using antibodies specific for a protein of the large (Rpl6) and the small (Rps8) ribosomal subunit. G indicates growth in galactose-containing medium, YPG; D indicates growth in glucose-containing medium, YPD. **(e)** mRNA extracted from *GAL1::CTK1-TAP* cells either grown in galactose or after an 18 h shift in glucose-containing medium is as translatable as wt mRNA under the same growth conditions. Translational activity was determined as described in Figure 22.

2.9 Ctk1 is needed for efficient translation *in vivo*

The above experiments suggested that loss of Ctk1 function leads to a decrease in translation activity. However, *in vitro* systems are a lot more sensitive towards changes than *in vivo* systems. Therefore, the potential translation defect was also assessed by *in vivo* labelling of cells deleted for *CTK1* or depleted for Ctk1 for 8 or 18 hours with L-[^{35}S]-methionine in order to detect the rate of newly synthesized proteins. Both, *Δctk1* cells as well as cells depleted for Ctk1 for 8 hours, synthesize less protein than the corresponding wt strain (Figure 25, compare lanes 1 to 2 and lanes 3, 4 and 5 to 6). This effect is increased when the cells were depleted of Ctk1 for 18 hours (Figure 25, compare lanes 6 and 7 to 8). Thus, loss of Ctk1 function also leads to a reduced translational activity *in vivo*. Even though it cannot be excluded that this reduced level of protein synthesis is due to lower levels of mRNA or ribosomes, based on the *in vitro* translation assays it is conceivable that at least a part of this protein synthesis defect is due to a function of Ctk1 in translation that cannot be accomplished in cells lacking Ctk1.

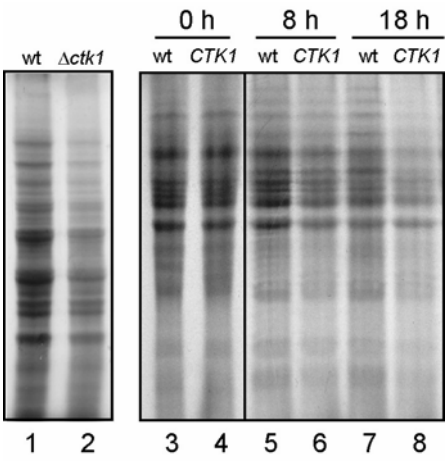


Figure 25: Translation activity in cells lacking Ctk1 is also reduced *in vivo*.
Deletion of Ctk1 causes a decrease in translation (lane 1, 2). *GAL1::CTK1-TAP* and wt cells grown in galactose do not show a defect in protein synthesis (lane 3, 4), whereas depletion of Ctk1 for 8 h (lane 5, 6) and 18 h (lane 7, 8) in glucose-containing medium results in a decrease in protein synthesis in comparison to wt. Cells were labelled with L-[^{35}S]-methionine, whole cell extracts prepared and analyzed by SDS-PAGE and autoradiography.

2.10 The function of Ctk1 in translation

The finding that Ctk1 is associated with translating ribosomes and that cells deleted or depleted for Ctk1 show a reduced translational activity *in vivo* and *in vitro*, pinpointed to a novel function of Ctk1 in the translation of mRNA. However, by which means Ctk1 precisely exhibits its function in translation was unclear.

2.10.1 Ctk1 is not involved in ribosome biogenesis

Even though it could be demonstrated that the mRNA of *Δctk1* cells is exported to the cytoplasm (Figure 14) and that these mRNAs were fully translatable in *in vitro* translation assays (Figure 22), one likely explanation for the deficient translation could be that Ctk1 functions in ribosome biogenesis, especially since it was shown that the RNAP I transcription pattern and most likely as an effect the structure of the nucleolus is altered in *Δctk1* cells (Bouchoux et al. 2004; Grenetier et al. 2006). Defects in ribosome biogenesis can be investigated by several methods. It is known that polysome profiles of dissociated ribosomes can be indicative for a defect in ribosome biogenesis as the ratio of 40S to 60S would change in case of ribosome maturation defects (Dong et al. 2005). When gradients of EDTA-treated extracts from wt and *Δctk1* cells were analyzed with regard to the relative ratio between 40S and 60S, it turned out that this ratio did not change significantly in *Δctk1* cells (Figure 26a), suggesting that the deletion of *CTK1* does not lead to a severe defect in 40S or 60S ribosome biogenesis.

Furthermore, defects in early ribosome biogenesis can be observed by accumulation of GFP-tagged ribosomal proteins in the nucleolus or nucleoplasm, under conditions where the export is either directly blocked or maturation defects prevent the proper export of the ribosomal particles in the cytoplasm (for review see Tschochner and Hurt 2003). As the export of ribosomal subunits is dependent on the nuclear export receptor Xpo1, a ts mutant of Xpo1 served as a positive control for the nuclear accumulation of GFP-tagged reporter plasmids for the small, Rps2, and the large, Rpl25, ribosomal subunits (Stade et al. 1997; Moy and Silver 1999; Gadal et al. 2001). Figure 26b shows that in the *xpo1* mutant the proteins of the small and the large ribosomal subunits clearly accumulated inside the nucleus, whereas in *Δctk1* and wt cells the GFP signal was evenly distributed all over the cytoplasm, indicating that the loss of Ctk1 function does not lead to a nuclear export defect of the subunits. Although it cannot be completely ruled out that deletion of Ctk1 leads to a minor ribosome biogenesis defect, a major function of Ctk1 in ribosome biogenesis is rather unlikely.

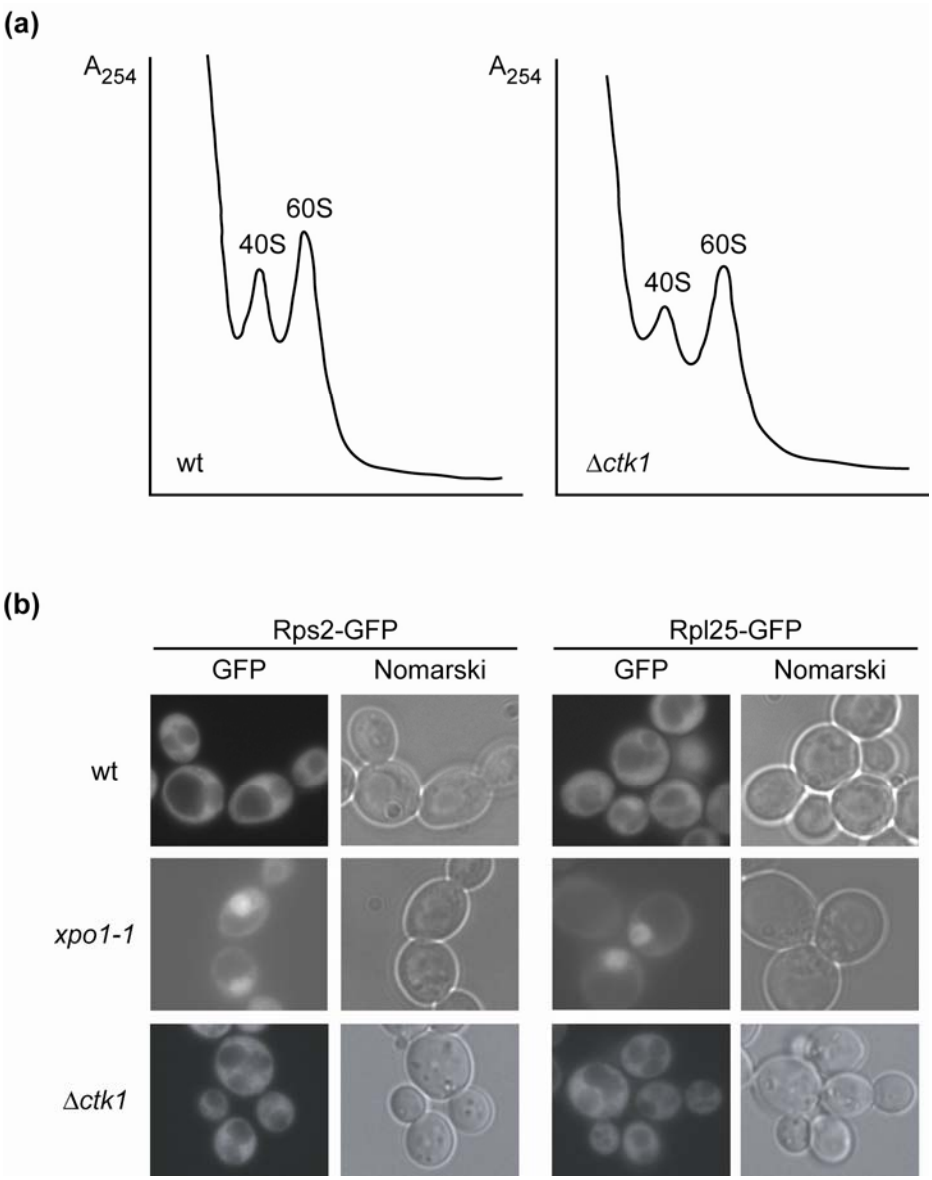


Figure 26: Deletion of Ctk1 does not lead to a defect in ribosome biogenesis.
(a) The ratio of 40S to 60S subunits does not change significantly in *Δctk1* cells in comparison to wt cells. 15-30% sucrose density gradients were performed with extracts of wt and *Δctk1* cells treated with EDTA to disrupt the ribosomes into the subunits. **(b)** Ribosomal subunits are exported into the cytoplasm in *Δctk1* cells. The localization of GFP-tagged Rps2 as a protein of the small ribosomal subunit and GFP-tagged Rpl25 as a protein of the large ribosomal subunit was analyzed in living cells.

2.10.2 Ctk1 influences translation initiation

The *in vitro* translation system described above mainly measures translation initiation as prior to addition of L-[^{35}S]-methionine no methionine is present in the extract and consequently, the translation process is mainly arrested at the stage of initiation when the assay is started. Defects in initiation can be visualized by profiles of polysome density gradients because a

defect in translation initiation results in an increased 80S peak and reduced polysome level (Zhong and Arndt 1993; Nielsen et al. 2004; Dong et al. 2005). When polysome profiles of wt and *Δctk1* cells were compared, it was obvious that the deletion of Ctk1 lead to an increase of monosomes (80S) and a concomitant reduction in polysomes (Figure 27), suggesting that Ctk1 has a function in initiating translation.

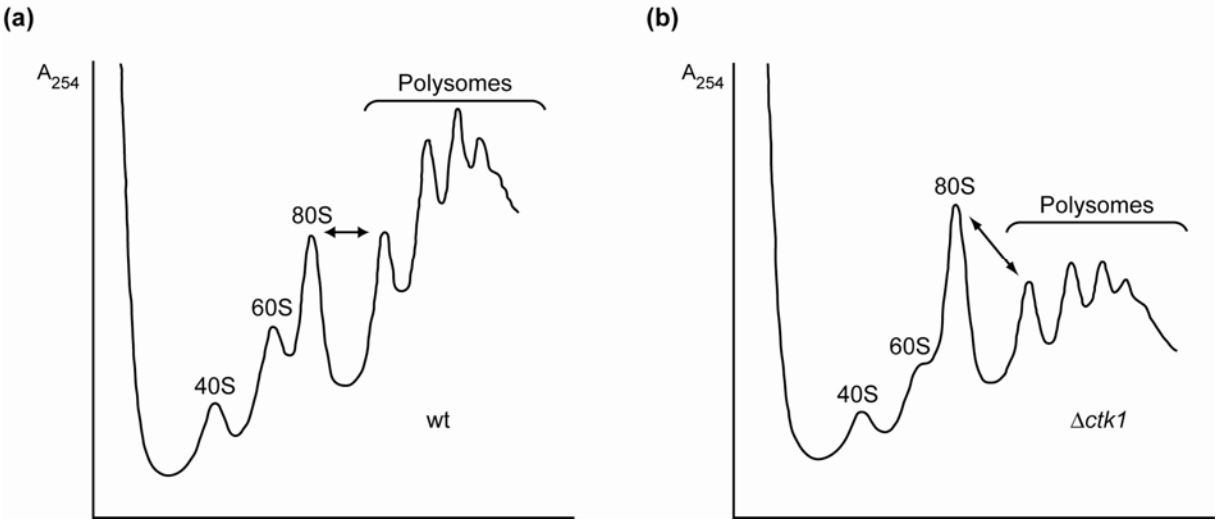


Figure 27: Deletion of Ctk1 leads to an increase in 80S monosomes and a simultaneous reduction in polysomes.
Extracts of WT **(a)** and *Δctk1* cells **(b)** were analyzed by sucrose density centrifugation.

2.10.3 Ctk1 depleted cells are sensitive towards drugs that influence translation

To uncover an unknown function of a protein, it is a common approach to analyze sensitivity or resistance of a mutant strain towards drugs that affect the process of interest. Thus, the sensitivity of wt and *GAL1::CTK1-TAP* cells grown for 18 hours in YPD, *i.e.* Ctk1 depleted, were tested for sensitivity towards the translational inhibitors paromomycin, hygromycin B, geneticin, cycloheximide and anisomycin. Cells depleted for Ctk1 are significantly more sensitive to all drugs tested than an isogenic wild type strain (Figure 28). Hypersensitivity towards paromomycin, hygromycin B and geneticin has often been linked to loss of translational accuracy, as these aminoglycoside antibiotics bind to the decoding region of the ribosomal A-site and stimulate the stable association of a near-cognate aminoacyl tRNA (Palmer et al. 1979; Singh et al. 1979; Moazed and Noller 1987). This result suggests that

besides the putative function in translation initiation, Ctk1 might be needed for the correct decoding of the mRNA.

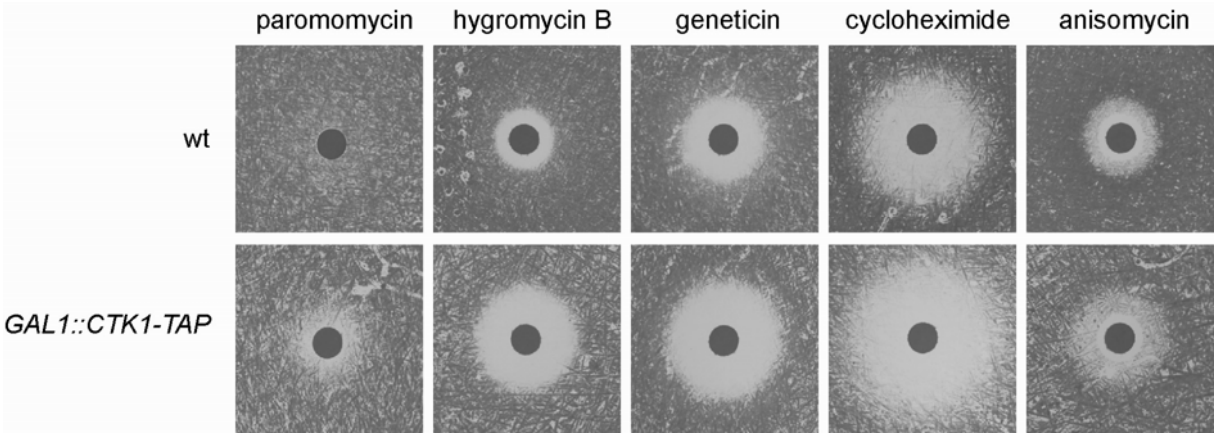


Figure 28: Cells that are depleted for Ctk1 are sensitive towards translation inhibitors.
Wt and *GAL1::CTK1-TAP* cells were grown for 18 hours in glucose-containing medium and then spread on YPD plates containing a filter on which paromomycin, hygromycin B, geneticin, cycloheximide, or anisomycin were applied. The size of the halo indicates the sensitivity of the strain towards this drug.

2.11 Ctk1 functions in translation elongation

The so far obtained results suggested that Ctk1 is not only important for translation initiation but might be also responsible for the correct decoding of the message during translation elongation. Thus, the defect observed in the *in vitro* translation assay with L-[^{35}S]-methionine most likely resulted from a combination of defects in both translation initiation and elongation. A versatile assay to study elongation apart from initiation is to perform *in vitro* translation assays with poly(U) homopolymers as mRNA. In this assay peptide formation is independent of translation initiation factors (Wyers et al. 2000) and the incorporation of radioactive labelled L-[^{14}C]-phenylalanine, resulting in poly-phenylalanine peptides, can be measured (Figure 29a, left arrow). This system allows not only to assess translation elongation but also to determine a potential miscoding frequency using radioactive labelled L-[^{14}C]-leucine. Leucine is incorporated into the poly-phenylalanine product due to pairing of the UUU codon with the near-cognate UAA or CAA of Leu-tRNAs (Figure 29a, right arrow).

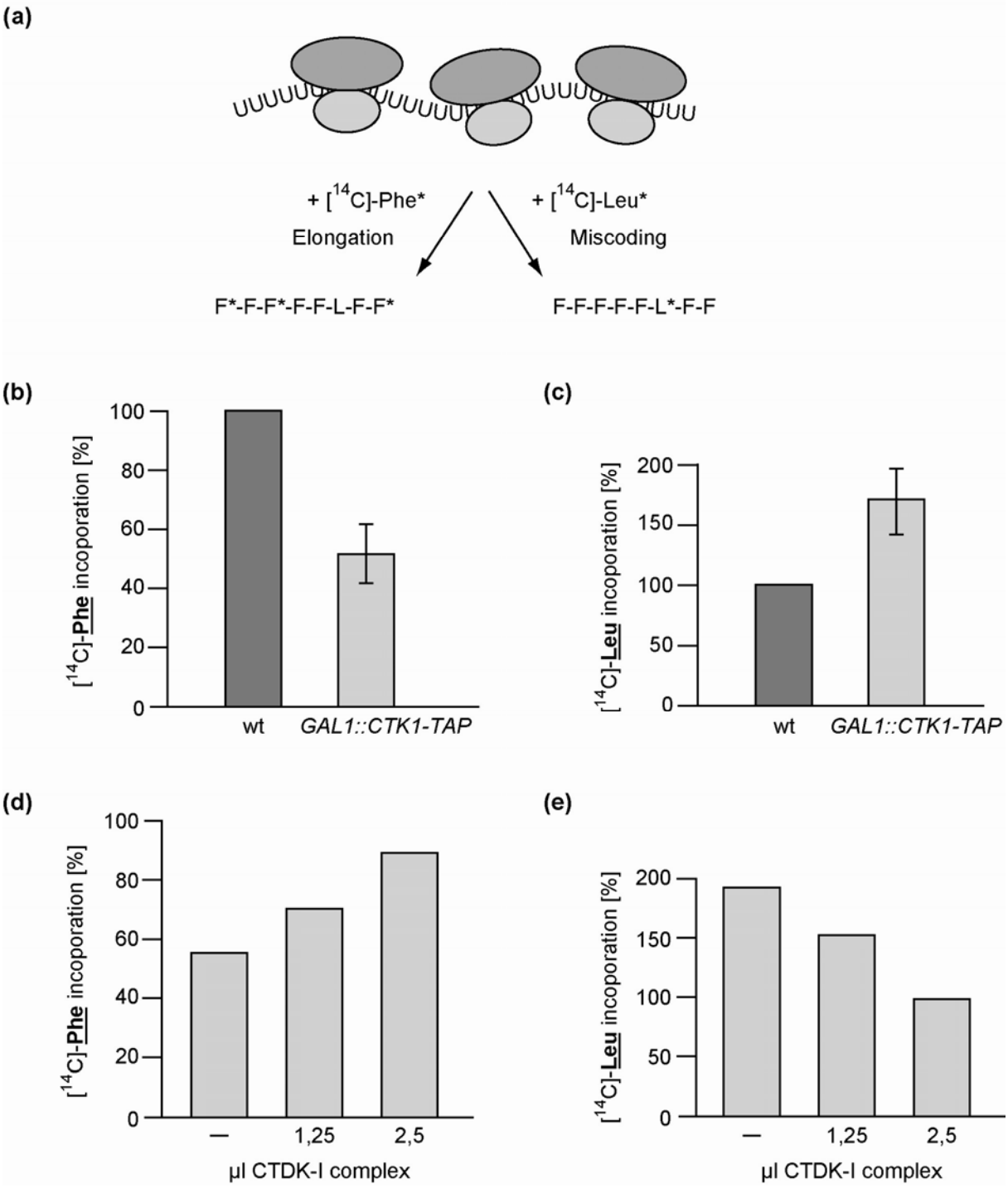


Figure 29: Ctk1 is important for translation elongation and maintenance of translational accuracy.

(a) Schematic illustration of the poly(U) assay. The efficiency of translation elongation was determined by measuring the incorporated L-[^{14}C]-phenylalanine (F*) and miscoding events were determined by the incorporation of L-[^{14}C]-leucine (L*). **(b)** Depletion of Ctk1 leads to a decrease in translation elongation. The incorporation of L-[^{14}C]-phenylalanine into the polypeptide chain of nuclease treated extracts prepared from wt and *GAL1::CTK1-TAP* cells after growth for 18 hours in glucose-containing medium was determined. **(c)** Cells depleted for Ctk1 show an increase in miscoding events in comparison to WT as determined by the ratio of incorporated cpm L-[^{14}C]-leucine divided by cpm L-[^{14}C]-phenylalanine. The activity

of wt was set to 100% in both experiments. **(d)**, **(e)** The defect of Ctk1 depleted cells in translation elongation and translational accuracy can be restored by addition of purified CTDK-I complex in a dose-dependent manner. Purified CTDK-I complex was added to extracts of cells depleted for Ctk1 for 18 h or wt cells and the efficiency of elongation and miscoding was determined as described. As negative control an eluate of a non-tagged wild type strain was added to the translation assays, and the activities of the CTDK-I treated extracts were calculated relative to the mock treated extracts. Due to variations in the CTDK-I purification, one representative experiment out of three independent experiments is shown.

When the translation activity of wt extracts and extracts depleted for Ctk1 (*GAL1::CTK1-TAP*, grown for 18 h in YPD) was analyzed for incorporation of phenylalanine, it turned out that the activity decreased to about 50% in comparison to wt (Figure 29b). However, this decrease in translation elongation might be due to a higher amount of misincorporated amino acids, because the miscoding activity as measured by the ratio of incorporated leucine versus phenylalanine was nearly doubled in cells when Ctk1 was depleted (Figure 29c), indicating that Ctk1 is indeed important for the efficiency and accuracy of the elongation process.

The use of the poly(U) assay allowed a differentiation between initiation and elongation. When the incorporation of L-[^{35}S]-methionine was measured, the translation activity of a *GAL1::CTK1-TAP* extract grown in YPD could be only rescued from 20 to 30% by addition of the CTDK-I complex to the *in vitro* translation assays (data not shown). However, addition of increasing amounts of CTDK-I complex in the poly(U) elongation/miscoding assay resulted in a concomitant increase from 50% elongation activity of the extract depleted for Ctk1 to almost wild type levels (Figure 29d). Accordingly, the amount of miscoding events decreased from almost double to about wild type levels when CTDK-I was added in increasing amounts (Figure 29e). Apparently, an isolated Ctk1-Ctk2-Ctk3 complex is able to restore a translation elongation defect and is responsible for a more accurate translation, proving that Ctk1 plays a direct role in translation elongation, *i.e.* Ctk1 promotes efficient and accurate decoding of the message.

2.12 Ctk1 is needed for correct decoding *in vivo*

The increased sensitivity of cells depleted for Ctk1 towards drugs that impair translation accuracy and the increase in misincorporation of leucine using a poly(U) template suggested that Ctk1 is involved in the decoding of the message. In order to test, whether the accuracy of translation is also affected *in vivo*, an assay system developed by Stahl and Cassan was employed (Stahl et al. 1995; Bidou et al. 2000). This system consists of a set of reporter plasmids encoding for β-galactosidase, a linker, and luciferase (Figure 30a). The linker contains either a readthrough sequence, where the luciferase sequence is translated as well as the β-galactosidase sequence, or one of the three stop codons or a +1 or -1 frameshift. Except for the readthrough construct, the luciferase gene is only expressed if a miscoding event occurs or if the ribosome fails to maintain the correct reading frame. For normalization of the amount of mRNA available for translation the luciferase activity is divided by the β-galactosidase activity. By this, any effects that might be due to a transcriptional or export defect, or degradation of the message are eliminated. This ratio is then normalized by the luciferase / β-galactosidase ratio of the construct with the readthrough linker. This excludes any effects that only affect the 3' end of the message, *e.g.* a decrease in transcription elongation efficiency.

When β-galactosidase and luciferase activity were determined for each of the reporter plasmids, it turned out that the depletion of Ctk1 lead to an approximately 1.5-fold increase in miscoding with each of the three stop codon constructs as compared to the same strain expressing Ctk1, *i.e.* grown in galactose (Figure 30b, columns UAA, UAG, UGA). In contrast, the frequency of a +1 or -1 frameshift (FS) event was not altered when Ctk1 was depleted (Figure 30b, columns -1 FS and +1 FS), suggesting that Ctk1 specifically affects translation fidelity *in vivo* by altering the specificity of the ribosome towards the correct amino acid.

The lower translational accuracy in Ctk1 depleted cells might also affect the translation elongation rate. *In vivo*, the relative speed of translation elongation can be assessed by polysome run-off experiments. General translation initiation was inhibited by glucose starvation and polysome profiles of cells after 30 s, 60 s and 120 s growth in medium without glucose were analyzed. Compared to wt cells, Ctk1 depleted cells showed a slightly faster run-off of polysomes as indicated by the faster increasing 80S peak, pointing to a more rapid but also more inaccurate elongation in Ctk1 depleted cells.

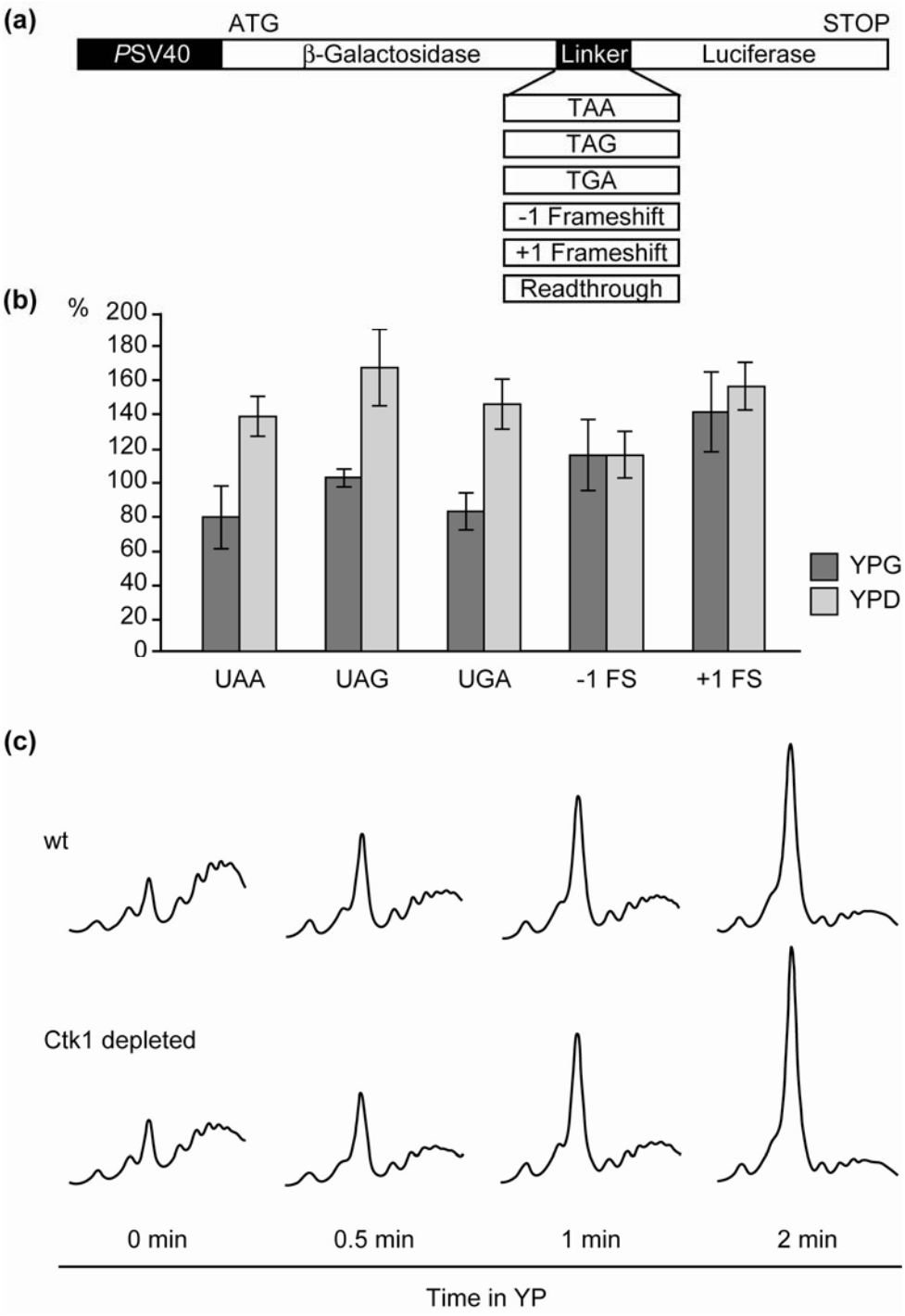


Figure 30: Depletion of Ctk1 leads to a misincorporation of amino acids at the site of stop codons.

(a) Assay system to measure translational accuracy (Stahl and Cassan). Each plasmid codes for β-galactosidase, a linker and luciferase. The linker contains a readthrough sequence, one of the three stop codons or a +1 or -1 frameshift. Luciferase is only expressed if the message is translated through a stop codon or the ribosome fails to maintain the correct decoding frame. **(b)** Cells depleted for Ctk1 (YPD) show an increase in the frequency of miscoding for all three stop codons (UAA, UAG and UGA) compared to cells expressing Ctk1 (YPG). An increased rate of frameshift events does not occur (-1 FS and +1 FS). **(c)** Depletion of Ctk1 leads to a faster polysome run-off after inhibition of translation initiation. Wt and *GAL1::CTK1-TAP* cells grown in YPD were shifted to medium without glucose (YP) and after the indicated time points polysome profiles were analyzed.

2.13 Ctk1 functions in translation accuracy by phosphorylating Rps2, a protein of the small ribosomal subunit

The above results suggested that Ctk1 is implicated in translation elongation and is probably needed for the correct decoding of the message. Since Ctk1 is the kinase of the CTDK-I complex and it was shown that the other two subunits are also associated with ribosomes (Figure 21), it seemed reasonable to assume that Ctk1 fulfils its function by phosphorylating a ribosomal protein.

2.13.1 Ctk1 phosphorylates a protein of the small ribosomal subunit

In order to identify the novel substrate of Ctk1, *in vitro* kinase assays with purified CTDK-I complex and 80S ribosomes were performed. CTDK-I (Ctk1-TAP) complex alone (together with radioactively labelled ATP) resulted in two bands, corresponding to autophosphorylated Ctk1 and phosphorylated Ctk3 (Lee and Greenleaf 1991). However, addition of 80S ribosomes produced a third band at around 30 kDa (Figure 31a), showing that Ctk1 can phosphorylate the ribosome *in vitro*. To distinguish whether Ctk1 phosphorylates the small or the large ribosomal subunit, 80S monosomes were separated into 40S and 60S subunits and incubated with CTDK-I in an *in vitro* kinase assay. Only the assay with 40S subunits showed an enrichment of the third band in the autoradiograph, indicating that Ctk1 most likely phosphorylates a ribosomal protein of the small subunit.

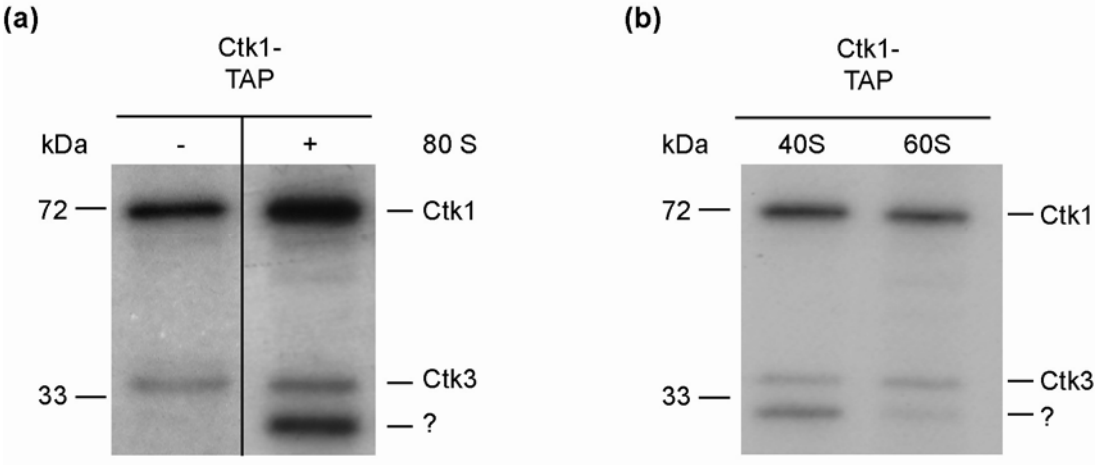


Figure 31: Ctk1 phosphorylates a protein of the small ribosomal subunit.
Ctk1 was purified by TAP in a high salt buffer and 80S, 40S and 60S subunits were purified by sucrose density centrifugation according to Algire et al. 2002. **(a)** *In vitro* kinase assays with CTDK-I, 80S ribosomes and γ-[^{32}P]-ATP. Ctk1 is autophosphorylated and phosphorylates Ctk3, upon addition of 80S ribosomes an additional band appears, indicated by a question mark. **(b)** *In vitro* kinase assays using CTDK-I as a kinase and 40S and 60S subunits as substrates show that Ctk1 phosphorylates a protein of the small ribosomal subunit.

2.13.2 Ctk1 phosphorylates Rps2, a protein involved in translational accuracy

In order to uncover the identity of the substrate of Ctk1, ribosomal proteins were TAP-tagged and used in *in vitro* kinase assays. An upwards shifting of the phosphorylated band due to the reduced mobility of the calmodulin binding protein (CBP) tag remaining on the protein after tandem affinity purification should identify the substrate. The Ctk1-Ctk2-Ctk3 complex was purified using a Ctk1-TAP or Ctk3-TAP in a high salt buffer to avoid co-purification of ribosomal proteins. As negative controls, the Bur1-Bur2 complex, which is also essential for transcription elongation (Yao et al. 2000; Keogh et al. 2003), and a wt strain were purified. Ribosomes were purified using strains expressing TAP-tagged versions of protein of the large subunit (Rpl11a-TAP) or of the small ribosomal subunit (Rps2-TAP). Incubating Ctk1 with the eluate from the non-tagged wt strain resulted in the (auto)phosphorylation of Ctk1 and Ctk3 (Figure 32b, lane 3, 4) whereas incubation with Rpl11a-CBP ribosomes showed an additional band (Figure 32b, lane 7). This band shifted upwards when ribosomes were purified by TAP-tagged Rps2, indicating that Rps2-CBP is the substrate of Ctk1 in *in vitro* kinase experiments (Figure 32b, lane 8). The phosphorylation of Rps2 by Ctk1 is specific as the closely related Bur1 kinase did not phosphorylate Rps2 (Figure 32b, lane 9)

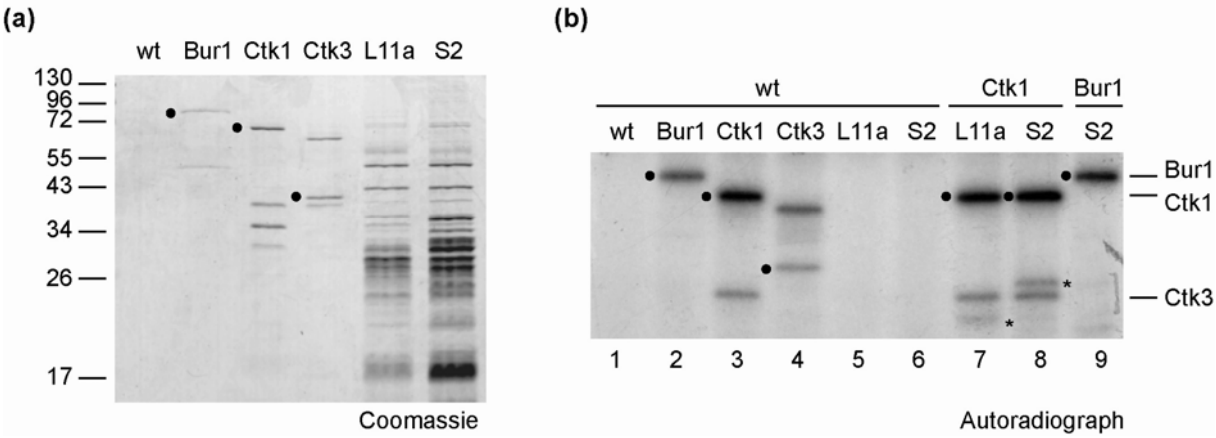


Figure 32: Ctk1 phosphorylates Rps2, a protein of the small ribosomal subunit.
(a) Coomassie staining of purified complexes. Ctk1-Ctk2-Ctk3 complex, Bur1-Bur2 complex, and a non-tagged wild type control were purified under high salt conditions to prevent co-purification of other proteins. Ribosomes were purified by either TAP-tagged Rpl11a or Rps2. **(b)** Identification of Rps2 as a novel substrate of Ctk1. Autoradiograph of *in vitro* kinase assays with kinases and substrates as indicated in the figure. The (auto)phosphorylated bands corresponding to Ctk1, Ctk3 and Bur1 (Yao et al. 2000) are indicated and TAP-tagged proteins are marked by a filled circle. The substrate, Rps2, is marked by a star.

Interestingly, Rps2 is known to be essential for translational accuracy (Eustice et al. 1986; All-Robyn et al. 1990; Synetos et al. 1996; Stansfield et al. 1998), and thus it is tempting to speculate that Ctk1 affects translational accuracy by phosphorylating Rps2.

2.13.3 Ctk1 phosphorylates serine 238 of Rps2

In order to see how Ctk1 could influence translational accuracy, the phosphorylation site on Rps2 was determined. Analysis of the amino acid sequence of Rps2 with regard to the presence of the CDK phosphorylation consensus motif [S/T]-P-X-[K/R] revealed three minimal (SP, TP) consensus sites: S181, S238 and T226 (Figure 33a).

Purification of TAP-tagged Rps2 containing either a serine 181 to alanine (S181A), or serine 238 to alanine (S238A) mutation yielded ribosomes comparable to ribosomes purified from wt Rps2-TAP (Figure 33b) as shown by Coomassie staining. When these mutant ribosomes were used as substrates in *in vitro* kinase assays, Rps2-S238A was not phosphorylated (Figure 33c), indicating that Ctk1 phosphorylates Rps2 on serine 238.

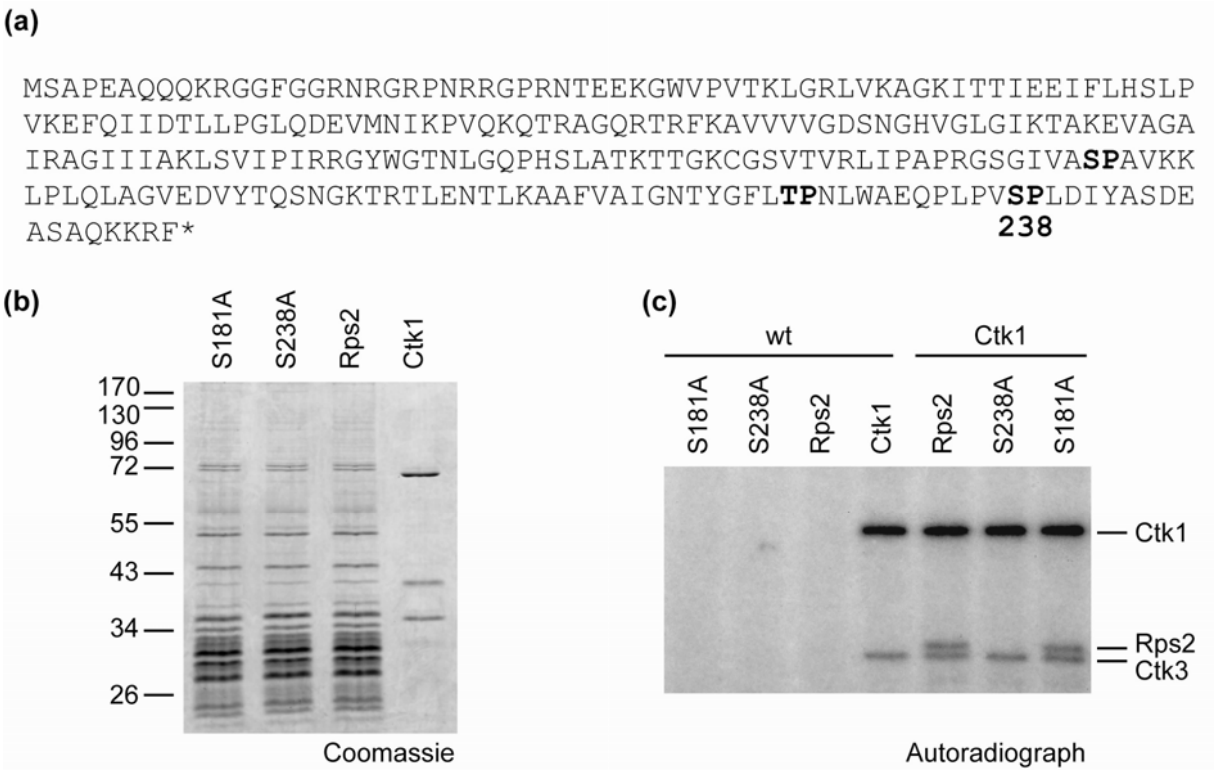


Figure 33: Ctk1 phosphorylates serine 238 on Rps2.
(a) Amino acid sequence of Rps2. The minimal CDK consensus motifs are highlighted in bold letters. S181, S238 and T226 of Rps2 were substituted with alanine. **(b)** Coomassie staining of purified Ctk1-Ctk2-Ctk3 complex and ribosomes containing either wt Rps2, rps2-S181A, or rps2-S238A. **(c)** Ctk1 phosphorylates Rps2 on S238 as purified ribosomes containing the rps2-S238A mutation are not phosphorylated.

Assuming that Ctk1 phosphorylates this residue and contributes thereby to translational accuracy, the S238A mutation should lead to an increase in miscoding as Ctk1 is not able to phosphorylate S238. Thus, translation extracts were prepared from wt and *rps2-S238A* cells and used in poly(U) *in vitro* translation assays. The translation elongation efficiency was only slightly affected in extracts from cells carrying the *rps2-S238A* mutation as determined by the incorporation of L-[^{14}C]-phenylalanine (Figure 34a). However, the relative amount of miscoding events was significantly increased (Figure 34b), suggesting that phosphorylation of Rps2 on S238 by Ctk1 is important to maintain translational accuracy.

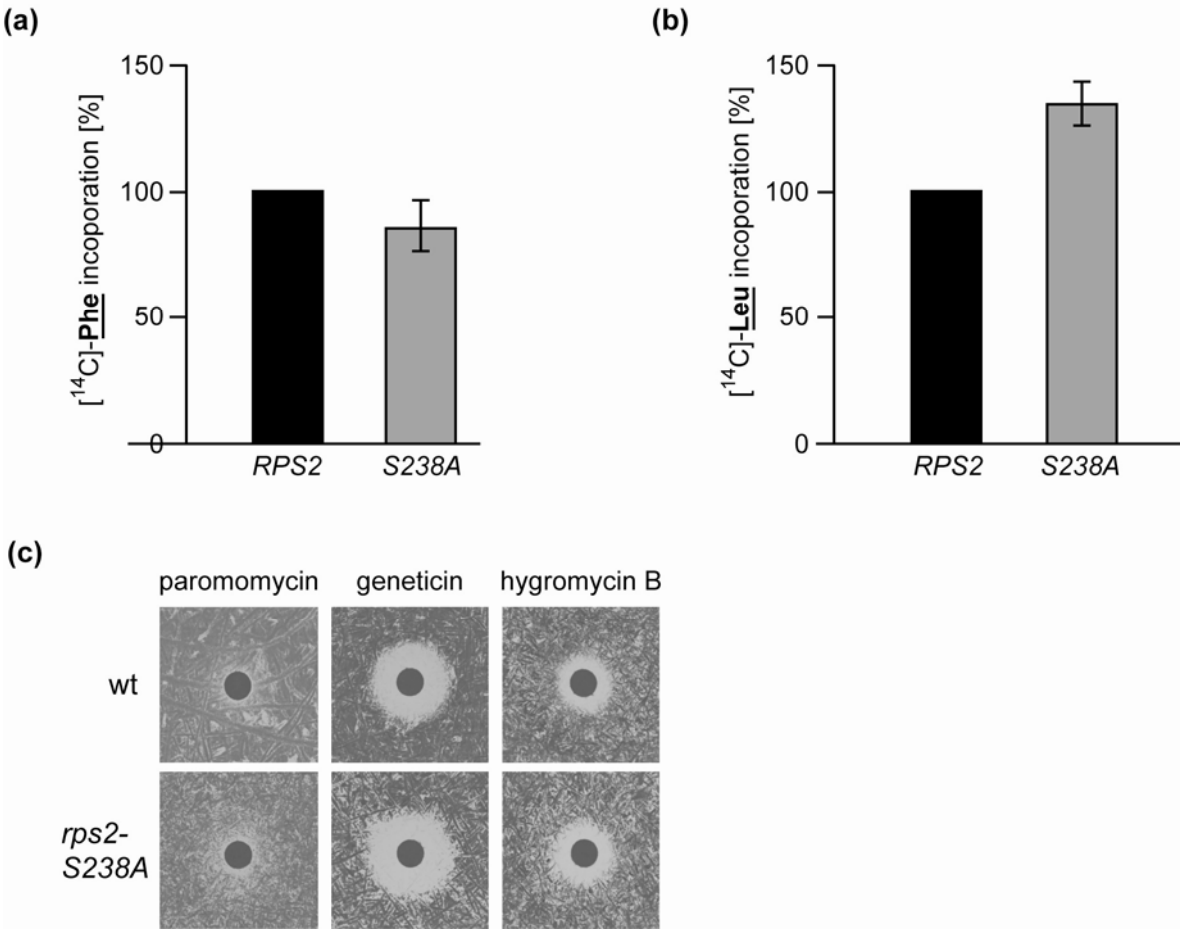


Figure 34: S238 is needed for translational accuracy *in vitro* and *in vivo*. (a), **(b)** *rps2-S238A* extracts show a minor decrease in translation elongation as determined by incorporation of L-[^{14}C]-phenylalanine and a significant increase in miscoding when the ratio between incorporation of L-[^{14}C]-leucine and L-[^{14}C]-phenylalanine was measured. **(c)** *rps2-S238A* cells are sensitive to drugs affecting translation fidelity. Wt or *rps2-S238A* cells were spread on YPD plates containing a filter with paromomycin, hygromycin B and geneticin. The size of the halo indicates the sensitivity of the strain towards this drug.

Importantly, *rps2-S238A* cells show, similarly to Ctk1 depleted cells, an increased sensitivity towards paromomycin, hygromycinB and geneticin (Figure 34c), suggesting that Ctk1

contributes to translation fidelity *in vivo* by phosphorylating S238 of Rps2. How precisely the phosphorylation of this residue by Ctk1 influences the decoding process will be subject of further investigation. Most likely, the phosphorylation event leads to a conformational change in the ribosome structure and thereby influences the selection process of the aminoacyl tRNA (see also discussion).

2.14 CDK9 co-migrates with translating ribosomes

As Ctk1 is a protein that is highly conserved throughout all eukaryotic organisms, it could be that its role in translation is conserved as well. CDK9, the potential mammalian homologue of Ctk1, together with one of the cyclins T1, T2 or K was originally identified as a factor enhancing transcription of RNA polymerase II and was therefore named positive transcription elongation factor P-TEFb (Marshall and Price 1995). Like Ctk1, P-TEFb is needed for efficient transcription elongation and phosphorylates S2 of the CTD of RNA polymerase II (Marshall et al. 1996; Price 2000). To gain a first insight into the potential conservation of Ctk1 function, extracts of the human embryonic kidney cell line 293T (HEK293T) were loaded onto sucrose density gradients to separate soluble proteins from (poly)ribosomes. The presence of CDK9 in each fraction was assessed by Western blotting using an antibody directed against CDK9. CDK9 was found mainly in the soluble pool, but a subpopulation of CDK9 clearly co-migrated with ribosomal subunits, monosomes and polysomes (Figure 35a). Disruption of polysomes with EDTA lead to a shift of CDK9 to the fractions containing the ribosomal subunits, showing that CDK9 most likely also associates with polysomes (Figure 35b).

The co-migration of CDK9 with translating ribosomes is an indication that the novel function of Ctk1 in translation is conserved throughout evolution. Further analysis with *e.g.* translation extracts of mammalian cells will help to prove the conservation of this process and to analyze whether Ctk1's function in translation is ubiquitously needed in all cells, or specifically needed for *e.g.* strongly proliferating cells.

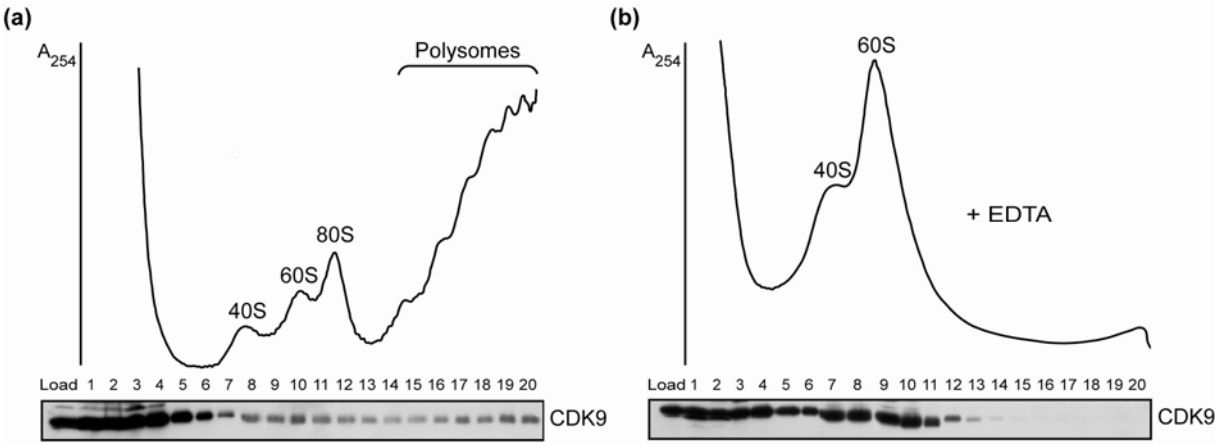


Figure 35: CDK9, the mammalian homologue of Ctk1 co-migrates with translating ribosomes and ribosomal subunits.
Sucrose density gradients were performed and ribosomal fractions (40S, 60S, 80S and polysomes) were determined by A_{254nm} measurement of the gradient fractions. Each fraction was analyzed by Western blotting using an antibody directed against CDK9. **(a)** CDK9 associates with polysomes, monosomes and ribosomal subunits. **(b)** Dissociation of the ribosome into the ribosomal subunits results in a shift of CDK9 to the ribosomal subunits.

3 Discussion

3.1 Ctk1 associates with translating ribosomes *in vivo*

In order to identify novel interacting proteins of Ctk1, tandem affinity purification was employed to purify native protein complexes. Besides the TREX components Gbp2 and Hrb1, Ctk1 interacts specifically with ribosomal proteins and proteins involved in translation: Arb1, a shuttling protein involved in ribosome biogenesis that might also be associated with 80S monosomes (Dong et al. 2005), the eIF3 components Prt1 and Rpg1 (Hinnebusch 2006), the eIF4F protein Tif4631 (Goyer et al. 1993) and Ded1 (Chuang et al. 1997; Iost et al. 1999), both involved in translation initiation, Yef3, a protein of the translation elongation machinery (Anand et al. 2003), Ssb1, important for accurate translation (Rakwalska and Rospert 2004) and Sro9, which associates with translating ribosomes and functions in the modulation of translation (Sobel and Wolin 1999). As some of the weaker bands could not be identified unambiguously, most likely there were more translation factors present in the purification, such as Nip1, Tif34, Tif35 and Hcr1 that form together with Prt1 and Rpg1 the very stable eIF3 complex (Hinnebusch 2006). Based on the sub-stoichiometric co-purification of translation factors with Ctk1 and the finding that Ctk1 phosphorylates a protein of the small ribosomal subunit, Ctk1 most likely interacts with translating ribosomes and thereby indirectly with ribosome-associated proteins or translation factors.

The specific association of the CTDK-I complex with ribosomal proteins was confirmed by the analysis of the migration behavior of Ctk1 in sucrose density gradients in comparison to ribosomal proteins. Various conditions such as omission of cycloheximide or the addition of puromycin resulted in a migration behavior of Ctk1 similar to that of ribosomal proteins, proving that Ctk1 interacts with translating ribosomes. Importantly, a co-migration with translating ribosomes could not be shown for representative proteins of the nuclear RNAP II transcription process Ctk1 is known to be involved in, or proteins important for ribosome biogenesis. In living cells, the main pools of Ctk1 (nucleus) and ribosomes (cytoplasm) are separated. However, when cells were crosslinked and the extract was prepared under high salt conditions, Ctk1 also co-sedimented with ribosomes, demonstrating that Ctk1 associates with translating ribosomes *in vivo*. In contrast, when the cells are disrupted to perform gradients or purifications without crosslinking, most likely any regulatory processes that might dissociate Ctk1 from the ribosome after phosphorylation of Rps2 are abolished and Ctk1 stays bound to the ribosome. This could be an explanation for the missing soluble fraction of Ctk1 in

gradients performed with non-crosslinked cells. The small amount of Ctk1 associated with translating ribosomes *in vivo* is therefore consistent with a highly regulated catalytic function of Ctk1 in translation.

3.2 Ctk1 promotes correct decoding of the message by phosphorylating a protein of the small ribosomal subunit

When translation elongation was studied, it could be shown that Ctk1 is directly involved in maintaining translational accuracy during decoding of the message as the observed defects in translation elongation and decoding *in vitro* could be restored upon addition of purified CTDK-I complex. The involvement of Ctk1 in the correct decoding of the message was not only demonstrated *in vitro* using poly(U) programmed ribosomes but also *in vivo*. Cells depleted for Ctk1 were more sensitive towards drugs that interfere with translation accuracy (such as paromomycin, hygromycin B and geneticin) and showed an increased frequency in misreading stop as sense codons *in vivo*. A molecular explanation for the function of Ctk1 during decoding of the message might be the finding that Ctk1 phosphorylates Rps2 on S238. Rps2 is a protein of the small ribosomal subunit that is known to be essential for translational accuracy. Mutation of S238, the residue phosphorylated by Ctk1 *in vitro*, to alanine leads to a decrease in translational accuracy *in vitro* and an increased sensitivity towards paromomycin, hygromycin B, and geneticin *in vivo*, indicating that this residue indeed is important for translation fidelity, most likely by being phosphorylated by Ctk1.

Research in *E. coli* showed that the accurate selection of the aa-tRNA depends not only upon the correct basepairing of three bases between the mRNA codon and the tRNA anticodon but the ribosome itself acts as sensor for the correct geometry of a Watson-crick base pairing. This interplay between codon-anticodon match and correct conformation assures a high accuracy of ribosomes (10^{-4}), important for the survival of the cell (for review see Rodnina and Wintermeyer 2001; Ogle et al. 2003). On a mechanistic level, aa-tRNA in a ternary complex together with *E. coli* EF-Tu (yeast eEF1a) and GTP is delivered to the ribosomal A-site. A reversible initial binding complex is formed that dissociates rapidly when there is no match between codon and anticodon. Upon recognition of a codon the complex is stabilized, and EF-Tu hydrolyzes GTP. After GTP hydrolysis, EF-Tu rearranges to the GDP-bound form, and thereby looses the affinity for the aa-tRNA. The aminoacyl end of the tRNA is then free to move into the peptidyl transferase center on the 50S subunit, a process termed

accommodation, where peptide-bond formation takes place (Figure 36). The selection of aa-tRNA in response to the codon in the ribosomal decoding center takes place before GTP hydrolysis (initial selection) and after GTP hydrolysis but before peptide-bond formation (proofreading).

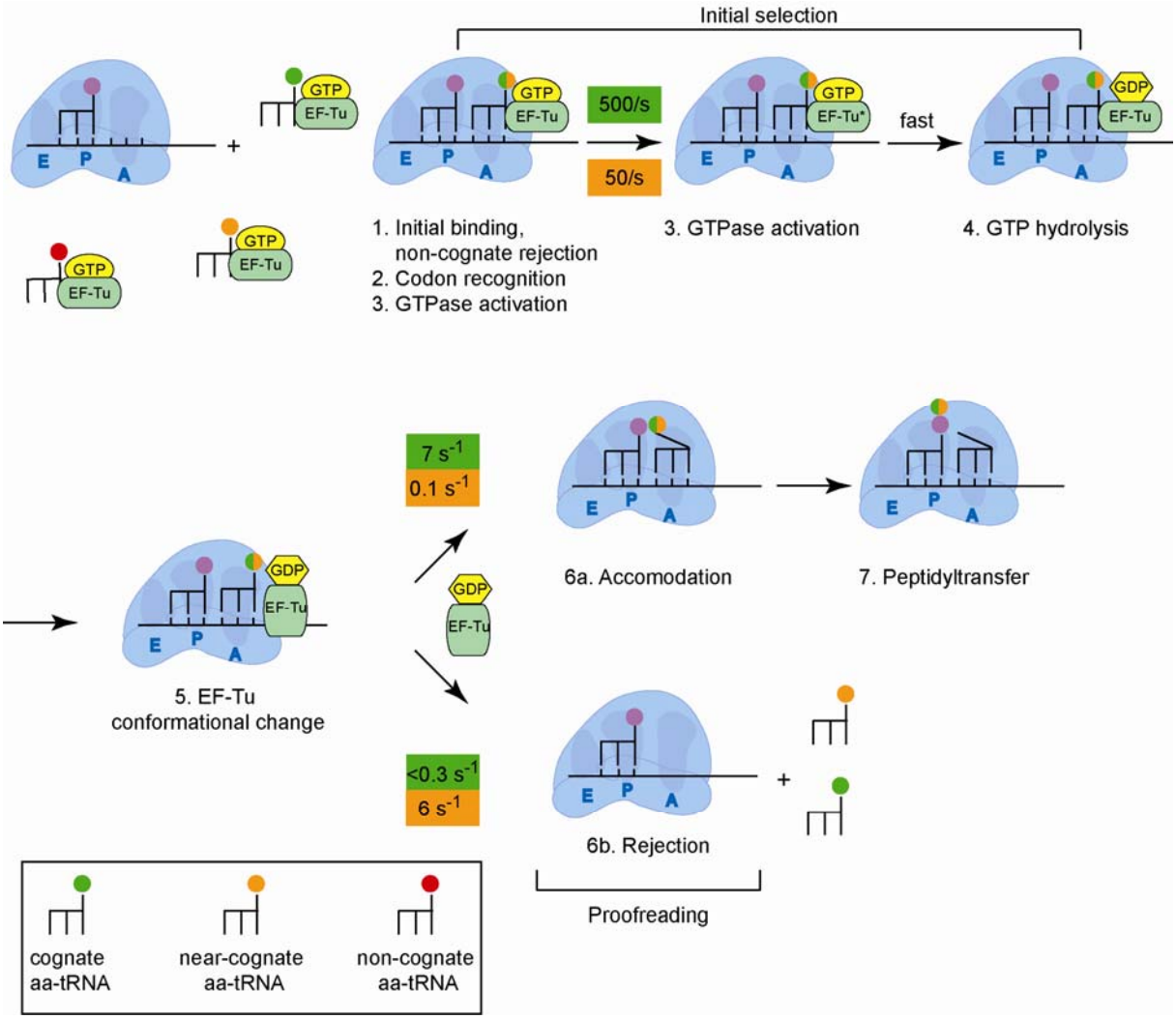


Figure 36: Mechanism of aa-tRNA binding to the A-site of the ribosome and discrimination against near-cognate ternary complexes.
Green: rate constants as determined for cognate aa-tRNA; orange: rate constants as determined for near-cognate tRNA. EF-Tu is depicted differently in the GTP-and GDP-bound conformations, GTP* denotes the GTPase state (Rodnina and Wintermeyer 2001).

Discrimination between a cognate and a non-cognate tRNA is mainly accomplished during the initial selection step. It has been shown that in the beginning all ternary complexes have the same chance to bind to the ribosome. The discrimination against near-cognate aa-tRNAs requires a more complex mechanism and is mainly based on kinetic parameters. Upon codon recognition, near cognate tRNA is stabilized and the hydrolysis of GTP is stimulated,

although not to the same extent as in the cognate case. Most of the near-cognate tRNAs are rejected during the subsequent proofreading step as the near-cognate codon-anticodon complex is less stable, and thus the probability that a near-cognate aa-tRNA will dissociate from the ribosome at this stage is higher. Furthermore, it has been shown that the efficiency of the initial selection is negatively influenced by the Mg^{2+} concentration in an *in vitro* translation assay, whereas the proofreading step is not much influenced by the buffer conditions. For Ctk1, the susceptibility to misincoporate the near-cognate amino acid leucine was more pronounced at a magnesium concentration of 10 mM (data not shown), suggesting that Ctk1 takes part in the initial selection process of aa-tRNAs.

The ribosome seems to be present in two different conformations: a binding conformation that accepts or rejects substrates on the basis of different binding stabilities and a productive conformation that favours GTP hydrolysis and peptide bond formation (for review see Ogle et al. 2003; Selmer et al. 2006). According to the current model in *E. coli*, three groups of structural elements influence the fidelity of protein synthesis: the decoding region on the small subunit, the subunit interface and a region on the large subunit. The decoding center is constituted of 16S rRNA and three proteins of the small ribosomal subunit. These elements are most likely involved in directly or indirectly sensing the structure of the codon-anticodon complex and in creating a conformational signal of codon-recognition. Parts of the 16S rRNA located at the interface of the two subunits most likely mediate the transmission of the conformational signal from the small ribosomal subunit decoding site to the active center on the large ribosomal subunit. Regions of the large ribosomal subunit most likely receive this signal and act as a "GTPase activating protein" (GAP) for EF-Tu, resulting in aa-tRNA accommodation.

Crystal structures have revealed that the 16S rRNA bases of the decoding center specifically contact the first two bases of a cognate codon-anticodon pair by induced fit. This results in a closed conformation of the 30S subunit, compared to a more open structure, when the A-site is unoccupied. The third position permits certain wobble base pairings, allowing some tRNAs to recognize several different codons as the contacts of the ribosome at this position do not depend upon perfect geometry. However, some non-Watson-crick base pairs are not allowed at the Wobble position, presumably because the ribosome imposes certain geometric constraints, for example upon the overall width of a base pair.

In addition to the 16S rRNA, three ribosomal proteins contribute to the accurate decoding of the message in *E. coli*. S12, S4 and S5 (the *E. coli* homologue of yeast Rps2), constitute the

highly conserved so-called decoding accuracy center (Figure 37) and have been shown to interact with a highly conserved 530 bp stem-loop region of the 16S rRNA (Powers and Noller 1991; Powers and Noller 1994). Mutations in these proteins may perturb the 16S rRNA structure, leading to an increased frequency of miscoding. Ribosomal protein S12 is localized close to the site of codon-anticodon interaction, and it has been shown that mutations in this protein confer a hyperaccurate phenotype (Bilgin et al. 1992). In contrast, mutations in ribosomal proteins S4 and S5, which are localized in the opposite site of the codon-anticodon locus, decrease the accuracy of decoding and were therefore called ram (ribosomal ambiguity) mutants (Cabezon et al. 1976; Piepersberg et al. 1979; Allen and Noller 1989).

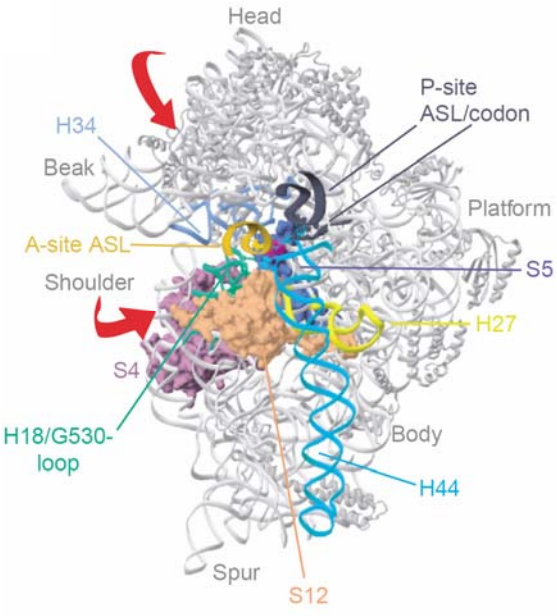


Figure 37: Overview of the decoding center in the 30S subunit structure, in complex with A-site tRNA anticodon stem loop (ASL, Ogle et al. 2003).
Red arrows indicate the movement of domains during the transition to the closed conformation. In the shoulder domain, the 530 bp stem-loop of the 16S rRNA is highlighted in green, Helix 44 important for correct decoding of the message is highlighted in turquoise and proteins S12 (orange), S4 (violet) and S5 (dark blue) are highlighted in space-filling representation.

Importantly, S5 is the *E. coli* homologue of yeast Rps2, the substrate of Ctk1, and mutation of yeast Rps2 was shown to decrease translation fidelity, consistent with an increase in sensitivity of *RPS2* mutants to paromomycin (Eustice et al. 1986; All-Robyn et al. 1990; Synetos et al. 1996; Stansfield et al. 1998 and this thesis). In *E. coli*, paromomycin binds to the 16S rRNA and induces a conformational change that stabilizes the binding of the aminoacyl tRNA in the A site not only when the anticodon is cognate, but also for non-cognate tRNAs, by allowing the domain closure around the near-cognate aa-tRNA (Fourmy et al. 1996; Fourmy et al. 1998; Ogle et al. 2003). Thus, in yeast, phosphorylation of Rps2 on S238 by Ctk1 could induce a structural change in Rps2 and thereby contribute to an accurate decoding process. Without Ctk1 Rps2 remains non-phosphorylated and thus the ribosome might be more prone to misreading.

In the *S. cerevisiae* 80S ribosome, Rps2 is positioned in the middle of the small ribosomal subunit right next to the mRNA entry tunnel (Figure 38). The position of Rps2 is based on the position of its *E. coli* homolog S5 in the crystal structure of the 70S ribosome (Spahn et al. 2001) and was modeled into the electron density of the yeast ribosome in red. The last 30 amino acids of yeast Rps2, including S238 are not present in the *E. coli* crystal structure, indicating a flexible structure in the C-terminus not present in *E. coli*. However, glycine 222, the last amino acid present in the structure points towards the solvent side, indicating that S238 is most likely accessible for phosphorylation by Ctk1.

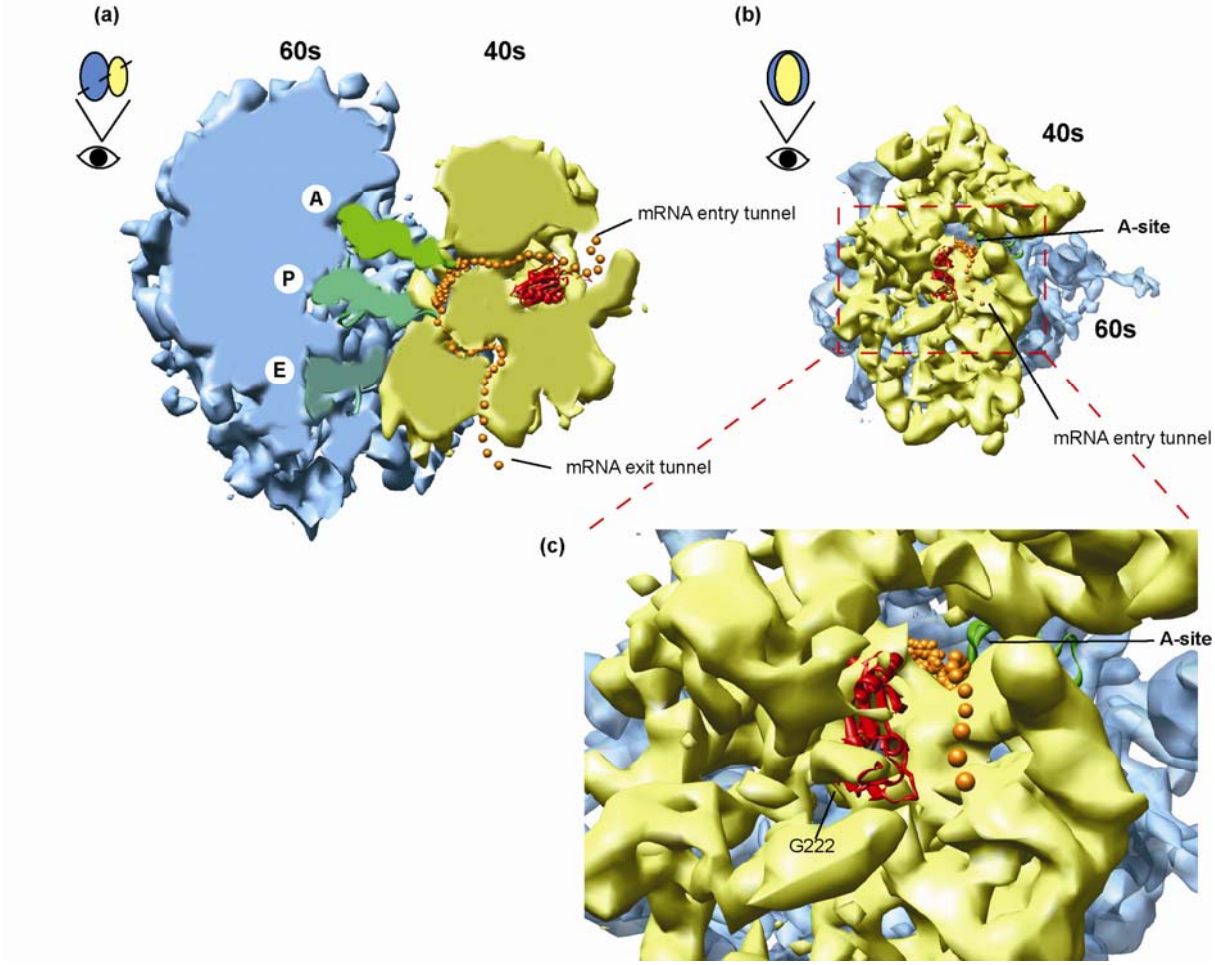


Figure 38: Cryo-EM model of an elongating 80S ribosome with messenger RNA, A-, P-, and E-site tRNA and Rps2 modelled in according to Spahn et al. 2001.
(a) Cross-section allowing a view on the elongation process. Rps2 is positioned at the entry site of the mRNA entry tunnel. **(b)** View on the 40S subunit from the solvent site. The transparency was reduced to visualize the path of the mRNA, the tRNAs and Rps2. Yeast Rps2 is present with reduced electron density in the 80S EM structure and as crystal structure of *E. coli* S5. **(c)** Close-up on the localization of Rps2 on the small ribosomal subunit at the entry tunnel of the mRNA. The large ribosomal subunit in yellow, mRNA in orange (single spheres show the likely path of the mRNA, which is not seen in the electron density), A-, P-, and E-site tRNAs in green and Rps2 in red.

Decoding of the message is also based on an extremely sensitive equilibrium between velocity and accuracy and the influence of these two parameters on translation is still an issue of debate. One the one hand, there are examples showing that the elongation rate does not necessarily influence accuracy (Andersson et al. 1982), but for other mutants an increase of misreading corresponds to an increase in elongation rate (Bjorkman et al. 1999) and hyperaccuracy slows down the elongation rate (Andersson et al. 1986; Carr-Schmid et al. 1999). Even though in this thesis the kinetic rate of translation elongation was not determined, the polysomal run-off experiments after glucose starvation suggest that the ribosomes of cells depleted for Ctk1 show a faster run-off in comparison to wt cells, indicating that the decoding defect in Ctk1 depleted cell leads to an increase in the elongation rate. At first glance this might seem contradictory to the observed elongation defect in *in vitro* translation assays with cells depleted for Ctk1, but this decrease is most likely due to misincorporation of cold amino acids present in the reaction, such as the near-cognate amino acid leucine. Taken together, depletion of Ctk1 most likely leads to a faster but more inaccurate translation of the message.

3.3 Phosphorylation by Ctk1 – a regulatory or a constitutive process?

Many processes in the living cells are regulated by phosphorylation. Translation is a prime target for regulation as it allows for a rapid response (Gebauer and Hentze 2004; Holcik and Sonenberg 2005). Since deletion of Ctk1 leads to a major decrease in translational activity in the *in vitro* translation assays with different kinds of mRNA templates, Ctk1 most likely regulates translation at a global level. This assumption is also supported by the identification of accuracy protein Rps2 as a substrate of Ctk1 and the *in vitro* translation experiments with poly(U) programmed ribosomes. Addition of the CTDK-I complex *only* was sufficient to restore the miscoding defect of Ctk1 depleted cells to wt levels. Even though it cannot be excluded that Ctk1 regulates translation by phosphorylation of a second protein in response to *e.g*. cellular stress, Ctk1 is most likely constitutively involved in the accurate decoding of the message.

So far, it has not been shown that phosphorylation can directly influence translation elongation, as control of translation by phosphorylation was restricted to the initiation process, where initiation factors or regulatory proteins are phosphorylated in response to changes in the environment. A prime example is the inhibitory phosphorylation of the GTPase eIF2α in its GDP-bound form (Gebauer and Hentze 2004). Upon phosphorylation, the guanine nucleotide exchange factor eIF2B is bound to eIF2α, but cannot exchange the GDP

for GTP and thereby prevents a new round of translation as the formation of the ternary complex of eIF2α, GTP and initiator tRNA is inhibited. Another example are eIF4E-binding proteins (4E-BPs) that bind to eIF4E and thereby prevent its association with eIF4G, which is needed for initiation (Richter and Sonenberg 2005). Phosphorylation of the 4E-BP leads to a release from eIF4E, and translation initiation can occur.

Interestingly, besides the function of Ctk1 during the decoding process, the *in vitro* translation assay that mainly measured the initiation of translation suggests that Ctk1 has a second function during initiation. This idea is also supported by the finding that gradients of *Δctk1* cells show an initiation defect as visualized by an increased 80S peak in comparison to wt cells and the unsuccessful rescue experiments when purified CTDK-I complex was added to the in *in vitro* translation assay with radioactively labelled methionine. Most likely, phosphorylation by Ctk1 of a so far unknown protein, which is not present in an extract prepared from cells depleted for Ctk1, is needed for efficient translation initiation. For example, SR or hnRNP proteins that are part of the mRNP might be phosphorylated by Ckt1 leading to an initiation-competent rearrangement of the mRNP, which might involve the release of a negative factor or, even more likely, the recruitment of a factor essential for efficient translation initiation. In humans, it was shown that hnRNP K binds to a 3' regulatory element of the LOX mRNA and represses its translation, but phosphorylation leads to release of hnRNP K and the message is translated (Ostareck-Lederer et al. 2002). In *S. cerevisiae* the phosphorylation of the shuttling SR protein Npl3 by the cytoplasmic kinase Sky1 is needed to dissociate Npl3 from the mRNP for efficient translation to take place (Windgassen et al. 2004). It will be extremely interesting and challenging to investigate the molecular mechanism of Ctk1's function during initiation of translation in the future. Even though the basic mechanism of translation initiation is subject to research since decades, the regulation of this highly important process in eukaryotes still needs to be investigated.

3.4 Ctk1 - a shuttling protein?

A seemingly paradox with this newly discovered function of Ctk1 is its mainly nuclear localization in contrast to the cytoplasmic presence of ribosomes in the cytoplasm. However, these steady-state distributions do not permit conclusions about the *in vivo* dynamics and shuttling experiments can help to assess a potential shuttling ability of a protein. Unfortunately, it could not be assessed whether Ctk1 shuttles between nucleus and cytoplasm, because after stopping Ctk1 expression, the signal of Ctk1 (either by immunofluorescence or

fluorescence microscopy of GFP-tagged Ctk1) was lost too quickly to be employed in shuttling assays.

Even though a shuttling of Ctk1 could not be shown, several lines of evidence argue in favour of this hypothesis. First, Ctk1 associates with translating ribosomes *in vivo* and the small amount of Ctk1 that is associated with cytoplasmic ribosomes when cells were crosslinked prior to sucrose density gradients is consistent with the proposed catalytic function of Ctk1 during translation and hence, a short "residence time" at the ribosome. Second, a translation elongation defect of Ctk1 depleted cells can be directly restored by addition of purified CTDK-I complex *in vitro*. Third, the potential metazoan homolog CDK9 was shown to shuttle between nucleus and cytoplasm (Napolitano et al. 2002; Napolitano et al. 2003; also see below). Thus, Ctk1 probably travels to the cytoplasm, enhances translation elongation and fidelity by phosphorylating Rps2 (Figure 39, 1) and rapidly returns to the nucleus (Figure 39, 4).

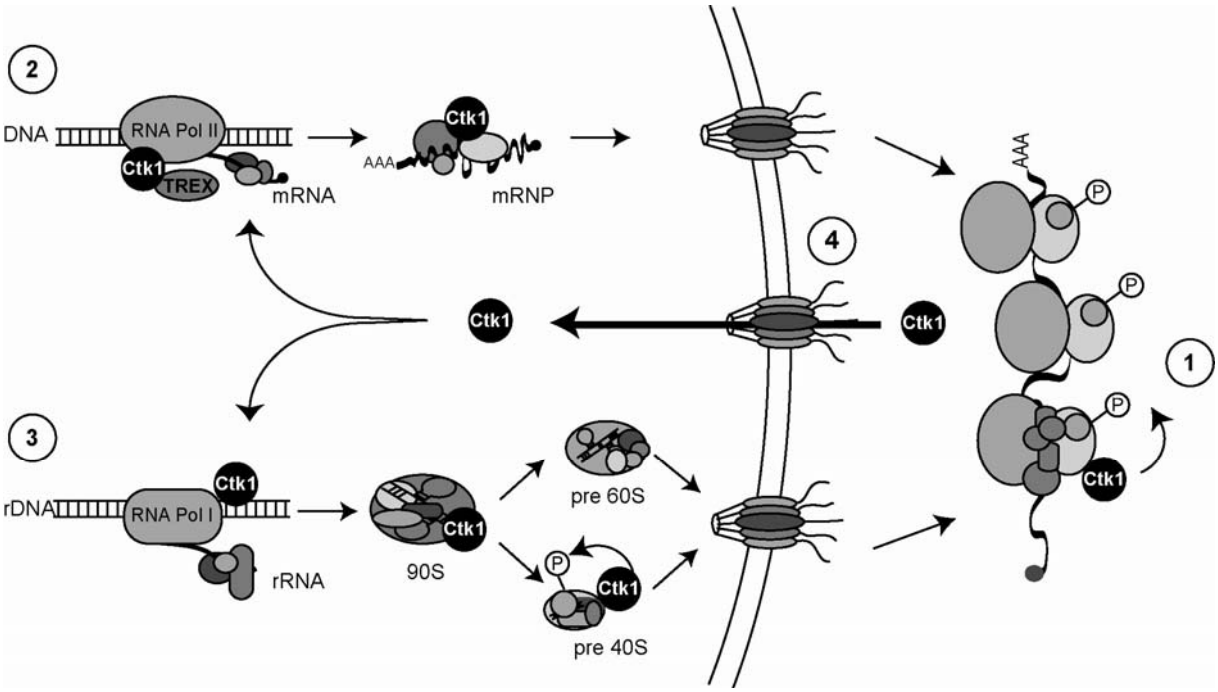


Figure 39: Model of Ctk1's function in translation.
(1) Phosphorylation of Rps2 on S238 by Ctk1 is needed for higher accuracy of mRNA decoding. **(2)** Since Ctk1 is implicated in transcription of RNAP II and interacts with the TREX complex, it could exit the nucleus bound to correctly processed mRNPs. **(3)** Alternatively, Ctk1 could travel to the cytoplasm with pre-ribosomes, as Ctk1 has been implicated in RNAP I transcription. **(4)** After phosphorylation of Rps2 (1), Ctk1 most likely rapidly re-enters the nucleus.

In principle, two possible routes could be taken by Ctk1 to travel from the nucleus to the cytoplasm. Since Ctk1 is necessary for efficient transcription by RNAP I (Bouchoux et al. 2004; Grenetier et al. 2006), Ctk1 could remain associated with pre-ribosomal particles during maturation and export to the cytoplasm (Figure 39, 3). In this case, Ctk1 could also be part of the ribosome biogenesis pathway. However, this is unlikely, as in sucrose density gradients the ratio of 40S to 60S subunits does not change significantly as compared to an isogenic wild type strain, *i.e.* Ctk1 does not show a ribosome biogenesis defect, and Ctk1 shows a different migration pattern in sucrose density gradients than proteins involved in 90S, pre-40S and pre-60S biogenesis. Furthermore, the steady state level of ribosomal proteins does not change significantly in Ctk1 depleted extracts, and *Δctk1* cells do not exhibit an export defect of the small or large ribosomal subunit into the cytoplasm. Even though Ctk1 seems to have no essential function during ribosome biogenesis, it cannot be completely ruled out, that Ctk1 travels to the cytoplasm bound to the ribosomal subunits.

Alternatively, Ctk1 could travel from the nucleus to the cytoplasm via the exported mRNP (Figure 39, 2). This possibility seems more likely because of the observed genetic and physical interactions between Ctk1 and TREX and Mex67 (this thesis and Hurt et al. 2004). *CTK1* is synthetic lethal with the THO subunits *MFT1*, *HPR1* and *THO2*. As THO as well as Ctk1 are both important for efficient transcription elongation (Chavez and Aguilera 1997; Piruat and Aguilera 1998; Patturajan et al. 1999; Chavez et al. 2000; Cho et al. 2001; Strasser et al. 2002), it could be that the observed genetic interaction between Ctk1 and THO is based on the transcription process and deletion of both proteins results in cell death. Alternatively, deletion of both Ctk1 and THO could result in an aberrant mRNP that is degraded, not exported or not efficiently translated. *CTK1* interacts genetically with *YRA1* mutants in which the C-terminal domain that affects the efficiency of the mRNA export or one of the two domains that interact with the Mex67-Mtr2 mRNA export receptor is missing. These genetic interactions suggest that Ctk1 could be a constituent of the mRNP to be exported, which is also supported by a more prominent physical interaction of Ctk1 with Yra1 when the export was blocked in a Mex67 mutant. However, the physical interaction observed under this condition does not necessarily implicate a completely matured mRNP at the nuclear pore. It could also reflect the association of Ctk1 with Yra1 at the nascent transcript at the site of transcription as it was shown that defects in mRNA export can lead to an accumulation of non-matured mRNPs in transcription site foci (Jensen et al. 2001; Libri et al. 2002). *CTK1* interacts genetically with *MEX67* mutants with mutations in the domain important for protein-protein interactions in general between *i.e.* the interaction between Mex67 and an mRNP

protein could be affected when the deletion of *CTK1* is combined with these mutants. *CTK1* is also synthetically lethal with a mutant that lacks the C-terminal domain of *MEX67* that may modulate efficient nuclear export of the shuttling Mex67-Mtr2. Both genetic interactions support the idea that Ctk1 might exit the nucleus bound to the mRNP.

Even though synthetic lethality could not be observed between *CTK1* and *GBP2* and *HRB1*, respectively, Ctk1 physically interacts more strongly with these two proteins than with Hpr1 or Sub2 in tandem affinity purifications (Hurt et al. 2004), allowing for the speculation that Ctk1 is co-transcriptionally loaded onto the mRNP and interacts with Hpr1 and Sub2 by means of Gbp2 and Hrb1. While Sub2 and Hpr1 are most likely released from the mRNP before or shortly after the transport through the nuclear pore, Gbp2, Hrb1 and most likely Ctk1 remain associated with the mRNP throughout translation as all three proteins have been shown to be associated with translating ribosomes (this thesis and Windgassen et al. 2004), which could also be an explanation for the stronger interaction of Gbp2 and Hrb1 with Ctk1.

3.5 The function of Ctk1 might be conserved

The function of Ctk1 in translation is most likely conserved throughout evolution as indicated by the finding that its human homologue, CDK9, also associates specifically with polysomes. In 2003, $CDK9_{55}$, a longer isoform of CDK9 with an N-terminal extension, has been described (Shore et al. 2003; Shore et al. 2005). Interestingly, immunodecoration of Western blots with CDK9 antibodies of gradients with human cells always resulted in two signals with a slightly different migration behaviour, suggesting that both isoforms are present at translating ribosomes. Ctk1, but not Bur1, the other potential homologue of CDK9, also contains an extension N-terminal of its kinase domain, although the extensions of Ctk1 and $CDK9_{55}$ do not share any striking sequence similarity. One the one hand it could be that in higher eukaryotes the dual function of Ctk1 in transcription and translation is separated. On the other hand, it was always an issue of debate that both Ck1 and Bur1 are considered to be homologues of CDK9. Thus, it could be that the different functions of Bur1 and Ctk1 in yeast are reflected by the two isoforms of CDK9 in higher eukaryotes and it will be of high scientific interest to assess the common and different functions of the $CDK9_{42}$ and $CDK9_{55}$ isoforms with respect to Ctk1 and Bur1 in transcription and translation.

Importantly, CDK9 was shown to shuttle between the nucleus and the cytoplasm, while its cyclin T1, important for transcriptional activity of CDK9, is constantly found in the nucleus (Napolitano et al. 2002; Napolitano et al. 2003). Overexpression of cyclin T1 enhances the

nuclear localization of CDK9. Since CDK9 can associate with several cyclins and proteins apart of T1, such as cyclin T2 and K, Hsp90 and Hsp70 (O'Keeffe et al. 2000), it is possible that CDK9 associated with a different partner could exhibit a function different to transcription elongation, namely in translation.
Interestingly, the autophosphorylation at the C-terminus of CDK9 was shown to be important for the re-import of the protein into the nucleus (Napolitano et al. 2003). Even though the authors speculated that this phosphorylation event is only important for the re-import of the protein, in the context of the novel discovered function of yeast Ctk1 in translation it could be that this phosphorylation event is important for release of the protein from polyribosomes, and thus a prerequisite for the re-import. These autophosphorylation residues are also part of the C-terminus of Ctk1. Thus, it would be extremely interesting to see whether the removal or mutation of these residues in Ctk1 would result in the same cytoplasmic localization as described for CDK9, especially since the truncation of the C-terminal tail apparently destroyed the autophosphorylation without affecting the binding to cyclin T1 or the CTD kinase activity.

Rps2 is also highly conserved over all kingdoms of life, underlining the importance of this protein in maintaining the fidelity of translation (http://www.ncbi.nlm.nih.gov/BLAST/). S238, the residue of Rps2 phosphorylated by Ctk1 is well conserved in eukaryotes, with the exception of *e.g. P. tetraurelia*, *D. discodideum*. *A. gambiae*, *D. melanogaster* and *S. pombe*. In these species the serine residue is exchanged for a threonine, which could also serve as phosphorylation site. However, in eubacteria and archaea, this C-terminal part of the protein, including S238, is not present, indicating that eukaryotes obviously developed an additional layer of regulation, most likely in concordance with the separation of transcription in the nucleus and translation in the cytoplasm.

3.6 Ctk1 – coupling of transcription to translation?

Even though coupling of the various nuclear processes such as transcription, processing of the mRNA and mRNA export is undisputed in general (for review see Maniatis and Reed, 2002, Proudfoot et al. 2002), examples connecting nuclear events with translation are still scarce. One example for such a connection is the human homologue of the Mex67-Mtr2 mRNA exporter. Tap-p15 is nuclear at steady state, but shuttles between the nucleus and the cytoplasm and promotes the translation of its exported unspliced viral CTE mRNA (Jin et al.

2003). In HeLa cells, it was shown that shuttling SR proteins, which function primarily in splicing, have also an additional role in mRNA translation (Sanford et al. 2004). Third, as already mentioned above, the SR-like protein Npl3 accompanies the mRNA to the ribosome, but needs to be dissociated before efficient translation (Windgassen et al. 2004).

In the future, it will be extremely interesting and challenging to further corroborate Ctk1's role during mRNP assembly and subsequent translation of the message, *i.e.* to investigate how and when Ctk1 is loaded onto the mRNP and how and when and to which position of the ribosome Ctk1 binds to. The binding site of Ctk1 on the ribosome could be identified by *e.g. in vitro* reconstitution experiments with subunits or monosomes and subsequent cryo-electron microscopy. In addition, it would be interesting to see how Ctk1 is dissociated from the ribosome. It could be, that the release is facilitated by the autophosphorylation of Ctk1, as it was shown for its shuttling metazoan homologue CDK9 that autophosphorylation of the C-terminal residue is important for its re-import into the nucleus. As Ctk1 also seems to be important for efficient translation initiation, Ctk1 most likely phosphorylates a factor that is either directly or indirectly (by recruiting a second factor) important for efficient translation initiation and it will be extremely interesting to identify the potential substrate and the molecular mechanism of Ctk1's function during initiation.

Even though it cannot be excluded that the observed function of Ctk1 during translation might be independent of its nuclear function, the obtained results indicate that Ctk1 binds co-transcriptionally to the nascent transcript, travels together with the mRNP to the ribosomes, phosphorylates Rps2 and thereby contributes to accurate translation. Apparently, Ctk1 plays a role in another layer of regulation of gene expression eukaryotes might have evolved. This study, together with the few examples mentioned above (Jin et al. 2003; Windgassen et al. 2004; Sanford et al. 2004) show that the composition of the mRNP might determine the translational fate of the mRNA, including efficiency and accuracy of translation.

4 Materials

4.1 Consumables and chemicals

Consumables and chemicals were purchased from the following companies:
Acros Organics (Geel, Belgium), Applichem (Darmstadt), Applied Biosciences (Darmstadt), Apollo Scientific Limited (Bredbury, UK), Axon (Kaiserslautern), Becton Dickinson, (Heidelberg), Beckman Coulter (Krefeld), Biaffin (Kassel), Biomol (Hamburg), Biorad (Munich), Biozym (Hess. Oldendorf), Chemicon (Temecula, Canada), Fermentas (St. Leon-Rot), Formedium (Norwich, UK), GE Healthcare (München), Gilson (Bad Camberg), Invitrogen (Karlsruhe), Macherey & Nagel (Düren), Medac (Hamburg), Medigenomix (München), Membra Pure (Bodenheim), Merck Biosciences (Darmstadt), Millipore (Molsheim, France), Mobitec (Göttingen), MP Biomedical (Illkirch, France), NEB (Frankfurt), Neolab (Heidelberg), Nunc (Wiesbaden), Peske (Aindling-Arnhofen), Promega (Mannheim), Qiagen (Hilden), Roche (Mannheim), Roth (Karlsruhe), Santa Cruz (Santa Cruz, USA), Sarstedt (Nümbrecht), Semadeni (Düsseldorf), Serva (Heidelberg), Sigma (Taufkirchen), Stratagene (Amsterdam, The Netherlands), VWR (Ismaning).

4.2 Commercially available kits

AmpliCap SP6 High Yield Message Maker Kit (Biozym, Hess. Oldendorf), DC Protein Assay (Biorad, München), ECL kit (Applichem, Darmstadt), Nucleobond AX PC100, Nucleospin Mini, Nucleospin extract (all Macherey & Nagel, Düren), RNeasy MinElute Cleanup Kit (Quiagen, Hilden).

4.3 Equipment

Beckman DU650 spectrophotometer, L80 Ultracentrifuge, SW32 rotor, SW40 rotor, (Beckman Coulter, Krefeld), T3 Thermocycler (Biometra, Goettingen), Gel dryer model 583, Mini-Protean II system (Biorad, München), DNA engine (Biozym, Hess Oldendorf), Lumat LB9507 (EG&G Berthold), bead mill (Fritsch, Idar-Oberstein), Thermomixer compact, Eppendorf centrifuge 5415D, 5415R (Eppendorf, Wesseling-Berzdorf), Äkta Basic System, peristaltic pump (GE Healthcare, München), Rotanda, 46R (Hettich, Tuttlingen), Ika Vibrax VXR basic (Ika, Staufen), Heraeus Hera Freeze (Kendro, Langenselbold), Kodak X omat M35 (Kodak), Kühner ISF-1-V (Kühner AG, Switzerland), Elektrophoresis Power Supply Consort E835, Heidolph shaker duomax 1030, Rotator, Thermostatcabinet Aqualytic, Vortex Genie (Neolab, Heidelberg), Innova 44 shaking incubator (New Brunswick Scientific,

Nürtingen), Olympus BX60 fluorescence microscope (Olympus, Hamburg), Semi-dry blotting device (Peqlab, Erlangen), Tri Carb Liquid Scintillation Analyzer (Perkin Elmer, Rodgau), Dissection microscope manual MSM (Singer, Somerset, UK), RP80AT-364, Sorvall Evolution, Sorvall RCM120Ex, SLC6000, SS34 rotor (Thermo Fisher Scientific)

4.4 Radioactivity

Radioactivity was obtained from GE healthcare:
[γ-^{32}P]-ATP (370 MBq/ml, 10 mCi/ml), L-[U-^{14}C]-leucine (1,85 MBq/ml, 50 µCi/ml), L-[^{35}S]-methionine (555 MBq/ml, 15 mCi/ml), L-[U-^{14}C]-phenylalanine (1,85 MBq/ml, 50 µCi/ml)

4.5 Enzymes

Calf Intestine Alkaline Phosphatase (1U/µl), restriction endonucleases (*Bam*HI, *Bgl*II, *Bsr*BI, *Cla*I, *Dpn*I, *Eco*RV, *Eco*RI, *Hind*III, *Hpa*I, *Kpn*I, *Nsi*I, *Nco*I, *Nde*I, *Nhe*I, *Not*I, *Sph*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Spe*I, *Sph*I, *Xba*I, *Xho*I), M-MuLV Reverse Transcriptase, T4-DNA Ligase, *Taq*-Polymerase for colony PCR and KNOP PCR (5 U/µl, all Fermentas, St. Leon-Rot), Micrococcal Nuclease (Roche, Mannheim), *Taq* Polymerase for RT-PCR (Axon, Kaiserslautern) TEV protease, Vent-Polymerase for KNOP-PCR (NEB, Frankfurt), Zymolyase 20000T / 100000T (Medac, Hamburg)

4.6 Antibodies

Primary Antibodies

	Source	Dilution	Company
anti-actin	mouse	1:1000	Chemicon, Canada
anti-CDK9	rabbit	1:200	Santa Cruz, USA
anti Rpl6	rabbit	1:5000	G. Dieci, Parma, Italy
anti-Rps8	rabbit	1:5000	G. Dieci, Parma, Italy
PAP	rabbit	1:5000	Sigma, Taufkirchen

Secondary antibodies

anti-mouse IgG-HRPO	goat	1:3000	Biorad, München
anti rabbit IgG HRPO	goat	1:3000	Biorad, München

4.7 Oligonucleotides

Oligonucleotides were obtained from Thermo Electron, Ulm.

Purpose	**Primer**	**Sequence (5'→ 3')**
Amplification of *ADH1* terminator	5'-*ADH1*$_T$-*Xho*I	gggCTCGAGCCAAGCTAATTCCGGGC
	3'-*ADH1*$_T$-*Sal*I	gggGTCGACCGGTAGAGGTGTGGTCAATAAG
Amplification of TAP-tag	5'-*TAP*-*Xho*I	gggCTCGAGAAGAGAAGATGGAAAAAGAATTTC
	3'-*TAP*-*Sal*I	gggGTCGACTCAGGTTGACTTCCCCGC
Disruption of *CTK1*	5'-*Not*I-*CTK*1-Prom	gggGCGGCCGCGCGCTAATCTAAAAATCGATG
	3'-*Bam*HI-*CTK1*-Prom	gggGGATCCTGTTACCAAATAATTTATGCTGTATG
	5'-*Bam*HI-*CTK1*-Ter	gggGGATCCGGATATATAGCCAATTGAAATAAGTAG
	3'-*Pst*I-*CTK1*-Te	gggCTGCAGGAGGACACAAAGAATTTCAGGC
To control disruption of *CTK1*	5'-*CTK1*-Prom-colony-PCR	GCCAACTCTTCGACAACTG
Point mutation in the active center	5'-*ctk1*-D306A	AAGATTTTACACCGTGCTGTTAAAGGCTCAAAC
	3'-*ctk1*-D306A	GTTTGAGCCTTTAACAGCACGGTGTAAAATCTT
Point mutation in the active center	5'-*ctk1*-K212A	AAGTTAGTAGCGCTGGCTAAATTGAGATTACAA
	3'-*ctk1*-K212A	TTGTAATCTCAATTTAGCCAGCGCTACTAACTT
Sequencing of *CTK1*	*CTK1-down2*	TAATAATACGTCGTATGAAAATAGGCC
TAP-tagging	*CEG1*-oligo1	CAAGTAATGATAAGGAGCCAAAATATGTAGACG AGGATGATTGGTCGGATtccatggaaaagagaag
	CEG1-oligo2	TGCTTTTGCTTTTCTATCTTATTCATACACAAAAT TCTTACACCGATATAtacgactcactataggg
Integration of *GAL1* promotor	*CTK1*-F4	AGTACTTTTAAAAAACCTATTTGTAAATAAAACT AATTCTAGCACTATTCGAATTCGAGCTCGTTTAAAC
	CTK1-R2	TTATTTCTGCTATAACTCTTTGAATAAGTATTGCC ATTATTGTAGGACATTTTGAGATCCGGGTTTT
TAP-tagging	*CTK1*-oligo1	GGTAATAGTAATAATAATAATAATAATAATG ACGATGATGATAAAtccatggaaaagagaag
	CTK1-oligo2	CGGGTATCGCGTAATAAATAAGTTATTAATCTAT TTTTTGTGTCTACtacgactcactataggg
TAP-tagging	*CTK2*-oligo1	TGGAGCTAATTAATGGCGAGTCCAGCATAAATAG TTCTACAAGACATGCAtccatggaaaagagaag
	CTK2-oligo2	AAAAAGGTACTTATTACTTGTGTAAAAGAAAGTT TATAATGATGATCTTAtacgactcactataggg
TAP-tagging	*CTK3*-oligo1	ACAAAAATATCAAGGCTTTAAATGACATTGCGAA GGCATCTTACATATATtccatggaaaagagaag
	CTK3-oligo2	ATTGATACGTATATACGTATATATAATACAGATA CGGATTCTCTTCACAGtacgactcactataggg
TAP-tagging	*RPL11a*-oligo1	ACACCGTCTCTTGGTTCAAGCAAAAGTACGATGC TGATGTTTTGGACAAAtccatggaaaagagaag
	RPL11a-oligo2	TTTTATTAATTGGTTACAAAAAAAACATAGATAT GAGTATATAAATGTATtacgactcactataggg
Cloning of *RPS2*	5'-*Bam*HI-*RPS2*	gggGGATCCGCTTATTCACTAAGGATTCTTAAG
Cloning of *RPS2*, endogenous ter	3'-*Xho*I-*RPS2* endogen	gggCTCGAGCTATTGTAGTCGCCTAATCTTG
Cloning of *RPS2*, without terminator	3'-*Xho*I-*RPS2* vor STOP	gggCTCGAGGAATCTCTTCTTTTGAGCAG
Mutation of CDK consensus site	5'-*RPS2*-S181A	TCTGGTATCGTCGCTGCTCCAGCTGTCAAAAAG
	3'-*RPS2*-S181A	CTTTTTGACAGCTGGAGCAGCGACGATACCAGA

Mutation of CDK consensus site	5'-*RPS2*-T226A	ACATACGGTTTCTTGGCTCCAAACTTGTGGGCC
	3'-*RPS2*-T226A	GGCCCACAAGTTTGGAGCCAAGAAACCGTATGT
Mutation of CDK consensus site	5'-*RPS2*-S238A	CAACCATTGCCAGTTGCTCCATTGGACATCTAC
	3'-*RPS2*-S238A	GTAGATGTCCAATGGAGCAACTGGCAATGGTTG
Mutation of CDK consensus site	5'-*RPS2*-S200A	GATGTCTACACCCAAGCTAACGGTAAGACTAGA
	3'-*RPS2*-S200A	TCTAGTCTTACCGTTAGCTTGGGTGTAGACATC
Sequencing of *RPS2*	*RPS2* seq	CCCAATCAGAAGAGGTTACTG
TAP-tagging	*RPS2*-oligo1	CTCCATTGGACATCTACTCCGATGAAGCTTCTGCT CAAAAGAAGAGATTCtccatggaaaagagaag
	RPS2-oligo2	AGTGTTATTATAAGTGTACAAAATTATTTAATGGT TGATTATAAAAAACTtacgactcactataggg

4.8 Plasmids

pBSKS(+)-5'-*Δctk1-HIS3*-3'	see 5.2.5
pBSKS(+)-*CTK1*	The *Xho*I-*Bam*HI fragment of pRS315-*CTK1* was subcloned into the respective sites of pBSKS(+).
pBSKS(+)-*ctk1*-D306A	Aspartate 306 was substituted for alanine by quick change mutagenesis using pBSKS(+)-*CTK1* as template.
pBSKS(+)-*ctk1*-K212A	Lysine 212 was substituted for alanine by quick change mutagenesis using pBSKS(+)-*CTK1* as template.
pBSKS(+)-*RPS2*	The coding sequence of *RPS2* plus ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating *Bam*HI and *Xho*I sites and cloned into the same sites of pBSKS(+).
pBSKS(+)-*rps2*-S200A	Serine 200 was substituted for alanine by quick change mutagenesis using pBSKS(+)-*RPS2* as template.
pBSKS(+)-*rps2*-S238A	Serine 238 was substituted for alanine by quick change mutagenesis using pBSKS(+)-*RPS2* as template.
pBSKS(+)-*rps2*-S181A	Serine 181 was substituted for alanine by quick change mutagenesis using pBSKS(+)-*RPS2* as template.
pASZ11-*CTK1*	*CTK1* plus ca. 600 bp promoter and ca. 250 bp terminator was subcloned using *Bam*HI and *Pst*I from pRS314-*CTK1* into the respective sites of pASZ11.
pRS313-*CTK1*	*CTK1* plus ca. 600 bp promoter and ca. 250 bp terminator was subcloned using *Bam*HI and *Xho*I from pRS315-*CTK1* into the respective sites of pRS313.
pRS314-*CTK1*	*CTK1* plus ca. 600 bp promoter and ca. 250 bp terminator was subcloned using *Bam*HI and *Xho*I from pRS315-*CTK1*

	into the respective sites of pRS314.
pRS315-*ctk1*-D306A	The *Bam*HI-*Xho*I fragment of pBSKS(+)-*ctk1*-D306A was subcloned into the respective sites of pRS315.
pRS315-*ctk1*-K212A	The *Bam*HI-*Xho*I fragment of pBSKS(+)-*ctk1*-K212A was subcloned into the respective sites of pRS315.
pRS315-*HRB1*	*HRB1* plus 500 bp promoter and 300 bp terminator was amplified by PCR creating *Bam*HI and *Xho*I sites and cloned into the respective sites of pRS315.
pRS315-*RPS2-TAP-*T_{ADH1}	*RPS2* (no stop) plus 500 bp promoter was amplified by PCR creating *Bam*HI and *Xho*I sites and cloned into the respective sites of pRS315-*TAP-*T_{ADH1}.
pRS315-*rps2-S181A-TAP-*T_{ADH1}	*rps2* (no stop)-S181A plus 500 bp promoter was amplified by PCR creating *Bam*HI and *Xho*I sites and cloned into the respective sites of pRS315-*TAP-*T_{ADH1}.
pRS315-*rps2-S238A-TAP-*T_{ADH1}	*rps2* (no stop)-S238A plus 500 bp promoter was amplified by PCR creating *Bam*HI and *Xho*I sites and cloned into the respective sites of pRS315-*TAP-*T_{ADH1}.
pRS315-*rps2-S200-TAP-*T_{ADH1}	*rps2* (no stop)-S200A plus 500 bp promoter was amplified by PCR creating *Bam*HI and *Xho*I sites and cloned into the same sites of pRS315-*TAP-*T_{ADH1}.
pRS315-T_{ADH1}	The 230 bp *ADH1* terminator was amplified by PCR creating *Xho*I and *Sal*I sites and cloned into the *Xho*I site of pRS315.
pRS315-*TAP-*T_{ADH1}	The 540 bp TAP-tag was amplified using pBS1479 as template creating *Xho*I and *Sal*I sites and cloned into the *Xho*I site of pRS315-T_{ADH1}.
pRS316-*CTK1*	*CTK1* plus ca. 600 bp promoter and ca. 250 bp terminator was subcloned by *Bam*HI and *Xho*I from pRS315-*CTK1* into the respective sites of pRS316.

The following previously described plasmids were used:

pRS314-*MEX67*, pRS314-*mex67-5*, pRS314-*mex67-NES2**, pRS314-*mex67-ΔNES*, pUN100-*MEX67*, pUN100-*mex67-5* (Segref et al. 1997), pRS314-*mex67-Cts4*, pRS314-*mex67-Cts19*, pRS314-*mex67-N+LRRts1*, pRS314-*mex67-N+LRRts2*, pRS314-*mex67-N+LRRts3*, pRS314-*mex67-N+LRRts4*, pRS314-*mex67-N+LRRts5 (mex67-6)*, pRS314-*mex67-N+LRRts6*,

pRS314-*mex67-N+LRRts7*, pRS314-*YRA1*, pRS314-*YRA1* no intron, pRS314-*yra1-451*, pRS314-*yra1-635*, pRS314-*yra1-667 = yra1-1*, pRS314-*yra1-ΔN* with intron, pRS314-*yra1-ΔN* no intron, pRS314-*yra1-Δ*RGG, pRS314-*yra1*-ΔRBD, pRS314-*yra1*-ΔRGG+RBD, pRS314-*yra1*-ΔNLS, pRS314-*yra1*-ΔC w/ intron, pRS314-*yra1*-ΔC, pRS314-*yra1*-ΔC (big), pRS315-*GBP2*, pRS315-*CTK1*, pRS315-*GBP2*, pRS315-*MFT1* (Strasser et al. 2000; Strasser and Hurt 2000; Hurt et al. 2004), pRS315-*RPL25eGFP*, pRS315-*RPS2eGFP* (Daniela Strauss), pRS315-*THO2* (A. Aguilera), pUN100-*HPR1* (Joris Braspening / Jochen Baßler) pRS313-*XPO1*; pRS313-*xpo1-1* (Stade et al. 1997), pACTy, pAC1789, pACTMV, pACTGA, pACTAA, pACTQ (Stahl et al. 1995; Bidou et al. 2000), pASZ11 (Stotz and Linder 1990), pRS313, pRS314, pRS315, pRS316 (Sikorski and Hieter 1989), pBSKS (+) (Alting-Mees et al. 1992), pBS1479, pBS1539 (Puig et al. 1998), pFA6a-*HIS3*MX6-P*GAL1* (Longtine et al. 1998), pSP6P (Verge et al. 2004), YdpH (Berben et al. 1991).

4.9 Strains

Escherichia coli

DH5α	F^-, endA1, hsdr17, (r_k^-, m_k^-), supE44, thi-1, recA1, gyrA96, RelA1, 80dlacZ, ΔM15

Saccharomyces cerevisiae

BUR1-TAP	*MATa*; as RS453; *BUR1-TAP::TRP1-KL*	Katja Sträßer
CEG1-TAP	*MATa*; as RS453; *CEG1-TAP::TRP1-KL*	*CEG1-TAP::TRP1-KL* PCR product was integrated C-terminal of *CEG1* into RS453.
CTK1 shuffle	*MATα/a*; as W303; *CTK1:: HIS3* (pRS316-*CTK1*)	see 5.2.5; spore VIIa (*MATα*); spore VIIc (*MATa*).
CTK1 shuffle *Δgbp2*	*MATα/a*; *his3*; *leu2*; *ura3*; *kan1*; *YCL011c::kanMX4*; *CTK1::HIS3* (pRS316-*CTK1*)	*CTK1* shuffle was mated to *Δgbp2*;spore Id (*MATα*); spore IIb (*MATa*).
CTK1 shuffle *Δhpr1*	*MATα/a*; *ura3*; *trp1-1*; *his3; leu2*; *ade2-1*; *can1-100*; *ctk1::ADE2*; *hpr1::HIS3* (pRS316-*CTK1*)	*CTK1* shuffle was mated to *Δhpr1*; spore IId (*MATα*), spore Ia (*MATa*).
CTK1 shuffle *Δhrb1*	*MATα/a*; *ade2-1*; *his3*; *leu2*; *ura3*; *can1*; *ynl004w::kanMX4*; *CTK1:: HIS3* (pRS316-*CTK1*)	*CTK1* shuffle was mated to *Δhrb1* shuffle; spore Ic (*MATα*); spore IIIb (*MATa*).
CTK1 shuffle *Δmft1*	*MATα/a*; *ura3*; *trp1-1*; *his3*; *leu2*; *ade2-1*; *can1-100*; *ctk1::ADE2*; *mft1::KAN* (pRS316-*CTK1*)	*CTK1* shuffle was mated to *Δmft1*; spore Ia (*MATa*); spore IIb (*MATα*).
CTK1 shuffle *MEX67* shuffle	*MATa*; *ade2*; *trp1*; *leu2*; *his3*; *ura3*; *mex67::HIS3*; *ctk1::LEU2*;	*CTK1* shuffle was mated to *MEX67* shuffle; spore Xa (*MATa*), spore

	(pRS316-*CTK1*; pRS316-*MEX67*)	XIIc (*MATa*).
CTK1 shuffle *SUB2* shuffle	*MATa*; *trp1*; *can1*; *leu2*; *his3-11,15*; *ura3*; *YDL084w(4,1338)::kanMX4*; *CTK1::LEU2* (pYCG-*SUB2*)	*CTK1* shuffle was mated to *SUB2* shuffle; spore IIIb (*MATa*), spore IXa (*MATa*).
CTK1 shuffle *YRA1* shuffle	*MATα/a*; *ade2*; *trp1*; *leu2*; *his3*; *ura3*; *CTK1*::*LEU2*; *yra1*::*HIS3* (pRS316-*CTK1*, pRS316-*YRA1*)	*CTK1* shuffle was mated to *YRA1* shuffle; spore Ib, (*MATa*), spore IVd (*MATα*).
CTK1-TAP	*MATα*; as RS453; *CTK1-TAP*::*URA3*	*CTK1-TAP::URA3* PCR product was integrated C-terminal of *CTK1* into RS453.
CTK1-TAP MEX67	*MATa*; as RS453; *mex67::HIS3*; *CTK1-TAP::URA3* (pUN100-*MEX67*)	*CTK1-TAP::URA3* PCR product was integrated into *Δmex67* (pUN100-*MEX67*).
CTK1-TAP mex67-5	*MATa*; ; as RS453; *mex67::HIS3*; *CTK1-TAP::URA3* (pUN100-*mex67-5*)	*CTK1-TAP::URA3* PCR product was integrated into *Δmex67* (pUN100-*mex67-5*).
CTK2-TAP	*MATα*; as RS453; *CTK2-TAP::TRP1-KL*	*CTK2-TAP::TRP1* PCR product was integrated C-terminal of *CTK2* into RS453.
CTK3-TAP	*MATα*; as RS453; *CTK3-TAP::TRP1-KL*	*CTK3-TAP::TRP1* PCR product was integrated C-terminal of *CTK3* into RS453.
GAL1::CTK1-TAP	*MATα*;as W303; *CTK1-TAP::TRP1 HIS3-PGAL1::CKT1*	*CTK1-TAP* was transformed with *HIS3*MX6-P*GAL1* PCR product.
RIX1-TAP	*MATα;* as RS453 *RIX1-TAP::TRP1-KL*	Katja Sträßer
RPB1-TAP	*MATa*; as RS453; *RPB1-TAP::TRP1-KL*	Katja Sträßer
RPL11a-TAP	*MATa*; as RS453; *RPL11a-TAP::TRP1-KL*	*RPL11a-TAP::TRP1* PCR product was integrated C-terminal of *RPL11a* into RS453.
RPS2-TAP	*MATa*; as RS453; *RPS2-TAP::TRP1-KL*	*RPS2-TAP::TRP1* PCR product was integrated C-terminal of *RPS2* into RS453.
RS453	MATa/α; αde2-1; his3-11,15; ura3-52; leu2-3,112; trp1-1; can1-100; GAL+	
TSR1-TAP	*MATa*; as RS453; *TSR1-TAP::TRP1-KL*	Katja Sträßer
UTP9-TAP	*MATα*; ασ RS453 *UTP9-TAP::TRP1-KL*	Katja Sträßer
W303	*MATa/α*; *ura3-1*; *trp1-1*; *his3-11,15*; *leu2-3,112*; *ade2-1*; *can1-100*; *GAL+*	
XPO1	as W303; *xpo1::LEU2* (pRS313-*XPO1*)	Stade et al. 1997
xpo1-1	as W303; *xpo1::LEU2* (pRS313-*xpo1-1*)	Stade et al. 1997

Homo sapiens

HEK293T	Human embryonic kidney cell line

5 Methods

5.1 Standard methods

Cloning procedures such as restriction digest, dephosphorylation of fragments, ligations, and transformation of newly generated vectors in *Escherichia coli* and separation of DNA in agarose gels were done according to Sambrook and Russell, CSHL Press, 2001. Commercial available kits were used according to manufacturer's instructions.

Amplification of yeast genes or TAP-tags was usually done in a 50-100 µl KNOP-PCR reaction using 0.5 µM primer1, 0.5 µM primer2, 0.2 mM of each dNTP, 1 x KNOP buffer (50 mM Tris-HCl, pH 9.2, 16 mM $(NH4)_2SO_4$, 2.25 mM $MgCl_2$), 0.3 µl genomic DNA or 1 µl 1:20 diluted plasmid Midi prep and 1 µl KNOP polymerase (2U *Taq*, 0.56 U Vent). In general, the following amplification protocol was used: 2 min 94°C, [1 min 94°C, 30 s at the respective °C, 1 min / 1000 bp 68°C], 35 cycles, 10 min 68°C.

To check for the correct integration of the disruption cassette, colony PCR was performed. A 25 µl reaction was assembled, containing 1 µM primer1, 1 µM primer2, 62.5 µM of each dNTP, 750 µM $MgCl_2$ and 1x Taq buffer (Fermentas, St. Leon-Rot). Freshly growing cells were picked with a yellow tip and added to the reaction. The PCR reaction was boiled for 15 min, before 1.5 U *Taq* polymerase (Fermentas, St. Leon-Rot) were added. Amplification was performed using the following protocol: [30 s 95°C, 30 s 45°C, 60 s 72°C] 25 cycles, 2 min 72°C.

Point mutations were inserted by quick change mutagenesis using the extensor polymerase protocol (Axon, Kaiserslautern). The PCR product was then digested with 10 U *Dpn*I for 2 hours at 37°C. 50 µl of the reaction were then transformed into *E. coli* DH5α. Point mutations were sequenced by the in-house sequencing service (AG Blum, Gene Center Munich) or plasmids were sent for sequencing to Medigenomix (München).

5.2 Yeast-specific techniques

5.2.1 Culture of *S. cerevisiae*

Yeast strains were cultured in either full-medium or synthetic complete (SC) medium. Full-medium contained 1% yeast extract (Becton Dickinson, Heidelberg), 2% Bacto-Peptone (Becton Dickinson, Heidelberg) and either 2% glucose (YPD) or 2% galactose (YPG). Synthetic complete media contained 0.67% yeast nitrogen base (Formedium, Norwich, UK),

0.06% complete synthetic mix (including all essential amino acids except the four amino acids used as auxotrophy markers, *i.e.* leucine, tryptophane, histidine, uracil and adenine) and either 2% glucose (SDC) or 2% galactose (SGC). 5-FOA was added to a final concentration of 0.1%.

5.2.2 Transformation of yeast cells

50 ml of yeast were grown to an optical density of 0.5 to 0.8 and harvested by centrifugation for 3 min at 3600 rpm using a Rotanda 46R centrifuge. After washing with 10 ml H_2O, the pellet was resuspended in 500 µl of solution I (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM Li-acetate). After centrifugation, the pellet was resuspended in 250 µl solution I. 1 µl of Midi-prep DNA or 5 µl of Mini-Prep DNA was provided in a tube, 5 µl of single strand carrier DNA (DNA of salmon or herring testis, 2 mg/ml), 50 µl of cells in solution I and 300 µl of solution II (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM Li-acetate, 40% PEG-4000) was added and the mixture was incubated for 30 min on a turning wheel at room temperature. Transformations were then heat-shocked for 10 min at 42°C, followed by 3 min incubation on ice. 1 ml of H_2O was added and centrifuged. The pellet was resuspended in 50-µl H_2O and plated on selective plates. For genomic integrations of a galactose promoter, a TAP-tag or a disruption cassette, the pellets were resuspended in 1 ml YPD or YPG and incubated for 1 h at room temperature on a turning wheel prior to plating. To transform yeast cells grown on plate, 1 loop of freshly restreaked yeast cells was resuspended in 100 mM Li-acetate and vortexed. DNA, carrier DNA and solution II were added as described above. After incubation for 30 min on a turning wheel at RT, 35 µl DMSO were added prior to heat shock and transformations were then treated as described above.

5.2.3 Preparation of genomic DNA

10 ml of an overnight RS453 culture with an $OD_{600nm} > 1$ were centrifuged (3 min, 3600 rpm) and washed with 10 ml H_2O. The cells were resuspended in 500 µl H_2O and 200 µl lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). 300 µl glass beads and 300 µl phenol:chloroform:isoamylalcohol (25:24:1) were added and the mixture was vortexed for 3 min. After a centrifugation for 10 min at 16,000 g the upper phase was removed and extracted with an equal volume of chloroform. Genomic DNA was precipitated using 1.2 ml 100% ethanol and incubation of the solution for 10 min at -20°C. After a centrifugation for 30 min at 4°C and 16,000 g, the pellet was dried and resuspended in

400 µl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). To destroy RNA, 20 µl RNaseA (10 mg/ml) were added and incubated for 40 min at 37°C. Genomic DNA was precipitated by addition of 40 µl 3 M Na-acetate, pH 5.2 and 800 µl 100% ethanol and incubation of the mixture for 10 min at – 20°C. After centrifugation (16,000 g, 4°C, 30 min) the pellet was washed with 80% ethanol, dried and resuspended in 30 µl TE.

5.2.4 Genomic integration of a TAP (tandem-affinity-purification) tag

The genomic integration of the TAP-tag is achieved by homologous recombination of the C-terminal region of the respective gene with a transformed PCR-product containing the TAP-tag and an auxotrophy marker. For C-terminal tagging, the 5' primer consisted of 50 nucleotides of yeast genomic sequence flanking the integration site before the stop codon plus 5'-tccatggaaaagagaag-3' (*YXZ*-oligo1), which hybridizes at the 5' end of the CBP coding sequence. The 3' primer consisted of 50 nucleotides in the 3' UTR region, 30 nt downstream of the stop codon (-30 to -80) plus 5'-tacgactcactataggg-3', which anneals downstream of the selection marker (*XYZ*-oligo2). These primers were used to amplify the TAP-tag using pBS1539 (*URA3*) or pBS1479 (*TRP1*) as template (Puig et al. 2001), following the Knop-PCR protocol. The PCR-product was then purified via phenol chloroform extraction. 100 µl PCR-product was mixed with 60 µl phenol:chloroform:isoamylalcohol (25:24:1). After 10 min centrifugation at 16,000 g, the upper phase was removed, mixed with an equal volume of chloroform and centrifuged for 5 min at 16,000 g. The upper phase was removed, mixed with 1 / 10 volume of 3 M Na-acetate and 2 volumes of 100% ethanol. After incubation for 1 h at -20°C, the DNA was precipitated and washed with 70% ethanol. The pellet was dried and resuspended in 10 µl TE. The DNA was then transformed into the yeast cells (5.2.2) to achieve integration into the genome by homologous recombination. Transformants were then tested for the presence of the tag by Western Blotting (5.13).

5.2.5 Deletion of *CTK1*

In order to disrupt a gene completely, the coding sequence of the respective gene has to be replaced by an auxotrophy marker. This is achieved by homologous recombination using a construct carrying overlapping regions in the promoter and terminator region of the respective gene, separated by an auxotrophy marker. The *ctk1::HIS3* construct was cloned by PCR amplifications of a 500 bp *Not*I-*Bam*HI promoter and a 500 bp *Bam*HI-*Pst*I terminator fragment of the *CTK1* gene, which were ligated into the *Not*I and *Pst*I sites of

pBluescriptIIKS(+) resulting in pBS-5'-*Δctk1*-3'. The *Bam*HI *HIS3* fragment of YDp-H (Berben et al, 1991) was then inserted into the *Bam*HI site of pBS-5'-*Δctk1*-3'. The *CTK1* shuffle strain was generated by transformation of the *Not*I-*Pst*I *ctk1::HIS3* fragment in a diploid W303 strain and selection for HIS^+ transformants. Deletion of *CTK1* was assessed by colony PCR using primers that anneal in the promoter of *CTK1* and in the *HIS3* gene. Positive heterozygous transformants were transformed with pRS316-*CTK1*. Cells were sporulated and tetrads dissected using a tetrad dissection microscope. A 2:2 ratio of 2 spores growing and 2 spores dying on SDC-His plates proved for the correct deletion process and haploid *ctk1::HIS3* spores were selected.

5.2.6 Crossings of yeast strains to test for synthetic lethality

Synthetic lethality can be assessed by either combining the deletion of two non-essential genes or by analyzing combinations between different allelic mutations in case of essential genes. Thus, double knockout shuffle strains of the genes of interest needed to be created. Haploid parental strains carrying opposite mating types were mixed onto YPD plates. After several hours, the characteristic diploid cells were selected using a dissection microscope onto YPD plates. As soon as these cells formed colonies, they were first transferred to the respective plates (either YPD or SDC-ura) and on the following day transferred to a sporulation plate. On these plates, the diploid cells undergo meiosis and the genetic information is divided in four haploid spores, enclosed in a tetrad. The outer cell wall of the tetrad was destroyed by incubating 1 loop of cells in 10 µl Zymolyase 20 T (50 mg/ml, 1.2 M sorbitol, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). The destruction was stopped by addition of 30 µl of 10 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, and the spores could be dissected using the dissection microscope. Tetrads with 4 growing spores were then restreaked onto YPD and the respective drop-out plates. Correct double knockout strains were selected by checking for the correct 2:2 auxotrophy marker distribution. Two spores with the opposite mating type were chosen to test for synthetic lethality.

5.2.7 Depletion of Ctk1 by glucose repression and growth curve

A genomic depletion system for *CTK1* was designed based on a strain carrying a C-terminal TAP-tagged version of Ctk1 (allowing determination of protein levels using the anti-protein A antibody) driven by the *GAL1* promoter (*GAL1::CTK1-TAP*). Cells were grown in galactose-

containing media (YPG, Ctk1 expressed) to mid-log phase. Cells were then harvested (3 min, 3600 rpm), washed with H_2O and resuspended in glucose containing medium (YPD), where the expression of Ctk1 was repressed. For the growth curve, cells of three independent cultures were measured every hour and diluted to an OD_{600nm} of 0.3 as soon as the culture reached an OD > 1.

5.2.8 Dot spots

1 loop of freshly growing cells was resuspended in 1 ml H_2O. After performing five 10-fold dilutions, 10 µl of the cell suspensions were spotted onto the corresponding plate (YPD, YPG, SDC-X).

5.3 Cell culture

HEK293T cells were maintained in DMEM high glucose (Invitrogen, Karlsruhe), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicilline, 100 µg/ml streptomycine (all Invitrogen, Karlsruhe). Cells were splitted every other day to maintain a proliferating culture.

5.4 Oligo(dT)-*in situ* hybridization

10 ml of cells were grown to mid-log phase. 1.25 ml 37% HCOH were added and the cell suspension was incubated for 90 min on a turning wheel. For *mex67-5* cells, a 10 ml culture was shifted for 15 min to 37°C prior to addition of HCOH. Cells were pelleted (5 min, 3000 rpm) and washed twice with 5 ml 0.1 M K-phosphate, pH 6.4. After washing with 1 ml of spheroblasting buffer (0.1 M K-phosphate, 1.2 M sorbitol), the cells were resuspended in 200 µl spheroblasting buffer with 100 µg of Zymolyase 100T and incubated for 30 min at 30°C. Zymolyase treatment was stopped by centrifugation for 4 min at 2000 rpm and the spheroblasts were attached to pre-coated poly-lysine slides. After 5 min, non-adherent cells were removed by aspiration. Fixation of the adherent cells was achieved by putting the slide for 6 min into -80°C methanol and for 30 s into acetone. After drying, the cells were first re-hydrated for 10 min in 100 µl 2x SSC (0.3 M NaCl, 30 mM Na-citrate, pH 7.0), before 12 µl prehybridisation buffer (50% formamide, 10% dextran sulphate, 125 µg/ml of *E.coli* tRNA, 500 µg/ml herring sperm DNA, 4x SSC, 0.02% polyvinyl pyrrolidone, 0.02% BSA, 0.02% Ficoll-400) were applied to the cells. After an incubation for 1 h at 37°C, 0.75 µl of 1 pmol/µl

oligo $d(T)_{50}$ probe was added, and the slide was incubated overnight in a humid chamber at 37°C. Slides were then washed with 100 ml 0.5x SSC for 30 min at RT in a staining jar, 5 µg DAPI were added, followed by an incubation for 3 min and further washed with 0.5x SSC for 5 min at RT. The slide was dried at RT in the dark, a solution of 80% glycerol in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) was added and the slide was covered with a coverslip. Finally, slides were analyzed using an Olympus BX60 fluorescence microscope.

5.5 Tandem affinity purification (TAP)

TAP allows a rapid and clean purification of native protein complexes using a combination of two different tags, Protein A and calmodulin binding protein (CBP), separated by a TEV-cleavage site.

5.5.1 Cell harvest and lysis

For purification of native protein complexes of *S. cerevisiae* (Puig et al. 2001), a 2 l culture of an optical density of 3-4 was harvested (2 min, 5000 rpm). Cells were first washed with water, followed by a second washing step with 25 ml TAP-buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 0.15% NP-40) plus protease inhibitors (1.3 µg/ml pepstatin A, 0.28 µg/ml leupeptin, 170 µg/ml PMSF, 330 µg/ml benzamidine). Cells were then flash-frozen in liquid nitrogen. An equal volume of TAP-Buffer (containing 1 mM DTT, protease inhibitors) and a double volume of glass beads were mixed with the cells in a bead mill (Fritsch, Idar-Oberstein), and lysed by the following milling protocol: 3 x [4 min, 500 rpm, 2 min break]. The glass beads were removed and washed once with buffer, so that the lysate comprised 25 ml. After centrifugation for 10 min at 4°C and 4000 rpm (Hettich Rotanda, Germany), the supernatant was subjected to a 1 h 100,000 g ultracentrifugation at 4°C using an SW32 rotor. The top fatty phase was removed by aspiration and the clear lysate was collected. For storage, glycerol was added to the lysate up to a final concentration of 5% and the lysate was flash-frozen in liquid nitrogen.

5.5.2 Purification and TCA precipitation

0.4 ml IgG-sepharose were washed 3 x in TAP-buffer (2 min, 1800 rpm, 4°C) and added to the lysate. After incubation for 1 h on a turning wheel at 4°C, the beads were centrifuged

down and transferred to a mobicol column, containing a 35 µm filter. The beads were washed with 10 ml TAP-Buffer containing 0.5 mM DTT by gravity flow.

To cleave-off the protein complex, 6 µl TEV protease were added in 150 µl TAP-buffer plus 0.5 mM DTT and incubated for 1 h 20 min on a turning wheel at 19°C. For elution, the mobicol was centrifuged in a table top centrifuge for 1 min at 2000 rpm.

During the TEV cleavage, 0.5 ml calmodulin beads were washed 3 x with TAP-buffer containing 1 mM DTT and 2 mM $CaCl_2$. After removal of surplus buffer, the beads were incubated with 150 µl TAP-buffer containing 1 mM DTT and 2 mM $CaCl_2$ on ice. For calmodulin binding, the 150 µl TEV eluate were added, incubated for 1 h on a turning wheel at 4°C and washed with 7.5 ml of TAP-buffer plus 1 mM DTT and 2 mM $CaCl_2$.

To elute the protein complex, the beads were incubated in a thermomixer at 37°C for 2 x 7.5 min in 10 mM Tris-HCl, pH 8.0 and 5 mM EGTA. The eluate was obtained by centrifugation for 1 min at 2000 rpm.

To concentrate the samples, TCA was added to a final concentration of 10% and the samples were incubated for 20 min on ice. After 20 min centrifugation at 16,000 g and 4°C, the pellet was resuspended in 60 µl 1 x SB (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 0.05% bromophenolblue, 25 mM DTT), the resulting solution neutralized by the addition of Tris-Base, denatured and subjected to SDS-PAGE.

5.5.3 Purification of Ctk1-TAP and Ctk3-TAP

The purification of Ctk1 plus associated proteins was essentially done as described above, with some modifications.

As Ctk1 and Ctk3 are low abundant proteins, it was necessary to purifiy them out of a 10 l culture. Five 2 l cell pellets were lysed and after centrifugation, the lysates were combined and separated into four parts. After four single IgG-binding steps, these four TEV eluates (600 µl) were incubated with 500 µl calmodulin beads in 600 µl TAP-Buffer containing 1 mM DTT and 4 mM $CaCl_2$. Binding, washing, elution and precipitation were carried out as described.

To purify exclusively the Ctk1-Ctk2-Ctk3 complex, the IgG beads were washed after the incubation with 10 ml TAP-buffer containing 1 M NaCl, followed by a 5 ml washing step with TAP-Buffer. For *in vitro* kinase assays, the obtained calmodulin eluates were then concentrated to a final volume of about 150 µl using a 30 kDa Membra-spin concentration column. After addition of glycerol to 10%, the samples were frozen at – 20°C. For

reconstitution assays the obtained calmodulin eluate was dialysed against *in vitro* translation buffer (30 mM Hepes-KOH, pH 7.4, 100 mM K-acetate, 2 mM Mg-acetate, 2 mM DTT) over night and then concentrated using a 30 kDa Membra-spin concentration column to a final volume of about 40 µl. This preparation was then immediately used for reconstitution of translation. As negative control for the reconstitution assay, 10 l RS453 were purified using the same method as applied for Ctk1.

5.5.4 Purification of proteins for *in vitro* kinase assays

For *in vitro* kinase assays 2 l cultures of either wt or *BUR1-TAP* cells were purified. After the IgG binding step the beads were washed with 10 ml TAP-buffer containing 1 M NaCl, followed by a 5 ml wash with TAP-buffer. The obtained calmodulin eluates were then concentrated using a 30 kDa Membra-spin concentration column to a final volume of about 150 µl and glycerol was added to a final concentration of 10%. Ribosomal protein TAP (Rps2-TAP, Rpl11a-TAP) were washed after the IgG binding step with 10 ml TAP-buffer containing 250 mM NaCl, followed by a 5 ml washing step with TAP-buffer. TEV eluates were mixed with glycerol to a final concentration of 10% and stored at -20°C until use.

5.6 Sucrose density gradients

5.6.1 Sucrose density gradients with extracts of *S. cerevisiae*

5.6.1.1 Standard conditions

50 ml yeast cells were grown in YPD to an optical density of 0.5. Cycloheximide, blocking translation at the stage of elongation, was added to a concentration of 0.1 mg/ml and cells were further incubated for 10 min at 30°C. Cells were harvested by centrifugation (3 min, 3000 rpm) and resuspended in 500 µl yeast polysome buffer (20 mM Hepes-KOH, pH 7.6, 75-mM KCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT) plus 0.1 mg/ml cycloheximide. Cells were lysed by vortexing the cell suspension with 250 µl glass beads for 5 minutes at 4°C. After removal of cell debris by centrifugation (5 min, 16,000 g), extract corresponding to 250-µg RNA was loaded onto a 10 ml 10-50% linear sucrose gradient in yeast polysome buffer plus 0.1 mg/ml cycloheximide and centrifuged for 2 h at 257,000 g using an SW40Ti rotor. 0.5 ml fractions were obtained by connecting a peristaltic pump to the UV flow cell of the Äkta Basic System (Amersham Biosciences). Absorbance was measured at 254 nm. 125µl

of the fractions were mixed with 500 µl of 20% TCA and precipitated over night on ice. After 30 min centrifugation at 4°C and 16,000 g, the supernatant was removed and the pellet was washed with 500 µl of -20°C cold acetone and centrifuged for 15 min at 4°C and 16,000 g. The pellet was dried at RT, dissolved in 10 µl 1 x SB and subjected to SDS-PAPGE and Western analysis.

5.6.1.2 EDTA treatment

(Poly)Ribosomes can be disrupted into 40S and 60S subunits by the addition of EDTA. Yeast lysates were prepared as described under 5.6.1.1 except that additionally EDTA was added to a final concentration of 15 mM. Extract corresponding to 250 µg RNA was loaded onto a 10 ml 15-35% linear sucrose gradient in yeast polysome buffer plus 0.1 mg/ml cycloheximide and 15 mM EDTA and centrifuged for 2 h at 257,000 g in a SW40Ti rotor. Fractions were obtained and precipitated as described above (5.6.1.1).

5.6.1.3 Omission of cycloheximide

Omission of cycloheximide leads to a nearly complete loss of polysomes and a concomitant increase in monosomes. 50 ml yeast cells were grown in YPD to an optical density of 0.5. Cells were harvested by centrifugation (3 min, 3000 rpm) and resuspended in 500 µl yeast polysome buffer. Cells were lysed by vortexing with 250 µl glass beads for 5 minutes at RT, followed by incubation for 15 min at RT. After removal of cell debris by centrifugation (5-min, 16,000 g), extract corresponding to 250 µg RNA was loaded onto a 10 ml 10-50% linear sucrose gradient in yeast polysome buffer and centrifuged for 2 h at 257,000 g using a SW40Ti rotor. 0.5 ml Fractions were obtained and precipitated as described under 5.6.1.1.

5.6.1.4 Puromycin treatment

Addition of puromycin leads to complete loss of polysomes due to abolishment of the phosphotransferase reaction. Puromycin treatment was carried out by incubating 50 ml yeast culture of an optical density of 0.5 with 1 mM puromycin / 2 mM GTP for 15 min prior to harvesting. Lysis was carried out in 500 µl yeast puromycin buffer (20 mM Hepes-KOH, pH-7.6, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1 mM puromycin, 2 mM GTP) and 250 µl glass beads at 4°C. After removal of cell debris by centrifugation (5 min, 16,000 g, 4°C), extract corresponding to 250 µg RNA was loaded onto a 10 ml 10-50% linear

sucrose gradient in yeast puromycin buffer and centrifuged for 2 h at 257,000 g using a SW40Ti rotor. 0.5 ml fractions were obtained and precipitated as described under 5.6.1.1.

5.6.1.5 *In vivo* crosslinking with formaldehyde

HCOH crosslinking was performed as described (Nielsen et al. 2004). 200 ml cells were grown to an OD_{600nm} of 1.5. Cells were transferred to a tube containing 50 g of crushed ice and the tube was inverted 5 times. HCOH was added to a final concentration of 1%, the tube was inverted for 10 times and incubated for 1 h on ice. Cells were harvested (5 min, 9,000 g, 4°C), and washed with yeast polysome crosslinking buffer (20 mM Hepes-KOH, pH 7.6, 500-mM KCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT) containing protease inhibitors. After centrifugation (5 min, 4000 rpm, 4°C), one volume of cells was resuspended in 1.3 volumes of yeast polysome crosslinking buffer and vortexed eight times for 30 s with 30 s intervals on ice with 1.3 volumes of glass beads. The lysate was centrifuged for 5 min at 1500 g and the resulting supernatant was cleared by two consecutive centrifugations at 16,000 g for 5 and 10 min at 4°C. 250 µg RNA were loaded onto a 10 ml 10-50% linear sucrose gradient in yeast polysome crosslinking buffer and centrifuged for 2 h at 257,000 g using a SW40Ti rotor. 0.5 ml fractions were obtained and precipitated as described under 5.6.1.1. Samples were boiled for 10 min in 1 x SB to reverse the crosslink.

5.6.1.6 Polysome run-off after glucose starvation

Analysis of polysome run-off was done as described (Ashe et al. 2000). Extracts from wt and *GAL1::CTK1-TAP* cells after 18 h growth in YPD were prepared in polysome buffer after 0, 0.5, 1 and 2 minutes shift to YP medium. 250 µg RNA was loaded onto a 10 ml 10-50% linear sucrose gradient in yeast polysome buffer and centrifuged for 2 h at 257,000 g using a SW40Ti rotor. Fractions were obtained as described under 5.6.1.1.

5.6.2 Sucrose density gradients with extracts of mammalian cells

5.6.2.1 Standard conditions

For sucrose density gradients with extracts of mammalian cells, $2x10^7$ adherent HEK293T cells were treated with 50 µg/ml cycloheximide for 5 minutes at 37°C. Cells were harvested and washed twice in icy PBS plus 50 µg/ml cycloheximide (2 min 1200 rpm, Hettich Rotanda). Lysis was performed in 600 µl mammalian polysome buffer (20 mM Tris-HCl, pH

7.5, 5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT and 50 µg/ml cycloheximide) plus 0.3% NP-40. After 5 min incubation on ice, cell debris was removed by 5 min centrifugation at 10,000 g. The supernatant was loaded onto a 10 ml 10-50% linear sucrose gradient in mammalian polysome buffer and centrifuged for 2 h at 257,000 g in a SW40Ti rotor. 0.5 ml fractions were obtained as described under 5.6.1.1 and 0.45 ml were precipitated for Western analysis.

5.6.2.2 EDTA treatment

To disrupt (poly)ribosomes into 40S and 60S subunits, EDTA was added to mammalian polysome buffer to a final concentration of 15 mM. After lysis and centrifugation the supernatant was loaded onto a 15-35% linear sucrose gradient in mammalian polysome buffer plus EDTA and centrifuged for 2 h at 257,000 g in a SW40Ti rotor. 0.5 ml fractions were obtained as described under 5.6.1.1 and 0.45 ml were precipitated for Western analysis.

5.7 *In vitro* translation

5.7.1 Preparation of yeast cell-free extracts

Four liters of cells were grown to an OD_{600nm} of 1.2-1.5. Cells were pelleted by centrifugation for 5 min at 3000 g and washed with 50 ml H_2O in an SS34 tube. The resulting pellet weighted between 6 and 9.5 g. The cells were then incubated for 30 min with 40 ml 1 M sorbitol, 2 mM EDTA and 14 mM β-mercaptoethanol on a shaking device at RT. After centrifugation, the pellet was resuspended in 40 ml 1 M sorbitol containing 1 mg zymolyase 20000T per g cells. Spheroblasting was stopped when the OD_{600nm} dropped to 50% of the value prior to zymolyase addition by centrifugation for 5 min at 3000 g. The spheroblasts were then regenerated in 40 ml 1 M sorbitol in YPD for 1 h at RT with gentle shaking. After centrifugation for 5 min at 3000 g, the cells were resuspended in 1 ml translation buffer (30 mM Hepes-KOH, pH 7.4, 100 mM K-acetate, 2 mM Mg-acetate and 2 mM DTT) per g cell pellet and homogenized with 30 strokes of a Dounce homogenizer with a tight fitting pistil.
The homogenate was then centrifuged for 14 min in a SW40 rotor at 40,000 g. The supernatant was transformed to a new tube and centrifuged for 15 min at 110,000 g. The lipid layer was then removed by aspiration and 2.5 ml of the supernatant were applied onto a PD10 column, pre-equilibrated with 25 ml translation buffer, to remove low molecular weight components. 0.5 ml fractions were collected and 5 fractions with the highest A_{260} value were pooled, aliquoted, flash-frozen and stored in liquid nitrogen.

5.7.2 Determination of translation activity using endogenous mRNA as template

The translation activity of the prepared extracts was measured by incorporation of L-[^{35}S]-methionine into the nascent polypeptide using endogenous mRNA as template (Altmann and Trachsel 1997). 1.25 A_{260} of wt extract or extracts of cells grown in galactose or glucose-containing medium and 1.625 A_{260} of *Δctk1* extracts were used as starting material for the *in vitro* translation reaction. Extracts were incubated with 1.6 µg creatin kinase (5 mg/ml in 30-mM Hepes-KOH, pH 7.4, 50% glycerol) and 1 mM $CaCl_2$ for 10 min on ice in a final volume of 18 µl. 2.2 mM EDTA, translation cocktail (10 mM Hepes-KOH pH 7.4, 2 mM Mg-acetate, 76 mM KCl, 0.4 mM GTP, 1 mM ATP, 0.05 mM of each amino acid except methionine and 12 mM creatin phosphate) was added to a final volume of 30 µl and incubated for 7.5 min at 22°C, leading to a ribosome run-off. 5.6 µCi L-[^{35}S]-methionine were added and the reaction was incubated for 30 min at 22°C. 3 µl of the reaction were spotted onto Whatman filter discs and then soaked in 5% TCA to fix proteins. The discs were then transferred to fresh 5% TCA solution and boiled for 3 min to deacetylate charged met-tRNA, which would otherwise have increased the background incorporation of L-[^{35}S]-methionine. The discs were then rinsed twice with 5% TCA, followed by H_2O, ethanol and acetone, respectively. After drying, the discs were measured in a liquid scintillation counter. Wild type activity was then set to 100% and the activity of the deletion or depletion strain calculated accordingly. Results are the mean of two independent experiments of three independent extracts.

5.7.3 Determination of translation activity using exogenous RNA

5.7.3.1 Preparation of total RNA

The 110,000 g pellet obtained in 5.7.1 contained polysome-bound mRNA and rRNA and was used as source for an exogenous RNA to be added in the *in vitro* translation reaction. To extract the RNA (Gallis et al. 1975) the polysome pellet was carefully rinsed with translation buffer and then resuspended in 0.1 M Tris-HCl, pH 7.5, 0.1 M LiCl, 1 mM EDTA to approx. 50 A_{260}/ml. SDS was then added to a final concentration of 0.1%. An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added and vortexed for 1 min. After 10 min centrifugation at 16,000 g, the extraction of the aqueous phase was repeated. The upper phase was removed and extracted twice with chloroform. After the last centrifugation step the

aqueous phase was removed and Na-acetate pH 5.2 was added to a final concentration of 0.2-M, and mixed with the double volume of -20°C cold 100% ethanol. The RNA was precipitated over night at -20°C and then centrifuged for 30 min at 16,000 g at 4°C. After a second wash with -20°C cold 70% ethanol, the pellet was dried and dissolved in DEPC-treated water.

5.7.3.2 *In vitro* translation reaction

In order to assess whether the translation extract was able to translate exogenously added mRNA, it was crucial to destroy the endogenous mRNA by using micrococcal nuclease. Thus, 1.25 A_{260} of wt extract or extracts of cells grown in galactose or glucose-containing medium and 1.625 A_{260} of *Δctk1* extracts were incubated with 1.6 µg creatin kinase (5 mg/ml in 30 mM Hepes-KOH, 50% glycerol), 1 mM $CaCl_2$ and 1.5 U micrococcal nuclease for 15 min at 22°C. The nuclease treatment was stopped by addition of 2.2 mM EDTA and transfer of the reaction on ice. Translation cocktail and 4 U RNase inhibitor (RNasin, Promega) were added to a final volume of 25 µl and the reaction was incubated for 7.5 min at 22°C, leading to a ribosome run-off on mRNA fragments remaining after nuclease treatment. 5.6 µCi L-[^{35}S]-methionine was added and 12 µl of this reaction were then mixed with H_2O (negative control) or 1 µg of either wt or *Δctk1* RNAs to a final volume of 15 µl. After 30 min incubation, 3 µl of the reaction were analysed as described above (5.7.2). Due to variations in the nuclease treatment, the activity of the extracts was compared to the activity of a sample not treated with nuclease. Wt activity was then set to 100% and the activity of the deletion or depletion strain calculated accordingly. Results are the mean of two independent experiments for each of three independent extracts.

5.7.4 Determination of translation activity by measurement of luciferase activity

In order to determine the translation activity of the extracts with a 100% correctly processed mRNA-template, the mRNA was produced by *in vitro* transcription. The template pSP6P (Verge et al. 2004) was first linearized using *Bsr*BI. The reaction was then phenol-chloroform purified according to 5.2.4 and resuspended in 10 µl DEPC-treated H_2O. For the *in vitro* transcription reaction, the AmpliCap SP6 High Yield Message Maker Kit (Biozym, Hess. Oldendorf) with 1.5 µg linearized template was used accordingly to the manufacturer's

instructions. The luciferase mRNA was then purified using the RNeasy MinElute Cleanup Kit (Quiagen, Hilden) and resuspended in 10 µl DEPC-treated H_2O.

In vitro translation was essentially performed as described in 5.7.3.2, except that instead of L-[^{35}S]-methionine 1 µg luciferase mRNA was added to the reaction. After 30 min incubation at 22°C, the activity of the reaction was measured. 25 µl of the *in vitro* translation reaction were mixed with 100 µl of 25 mM glycyl-glycine, pH 7.8, 10 mM K-phosphate, pH 7.8, 12 mM $MgSO_4$, 3.2 mM EGTA, 2 mM ATP, 1 mM DTT, and 200 µM luciferin using a Lumat LB9507 Luminometer (EG&G Berthold), and the emitted luminescence was detected for 10 s. Wild type activity was set to 100% and the activity of the deletion or depletion strain calculated accordingly. Results are the mean of two independent experiments for each three independent extracts.

5.7.5 Determination of elongation activity and miscoding events

Translation elongation can be determined by incorporation of L-[^{14}C]-phenylalanine into polyphenylalanine using poly(U) as mRNA template. In parallel, the misincorporation of L-[^{14}C]-leucine can be measured due to pairing of the UUU codon with the near-cognate UAA or CAA of Leu-tRNAs. 3.25 A_{260} of wt extract or extracts of cells grown in galactose or glucose-containing medium were incubated with 4 µg creatin kinase (5 mg/ml in 30 mM Hepes-KOH, 50% glycerol), 1 mM $CaCl_2$ and 3.75 U micrococcal nuclease for 15 min at 22°C in a final volume of 45 µl. The nuclease treatment was stopped by the addition of 2.2-mM EDTA and transfer of the reaction on ice. The *in vitro* translation reaction was then splitted (2 x 18.3 µl) and incubated with 4 U RNase inhibitor (RNasin, Promega) and translation cocktail in a final volume of 25 µl. The translation cocktail for determination of translation elongation activity contained 2 mM Mg-acetate, whereas the translation cocktail for measuring misincorporation of leucine contained 10 mM Mg-acetate. For elongation measurements 0.075 µCi L-[^{14}C]-phenylalanine and for determination of miscoding activity 0.075 µCi L-[^{14}C]-leucine were added and 12 µl of the respective reaction were then mixed with 3 µl water (= negative control) or 15 µg poly(U) mRNA. After 30 min incubation, 3 µl of the reactions were analysed as described above (5.7.2). The result of the negative control was subtracted from the result of the reaction containing poly(U). Elongation activity of wt was set to 100% and the activity of the depletion or mutant strain calculated accordingly. Misincorporation was determined by calculating the ratio of incorporated leucine over phenylalanine, followed by the determination of the percentual increase in misincorporation

of the depleted or mutant extract in comparison to wt. Results are the mean of two independent experiments out of three independent extracts each.

5.7.6 Reconstitution of *in vitro* translation extracts with the CTDK-I complex

In order to investigate if Ctk1 is directly involved in the translation process, the Ctk1-Ctk2-Ctk3 complex and a non-tagged wt strain as negative control (RS453) were purified as described under 5.5.3. Buffer, 1.25 µl and 2.5 µl of Ctk1-Ctk2-Ctk3 or RS453 were incubated with 1.25 A_{260} wild type or depletion extract for 30 min at 22°C prior to the *in vitro* translation assay as described under 5.7.2, using endogenous mRNA as a template or as described under 5.7.5 to test the influence of a purified CTDK-I complex on translation elongation or miscoding. Due to variations in the purification of Ctk1 only one out of three representative experiments is shown.

5.8 *In vivo* labelling with L-[^{35}S]-methionine

Cells were grown to mid-log phase. 1.5 OD_{600nm} were pelleted and resuspended in 0.2 ml of YPD. Cells were labeled with 100 µCi L-[^{35}S]-methionine for 5 min at 30°C. After harvesting cells were lysed with 25 µl glass beads in 50 µl 1 x SB. 20 µl of the lysate were analyzed by SDS-PAGE and subsequent autoradiography.

5.9 Drug sensitivity assay

300 µl of a mid log phase of *RPS2* and *rps2-S238A* or wild type and *GAL1::CTK1-TAP* cells grown for 18 hours in glucose-containing medium were spread onto YPD plates on which filter papers were placed. The filter papers contained either 2.5 µg cycloheximide, 100 µg anisomycin, 0.5/5 mg paromomycin (Ctk1 depleted/rps2-S238A), 0.2/1 mg hygromycin B, or 200/500 µg geneticin. Plates were incubated for 3 days at 30°C and the size of the halo indicating dead cells was measured. Representative plates of three independent experiments are shown.

5.10 *In vivo* readthrough frequency analysis

The analysis of readthrough frequency was carried out as described (Kwapisz et al. 2005). Plasmids (Stahl et al. 1995; Bidou et al. 2000) pACTy (+1 frameshift), pAC1789 (-1 frameshift), pACTMV (UAG readthrough), pACTGA (UGA readthrough), pACTAA (UAA readthrough), pACTQ (in frame control), were transformed in W303 and *GAL1::CTK1-TAP*. Cells were grown either in galactose or for 18 hours in glucose-containing medium to an OD between 1.5 and 2.0. 65 OD and 6 OD were used in the β-galactosidase and luciferase assay, respectively. For the β-galactosidase assay, the cells were washed with 1 ml Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0). Lysis was carried out with 100 µl glass beads in 170 µl Z-buffer plus 40 mM β-mercaptoethanol for 30 min in the cold room using a Vibrax device (Ika, Staufen). After 5 min spin at 16,000 g at 4°C, 40 µl of the supernatant were added to 130 µl Z-Buffer plus 40 mM β-mercaptoethanol and 32 µl 4 mg/ml o-nitrophenyl-beta-D-galactopyranoside dissolved in Z-Buffer. Reactions were carried out in triplicates in 96 well plates (MicrotestTM96, Becton-Dickinson) and β-galactosidase activity was measured in a plate reader for 1 h at 30°C with interval-mixings (61 x 3 s). Cells for luciferase assay were washed with 1 ml luciferase lysis buffer (1% TritonX-100, 25 mM glycyl-glycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT) and lysed in 100 µl luciferase lysis buffer and 100 µl glass beads for 30 min at 4°C using a Vibrax device (Ika, Staufen). After 5 min centrifugation at 16,000 g at 4°C, 30 µl of the lysate were measured in a luminometer (Lumat LB9507, EG&G Berthold) according to 5.7.4. The frequency of miscoding, compared to the wild type expressed in percent, was calculated by dividing the luciferase/β-galactosidase ratio for the *GAL1::CTK1-TAP* strain after growth in YPD or YPG for each test plasmid by the ratio for the in-frame control, followed by normalization against the wild type strain. Results are the mean of three independent experiments.

5.11 *In vitro* kinase assay

For *in vitro* kinase assays 5 µl of each kinase and/or substrate were incubated in the presence of 0.5 mM ATP, 1 mM DTT, 100 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA and 1 Ci [γ-^{32}P]-ATP at 30°C for 30 min. Reactions were separated by 15% SDS-PAGE, followed by autoradiography.

5.12 Preparation of salt-washed 80S ribosomes and 60S and 40S subunits

5.12.1 Preparation of salt washed 80S ribosomes

The preparation of salt-washed 80S ribosomes and 60S and 40S subunits was performed as described (Algire et al. 2002). 1 l of W303 was grown in YPD to an OD of 2.6. The culture was cooled on ice for 1 h and harvested by centrifugation for 10 min at 8,000 g at 4°C. The pellet was resuspended in 30 ml ribosome buffer (100 mM K-acetate, 20 mM Hepes-KOH, pH7.6, 2.5 mM Mg-acetate, 1 mg/ml heparin, 2 mM DTT) plus protease inhibitors. After lysing the cells as described under 5.5.1, the lysate was centrifuged for 30 min at 4°C and 17,000 g. The top lipid phase was removed by aspiration and the clear lysate was subjected to a 400,000 g centrifugation using a RP80AT-364 rotor in a Sorvall RCM120Ex ultracentrifuge for 20 min at 4°C. The pellet was washed with 0.5 ml ribosome buffer and resuspended in 18 ml ribosome buffer containing 500 mM KCl and stirred gently on ice for 30-60 minutes. Afterwards, the solution was centrifuged for 10 min at 14,000 rpm to pellet insoluble material. The supernatant was transferred to new tubes and subjected again to a centrifugation. This procedure was repeated for a total of four centrifugations. 2.75 ml of the supernatant were placed on top of a 0.25 ml sucrose cushion (100 mM K-acetate, 20 mM Hepes-KOH pH 7.6, 2.5 mM Mg-acetate, 500 mM KCl, 1M sucrose, 2 mM DTT) and centrifuged for 20 min at 400,000 g using a RP80AT-364 rotor in a Sorvall RCM120Ex ultracentrifuge. After the centrifugation the supernatant was removed and the pellets were quickly washed with 0.5 ml of ribosome storage buffer (100 mM K-acetate, 20 mM Hepes-KOH pH 7.6, 2.5 mM Mg-acetate, 250 mM sucrose, 2 mM DTT). The pellets were resuspended in ribosome storage buffer, flash frozen in liquid nitrogen and stored at -80°C.

5.12.2 Preparation of 40S and 60S subunits

1l of W303 was grown in YPD to an OD_{600nm} of 2 and then pelleted by centrifugation at 6,000-g for 10 min at 4°C. The pellet was resuspended in an equal volume of breaking buffer (20 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA) plus protease inhibitors and lysed according to 5.5.1. The lysate was cleared by centrifugation at 14,000 g for 30 min, 4°C. The supernatant was then centrifuged for 3 h at 100,000 g at 4°C using a SW40 rotor. The resulting pellet was dissolved in 10 ml ribosomal salt wash buffer (20 mM Tris-HCl, pH 7.6, 500 mM KCl, 1 mM DTT, 0.1 mM EDTA, 250 mM sucrose) by gentle stirring for 1 h on ice. The resulting solution was centrifuged for 4 h at 4°C at

100,000-g in a SW40 rotor. The pellet was resuspended in subunit buffer (50 mM Hepes-KOH, pH 7.4, 2 mM $MgCl_2$, 500 mM KCl) to obtain a concentration of 50 A_{260}/ml. Puromycin was added to a final concentration of 1 mM and the mixture was incubated on ice for 15 min, at 37°C for 10 min and then returned to ice. 0.5 ml samples were loaded onto a 10-ml 5-20% sucrose gradient and centrifuged for 9 h at 4°C and 76,000 g in a SW40 rotor. 0.5 ml fractions were obtained by using a peristaltic pump connected to the UV flow cell of the Äkta Basic System (Amersham Biosciences). Absorbance was measured at 254 nm and fractions containing either 40S or 60S subunits were pooled. The pooled fractions were diluted 2 fold with water and centrifuged for 20,5 h at 145,000 g at 4°C. The resulting pellets were resuspended in subunit storage buffer (20 mM Tris-HCl, pH 7.6, 250 mM sucrose, 1 mM DTT, 0.2 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$), flash frozen in liquid nitrogen and stored at -80°C.

5.13 Whole cell extracts (WCE)

Analytical WCE were used to test for the correct integration of the TAP-tag. A 2 ml culture was inoculated with one loop of freshly grown cells and grown over night to saturation. Cells were harvested (3 min 3600 rpm), resuspended in 96°C 100 µl 1 x SB and vortexed with 100-µl glass beads as following: 3 x [1 min vortexing, 3 min 96°C]. After 5 min 16,000 g, 5-µl of this extract were subjected to SDS-PAGE and Western blotting and the presence of the TAP-tag was assessed using the PAP antibody.

For native WCE, cells were grown to mid-log phase and 10 OD_{600nm} were harvested and washed with 1 ml TAP-buffer. The pellet was resuspended in an equal volume of TAP-buffer and lysed with the double volume of glass beads (4 x 2 min vortex, 2 min ice). After a low spin (3 min 1500 g, 4°C), the supernatant was centrifuged for 30 min at 16,000 g, 4°C. The protein concentration was determined using the DC Protein Assay kit (Biorad, München) and 0.2 A_{750} were loaded onto SDS-PAGE and subsequent Western blotting.

5.14 SDS-PAGE and Western Blotting

SDS-PAGE was performed according to Laemmli using the Mini-Protean II system (Biorad, München). Proteins were transferred onto nitrocellulose membrane using a semi-dry blotting machine (Peqlab, Erlangen). After transfer, the membrane was blocked with blocking buffer (2% milk-powder in PBS) and incubated overnight at 4°C with the first antibody dissolved in blocking buffer. Excess of first antibody was removed by washing the membrane 3 times for

15 min with blocking buffer at RT. The membrane was incubated with secondary antibodies diluted in blocking buffer for 2 h at RT. Visualization of immunodecorated proteins was performed using either an ECL-Kit (Applichem), followed by exposure of the membrane to light-sensitive films and subsequent developing using a Kodak X omat M35 developing machine, or by incubating the membrane with 0.3 mg/ml chloronaphthol, 10% methanol, 0.03% H_2O_2 in PBS.

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7 Abbreviations

A-site	acceptor-site of the ribosome
aa-tRNA	aminoacyl tRNA
ASL	anticodon stem loop
ATP	adenosine 5’-triphosphate
CBP	calmodulin binding protein
CDK	cyclin dependent kinase
cpm	counts per minute
CTD	C-terminal domain
CTDK-I	C-terminal domain kinase 1
Cy	carbocyanine
d	day
DAPI	4’,6-diamidino-2-phenylindole
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
eRF1	eukaryotic release factor
E-site	exit-site of the ribosome
FG-repeats	phenylalanine-glycine repeats
5-FOA	5-Fluoroorotic acid
FS	frameshift
GAP	GTPase activating protein
GFP	green fluorescence protein
h	hours
HEK293T	human embryonic kidney cell line
hnRNP	heterogeneous nuclear ribonucleoprotein
LRR	leucine rich region
MBq	megabecquerel
MFC	multifactor complex
min	minute
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein
mut	mutated
NLS	nuclear localization signal

NPC	nuclear pore complex
nt	nucleotides
ORF	open reading frame
PAA	polyacrylamide
PCR	polymerase chain reaction
PIC	pre-initiation complex
P-site	peptidyl-site of the ribosome
P-TEFb	positive transcription elongation factor b
RNAP I	RNA polymerase I
RNAP II	RNA polymerase II
ram	ribosomal ambiguity
Rpl	ribosomal protein of the large ribosomal subunit
Rps	ribosomal protein of the small ribosomal subunit
RRM	RNA recognition motif
rRNA	ribosomal RNA
RT	room temperature
s	second
S2	serine 2
S5	serine 5
sb	sample buffer
sl	synthetically lethal / synthetic lethality
SR-proteins	serine-arginine rich proteins
TAP	tandem affinity purification
TCA	trichloro-acetic acid
TF	transcription factor
TREX	transcription and export
tRNA	transfer ribonucleic acid
ts	temperature sensitive
UTR	untranslated region
YP	yeast extract, peptone
YPD	yeast extract, peptone, glucose; glucose-containing medium
YPG	yeast extract, peptone, galactose; galactose-containing medium
wt	wild type

Curriculum Vitae

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