筑 波 大 学

博士（医学）学位論文

Cytoplasmic deadenylase Ccr4 is required for translational repression of *LRG1* mRNA in the stationary phase in *Saccharomyces cerevisiae*

(ポリ A 分解酵素 Ccr4 は定常状態での *LRG1* mRNA の翻訳抑制に関与する)

2018

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Abbreviations

3'-UTR	3'- UnTranslated Region
CAF1	Chromatin Assembly Factor 1
CWI	Cell Wall Integrity
POP2	PGK promoter directed OverProduction 2
CCR4	Carbon Catabolite Repression 4
ELM1	ELongated Morphology 1
GAPs	GTPase-Activating Proteins
LRG1	Lim-RhoGap homolog 1
MCM2	MiniChromosome Maintenance 2
MCM4	MiniChromosome Maintenance 4
MCM7	MiniChromosome Maintenance 7
miRNA	microRNA
mRNP	messenger RiboNucleoProtein
PAB1	Poly(A) Binding protein 1
PABP	Poly(A) Binding Protein
PAN2	Poly(A)-binding protein-dependent poly(A) riboNuclease 2
PAN3	Poly(A)-binding protein-dependent poly(A) riboNuclease 3
PAP	Poly(A) Polymerase
PBP1	Pab1-Binding Protein 1
PGK1	3-PhosphoGlycerate Kinase 1

PKA	Protein Kinase A
PUF	PUmilio and FBF
P-body	Processing body
qPCR	quantitative real-time Polymerase Chain Reaction
RHO1	Ras HOmolog 1
miRISC	microRNA-Induced Silencing Complex
SCR1	Small Cytoplasmic RNA 1
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
XRN1	eXoRiboNuclease 1
YPD	Yeast extract Peptone Dextrose

Chapter 1. Introduction

1.1. Poly(A) tail regulates mRNA fate

The central dogma of molecular biology explains the flow of genetic information from DNA to mRNA, to make functional protein. This flow is so-called gene expression process which is tightly and precisely regulated by many regulatory factors in adaptation to changing environmental conditions. In the eukaryotic nucleus, mRNAs are transcribed and then undergo modification steps include adding of the cap 7-methylguanosine (m7G) to the 5' end, splicing to remove introns, and adding of poly(A) tail to the 3' end [1]. The poly(A) is added to 3’ end of mRNA to a certain length, up to 70 nucleotides in yeast cell and to 250 nucleotides in animal cell, which is done by template-independent poly(A) polymerases (PAPs) reside in the nucleus [2, 3]. The mature mRNAs are then exported to cytoplasm and ready for translation. In the cytoplasm, mRNA poly(A) tail is supposed to be determinant of mRNA fate that refers to mRNA degradation and translational control (Fig. 1) [2-6]. If the mRNA poly(A) tail is long enough for poly(A) binding protein (PABP/Pab1) binds to and interacts with eIF4G-eIF4E, the components of the translational initiation complex, which also binds to 5’ end of mRNA to form the mRNP loop structure that consequently recruits ribosome subunits and initiates the translation [5, 7-10]. During the lifetime of a single mRNA molecule, the poly(A) tail is shortened gradually from the 3’ end until a certain length causing the disruption of mRNP loop and expose both mRNA ends to degradation enzymes. The mRNA 3’ end is targeted to 3’-5’ exosome exonulease while the mRNA 5’ end is targeted to decapping enzymes for removing of 5’ cap and subsequently degraded by 5’-3’ exonuclease Xrn1 (Fig. 1) [4-6]. For a very long time it has been believing that long poly(A) tail would enhance the translation of mRNA [3, 5, 10]. However, until now, there are a few evidences supporting this idea. The very first evidence is that long poly(A) tail facilitated the translation of reporter mRNA *in vitro* using yeast cell-free translation system [11]. Another work has also suggested that long poly(A) tail enhances the translation of reporter transcript *in vivo* [12]. With endogenous transcripts, the positive correlation between poly(A) tail length and protein level was also observed at specific time in the circadian cycle of mouse liver mRNA [13], and in neuron cell [14]. By contrast, in a recent global genome-wide study, the median poly(A) tail length of animal mRNAs is 50-100 nucleotides, and that the length does not correlate with translational efficiency [15]. Or in a global analysis of poly(A) tail lengths

done with multiple eukaryotic organisms, the correlation between poly(A) tail length and translational efficiency could not be found except for the case of embryonic cells [16]. Therefore, more extensive and careful studies need to be done to clarify the relation of poly(A) tail length and translation control. Whether poly(A) tail length is only important to the specific circumstances like in embryonic stage?

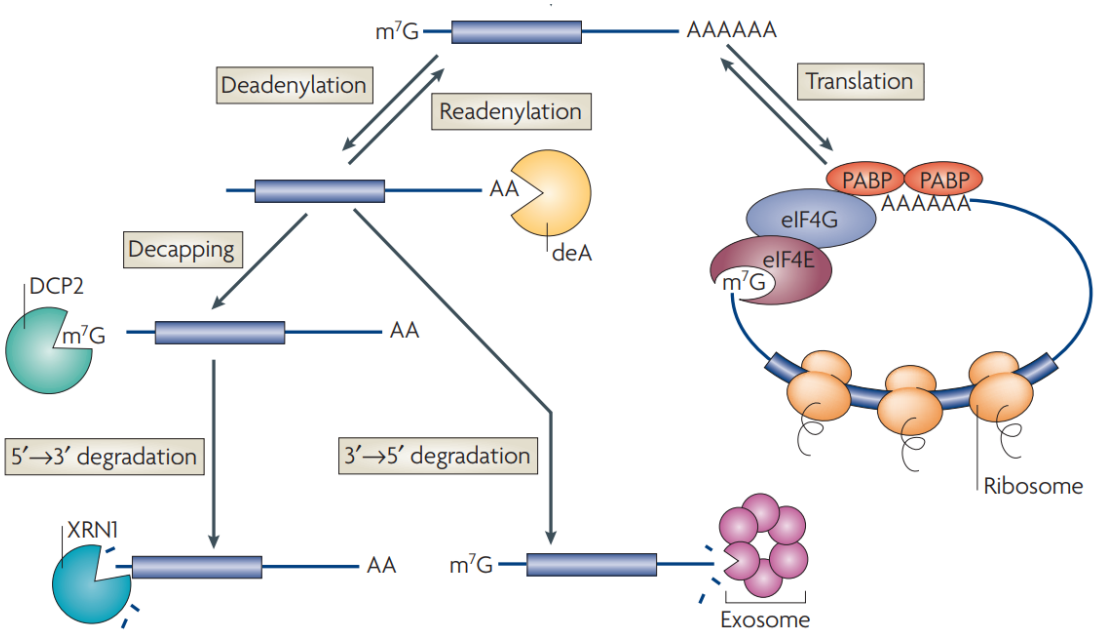

Figure 1. Pathways of translation and degradation of mRNA [6]. Long poly(A) tail would enhance translation of mRNA by promoting the mRNP loop structure formation which recruits ribosome subunits and initiates translation. On the other hand, mRNA poly(A) tail is gradually shortened by cytoplasmic deadenylase leads to disruption of mRNP loop, and then targets mRNA to exonucleases for degradation.

1.2. Cytoplasmic deadenylases shortens poly(A) tail of mRNA

The enzymes responsible for shortening mRNAs poly(A) tail are deadenylases. In the eukaryotic cell, there are two major cytoplasmic deadenylases: the Ccr4-Not complex and the Pan2-Pan3 complex; both are evolutionarily conserved from yeast to human [17-21].

1.2.1. The Ccr4-Not complex

The Ccr4-Not complex is a multi-subunit complex present in all eukaryotic organisms which involves in regulation of transcription, mRNA degradation, translational repression, and protein quality control [19, 21, 22]. It consists of a scaffold

protein Not1 and a number of highly conserved proteins that bind to Not 1 including the Ccr4, three Caf proteins and four Not proteins subunits (Fig. 2) [19, 21]. The Ccr4-Not complex has been detected both in the cytoplasm and in the nucleus. In the cytoplasm, Ccr4-Not subunits are present in polysomes [23-25] where mRNAs are being translated and they have been also detected in P-bodies [26] where mRNAs undergo inhibition of translation, decapping and/or initiation of degradation. In this research, I only focus on the deadenylase activity of this complex, which is very important to mRNA decay and translational repression.

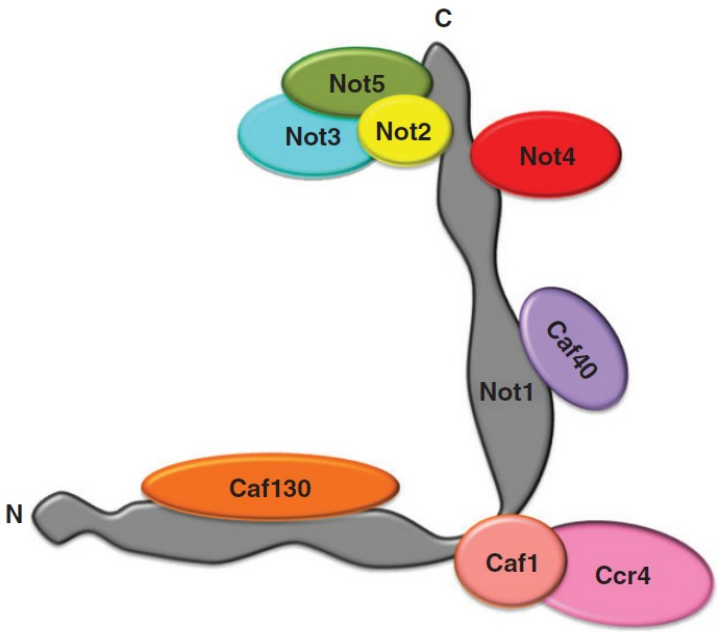

Figure 2. Cartoon represents the components of the Ccr4-Not complex [19]. The L-shape is based on electron microscopy. The position of Ccr4, Caf1/Pop2 and central fragment of Not1 is based on crystal structure. Ccr4 is tethered to Not1 via interaction with Caf1/Pop2 mediated by its N-terminal leucine-rich repeat (LRR) domain.

In yeast, two subunits of the Ccr4-Not complex, a 96 kDa Ccr4 subunit (CNOT6/6L in vertebrates) and a 50 kDa Caf1/Pop2 subunit (CNOT7/8 in vertebrates) have deadenylase activity. Ccr4 is an endonuclease-exonuclease-phosphatase (EEP) superfamily protein and contains a DNase I-like domain. Caf1/Pop2 is a DEDD (Asp-Glu-Asp-Asp) family protein and contains an RNase D-like domain. Even though containing two catalytic subunits, it is proven that Ccr4 is the main catalytic component of Ccr4-Not complex [17, 18, 27], and that Caf1/pop2 subunit acts as the linker that connects Ccr4 with Not1 (Fig. 2) [19, 21]. Deletion of *CCR4* or *CAF1/POP2* leads to

elongation of mRNA poly(A) tail length [17, 28], which may result in increasing in protein level. However, it is reported that the protein levels of septin genes, such as *CDC11* and *CDC42*, are not increased in the *ccr4Δ* mutant although these mRNAs have longer poly(A) tails than those in wild-type (WT) cells [28]. Besides, the *ccr4Δ* mutant shows pleiotropic phenotypes including cell checkpoint defect, aberrant septin organization, weak cell lysis, and cell growth defect. The multiple defects may be caused by the aberrant expression of the target mRNAs of Ccr4, and each of phenotypes can be suppressed by deletion of the related specific genes [28-32]. The Ccr4-Not complex recognizes its mRNA targets through interaction with RNA-binding proteins (for example Puf5 RNA binding protein, figure 3) or the microRNA machinery (in vertebrates), which bind to cis-elements mainly located in 3' UTR of the specific mRNAs [21, 22, 33].

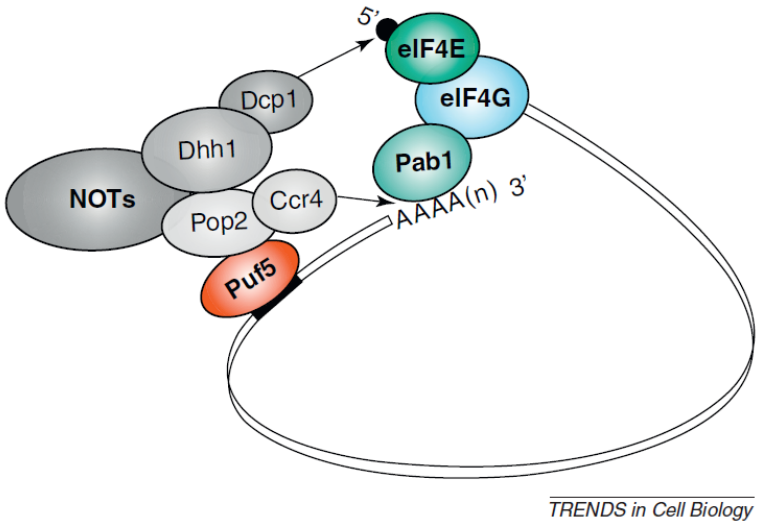

Figure 3. PUF proteins recruit the Ccr4-Not complex to mRNA for deadenylation [33]. Yeast Puf5 RNA binding protein binds to the recognition sequences in the 3' UTR of their target mRNAs and recruits the Ccr4-Not deadenylase complex via their interaction with the Pop2 subunit.

1.2.2. The Pan2-Pan3 complex

The poly(A) specific nuclease (PAN) was initially purified from a PABP/Pab1-dependent yeast strain as an exonuclease that requires PABP/Pab1 to remove poly(A) tail from a model mRNA substrate [34]. Both yeast and mammalian PAN have been shown to contain two subunits, a 127 kDa Pan2 subunit and a 76 kDa Pan3 subunit. The catalytic activity of the complex is due to Pan2, which is a member of the

DEDD/RNaseD family of hydrolytic 3'- exonucleases, the same type of domain found in the Caf1/Pop2 subunit. Whereas the Pan3 subunit acts as a regulatory subunit which contain a poly(A) binding protein (PABP) - interacting motif 2 (PAM2) at N-terminal region that binds to the C-terminal domain of PABP/Pab1, and that Pan2 deadenylase access to mRNA poly(A) tail through its interaction with Pan3 [20, 21]. In yeast, neither of Pan2 or Pan3 is essential for viability but their deletion results in an increased average poly(A) tail length in vivo [35]. The major recruitment of Pan2-Pan3 to mRNAs is via Pan3-PABP/Pab1 interaction which provides a generic mechanism of recruitment of this complex to cytoplasmic mRNA. However, it is not known whether Pan2-Pan3 complex interacts with every PABP/Pab1 bound mRNAs? There are evidences that Pan2-Pan3 complex can target a subset of specific mRNAs through interacting with protein GW182/TNRC6 subunit of microRNA-induced silencing complex (miRISC) in higher eukaryotes [36, 37], indicating that it may regulate the fate of a subset of mRNAs.

1.2.3. The cooperation between Ccr4-Not and Pan2-Pan3 complexes

It has been supposed that after exported to cytoplasm, mRNAs poly(A) tail is first trimmed by Pan2-Pan3 complex and then Ccr4-Not complex primarily shortens the tail in a rate limiting manner to trigger mRNA decay which followed by decapping and degradation of mRNA body by exonucleases [17, 38, 39]. Disruption of either *CCR4* or *PAN2* gene lead to increasing of poly(A) tail length, and deadenylation is completely eliminated when both genes are deleted [20, 28]. In yeast, disruption of *PAN2* caused no obvious phenotype whereas disruption of Ccr4 leads to pleiotropic phenotype, suggesting that Ccr4 may compensate for the loss of Pan2 activity, and that Ccr4 play the dominant role in poly(A) tail shortening [20, 21].

Unexpectedly, recent studies have shown that there is no correlation between the changes in decay rate of mRNAs in *ccr4*Δ mutant versus *pan2*Δ mutant, revealing that they share different subsets of mRNA targets [40, 41]. Interestingly, Ccr4 associates with approximately half of yeast transcriptome in which their abundance and stability are most affected by the deletion of *CCR4*, while the unassociated mRNAs are more strongly affected by the deletion of *PAN2* [41]. Thus, the different phenotypes of *ccr4*Δ and *pan2*Δ mutants can be explained by the difference in mRNA targets, and it seems to be that mRNA targets of Ccr4 are the mRNAs of essential genes. It also

suggests that Pan2-Pan3 complex has more impact on specific transcripts rather than acting as an initial general deadenylase.

In order to assess the role of deadenylases in mRNA regulation, the most common approach is generating the deadenylase dead, knock-down or knock-out mutants and then examine the phenotypes [19-21, 40, 41]. These mutants were also used as a background for study the effect of long poly(A) tail on mRNA fate due to the difficulty of introducing a long poly(A) tail reporter transcript into the cell in the presence of deadenylases. Moreover, until now, most of those studies were carried out at transcript level while few reports about the effect of the deadenylase or of the long poly(A) tail on endogenous proteins level. Thus, it is important to use those genetic backgrounds for analyzing the effect of deadenylase and poly(A) tail on protein level.

1.3. The poly(A) binding protein binding protein-1 (Pbp1) involves in regulation of mRNA poly(A) tail length

Beside deadenylases, other proteins are also involved in regulation of poly(A) tail length including Pab1 binding protein 1 (Pbp1) which was initially found through its interaction with Pab1 [42]. The cell extract of *pbp1*Δ enhances deadenylase activity [42], and that Pbp1 inhibits Pan2-Pan3 complex in yeast cell crude extract [43]. This inhibition is interpreted by that Pbp1 binds to Pab1 and interferes the binding of Pan3 to Pab1 (Fig. 4) [44]. Pbp1 is a member of Like-Sm (LSm) protein family, which participates in a large number of functions related to RNA processing and RNA metabolism [45]. In addition, in yeast Pbp1 associates with Mkt1 to repress the translation of *HO* mRNA and present in polysome fractions [46], and interacts with ribosomal protein Rpl12a and Rpl12b to regulate the cell growth [32], suggesting that Pbp1 not only involved in regulation of poly(A) tail length but also involved in translation regulation. Moreover, pbp1 is co-localized to stress granule where RNA molecules and proteins aggregate (mostly stalled translation initiation complex), and is used as a stress granule marker, however, its role in this type of foci still remains unclear. Disruption of *PBP1* has no obvious phenotype in rich media except for mitochondria dysfunction in stress condition [47].

Similar to other regulatory proteins of poly(A) tail, Pbp1 is highly conserved from yeast to human with the ortholog in mammals is ataxin-2, which is reported involves in neurodegenerative disorders [48]. Therefore, studying the role of Pbp1 using yeast model would give valuable information about cellular function of ataxin-2. Generally,

Pbp1 can reach most of mRNAs by interacting with Pab1. Surprisingly, in a recent report, ataxin-2 associate with a subset of mRNAs through binding to a consensus motif located in 3' UTR and enhance mRNA stability and protein expression [49], suggesting that it may regulate mRNA fates in a specific manner.

Previously, my laboratory has showed that deletion of *PBP1* could suppress the growth defect of *ccr4*Δ mutant [32] indicating that Pbp1 together with Ccr4 may regulate the proper cell growth through regulating of deadenylation, poly(A) tail length and translation.

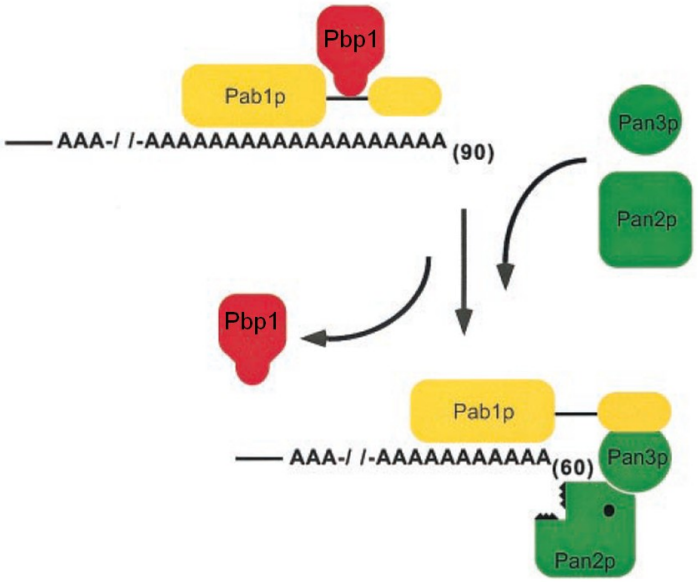

Figure 4. Pbp1 inhibits Pan2-Pan3 complex [44]. Pbp1 binds to Pab1 and hinders the Pan3-Pab1 interaction. In the absence of Pbp1, Pan3 binds to Pab1, which facilitates deadenylation of poly(A) tail by Pan2.

1.4. The Lim-RhoGap homolog 1 - Lrg1

1.4.1. Lrg1 is a GTPase activating protein of cell wall integrity pathway

The yeast cell has the cell wall which serves principal functions including providing protection from osmotic shock; protecting the cell against mechanical stress; establishing and maintaining the cell shape which is essential for the formation of cell budding and division; serving as a scaffold for cell-surface proteins [50]. The regulatory pathway employed by *Saccharomyces cerevisiae* to maintain cell wall integrity during growth, morphogenesis, and in the face of environmental challenges is cell wall integrity pathway (CWI). This pathway exists for the purpose of detecting and

responding to cell wall stresses that arise during normal growth conditions or through environmental changes. The CWI pathway responds to cell wall stress signals through a family of cell surface sensors coupled to a small G protein, Rho1, whose activity is also stimulated periodically through the cell cycle and regulated by both guanosine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Fig. 5). Rho1 is considered to be the master regulator of CWI signaling not only because it integrates signals from the cell surface and the cell division cycle, but also because it regulates a variety of outputs involved in cell wall biogenesis, actin organization, and polarized secretion (Fig. 5) [50]. One of the GAPs of Rho1 is Lrg1, which contains Rho1p-specific GAP activity that interacts with activated forms of Rho1p; functions as a negative regulator of the cell wall integrity signaling pathway, and of cell wall 1,3-beta-glucan biosynthesis [50, 51]. High level of Lrg1 protein inhibits Rho1 active form results in down-regulation of CWI pathway and reduces cell wall integrity, which causes the cell lysis at high temperature.

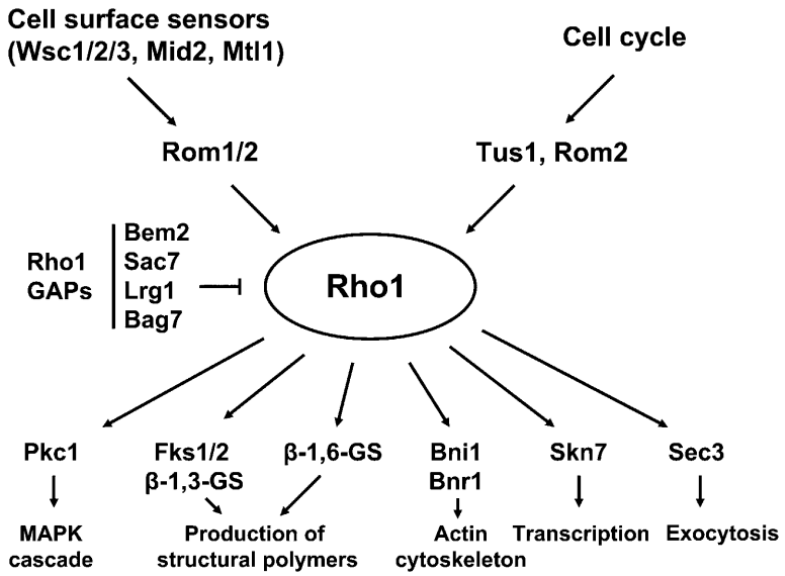

Figure 5. Rho1 regulators and effectors [50]. In response to cell wall stresses, Rho1 localization and activity are regulated by cell surface sensors, a family of GEFs (Rom1, Rom2, and Tus1), and a set of GAPs (Bem2, Sac7, Lrg1, and Bag7). Rho1 activity controls the downstream events including cell wall biogenesis through polymer synthesis, polarization of the actin cytoskeleton, directed secretion, and transcription.

1.4.2. *LRG1* mRNA is a target of Ccr4 deadenylase and Puf5 RNA binding protein

My laboratory has previously shown that *LRG1* mRNA is stabilized in the *CCR4* knock-out mutant (Fig. 6) [31, 52]. It indicates that Ccr4 negatively regulate *LRG1* mRNA, maybe through regulating *LRG1* poly(A) tail length, which is the determinant of mRNA degradation. However, it is not know whether *LRG1* poly(A) tail length is shortened by Ccr4 or not. In addition, *LRG1* mRNA is a target of Mpt5/Puf5 RNA binding protein which belongs to PUF (Pumilio and FBF) protein family known for its roles in cell division, differentiation and development [33, 53, 54]. Puf5 binds to a subset of mRNAs, approximately 17% of yeast transcriptome [54] including *LRG1* transcript, at a consensus motif located in the 3' UTR [53, 54], and recruits Ccr4-Not deadenylase complex through physical interaction with Caf1/Pop2 subunit (Fig. 3) [55, 56].

The *ccr4*Δ mutant shows high-temperature sensitive defect, which may be caused by the abundance of Lrg1 protein level, and that this phenotype could be suppressed by deletion of *LRG1* gene [52]. Interestingly, deletion of *PBP1* also confers the high temperature sensitive defect of *ccr4*Δ mutant [32] suggesting that Pbp1 may contribute to the regulation of *LRG1* expression together with Ccr4.

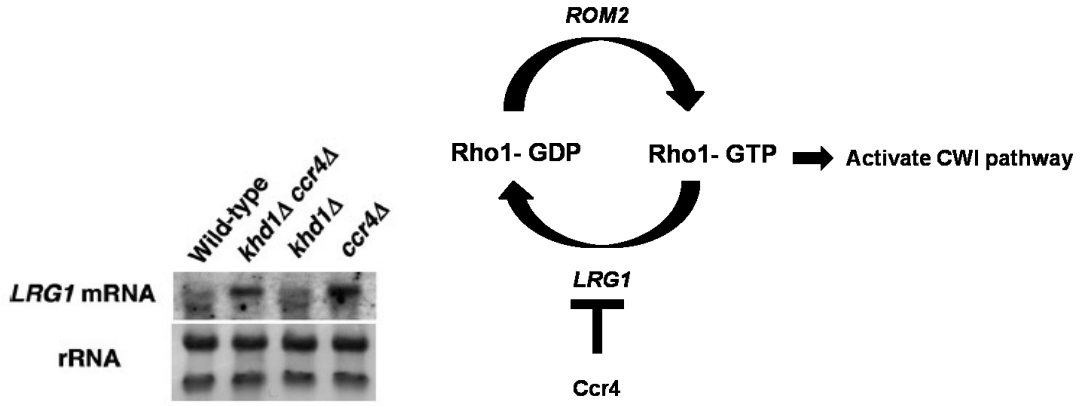

Figure 6. Ccr4 negatively regulates *LRG1* mRNA involved in CWI pathway [31]. *LRG1* mRNA encoding for GTPase activating protein of small GTPase Rho1 involved in cell wall integrity pathway. In the absence of Ccr4, *LRG1* is up-regulated leads to down-regulation of CWI pathway by converting Rho1-GTP active form into Rho1-GDP inactive form, and results in high-temperature sensitive growth defect.

1.5. The aim of this study

To my knowledge, most of studies examined the role of deadenylation with reporter transcripts rather than endogenous transcripts, and at mRNA level rather than protein level, of the growing phase cells when the nutrients are abundant in the culture. In a rare work done with endogenous transcripts and examined at protein level, unexpectedly, Traven et al. has found no positive correlation in between poly(A) tail length and protein level of endogenous septin mRNAs in the log phase *ccr4*Δ mutant cells [28]. Therefore, I aim to use *LRG1* transcript as a candidate to study the effect of Ccr4 and Pbp1 on its expression, from poly(A) tail length to transcript level and protein level, from the log phase to the stationary phase when nutritions in the media depleted, in order to get further insights into cellular function of Ccr4, Pbp1 and poly(A) tail length in gene expression regulation.

Chapter 2. Materials and Methods

2.1. Strains and media

Escherichia coli DH5α strain was used for DNA manipulations. The yeast strains used in this study are isogenic derivatives of the W303 background and are listed in Table 1. The deletion mutants were generated by a PCR-based method, as described previously [57], and were verified by PCR to confirm complete deletion at the expected locus. Yeast strains were manipulated according to standard procedures [58]. The media used in this study including rich medium (YPD) and synthetic complete medium (SC). SC media lacking amino acids or other nutrients (e.g. SC-Trp corresponding to SC lacking tryptophan) were used to select the transformants. The glucose level in the media was measured by using the Glucose (GO) Assay Kit (Sigma), and ethanol level was measured by using the Ethanol Assay Kit (DIET-500) (BioAssay Systems).

2.2. Plasmids

Plasmids used in this study are listed in Table 2. The pRS314-3FLAG-LRG1 plasmid was constructed as follow. The fragment encoding *LRG1* promoter and the fragment encoding *LRG1* ORF - *LRG1* terminator were obtained by PCR from genomic DNA using two pairs of primers (CTAAAGGGAACAAAAGCTGGGTACCTATGGGCAAACAATATAACCC and GATAACCAGCAGAATTTTGAACCATGGCTCACCTCCGGTACTTGT; ACAAGTACCGGAGGTGAGCCATGGTTCAAAATTCTGCTGGTTATC and CTCACTATAGGGCGAATTGGAGCTCATATTCAATGGTGTCATTAAT) to introduce an additional *Nco*I site right after the start codon. Two fragments were inserted into between *Kpn*I and *Sac*I sites of the pRS314 plasmid using gap repair cloning [59]. The synthetic fragment encoding 3xFLAG with two flanking *Nco*I sites (5'-CATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGG ATGACGATGACAAGGG-3' and 3'-CTGATGTTTCTGGTACTGCCACTAATATTTCTAGTACTGTAGCTAATGTTCCTACTG CTACTGTTCCCGTAC-5') was then annealed and inserted into the N-terminal of *LRG1* ORF. The plasmid YEplac195-LRG1 and YEplac195-PAN2 were used to over-express *LRG1* and *PAN2* genes, respectively. The plasmids YCplac33-CCR4 and YCplac33-CCR4-D713A express the wild-type *CCR4* allele and the deadenylase-dead *CCR4*

(D713A) allele [27], respectively. The plasmids pCgLEU2, pCgHIS3, and pCgTRP1 are pUC19 carrying the *Candida glabrata LEU2, HIS3,* and *TRP1* genes respectively, were used for gene deletion experiments [60].

2.3. RNA extraction, qRT-PCR, and poly(A) tail length assay

Cells were grown from the exponential phase to the stationary phase in YPD medium or SC-Trp medium and then harvested at the indicated times. Total RNAs were then prepared using ISOGEN reagent (Nippon Gene) and the RNeasy Mini kit (Qiagen). First strand of cDNAs were generated using the Prime Script RT reagent Kit (Takara). The cDNAs were quantified by a quantitative real-time RT-PCR (qRT-PCR) method using a 7500 fast real-time RT-PCR system (Applied Biosystems) with SYBR Premix Ex Taq (Takara). The *LRG1* primers (ACCTGCCAAGACTGTCAGAAAC and TAATCCACGCAATGGGGTATC) and *SCR1* primers (AACCGTCTTTCCTCCGTCGTAA and CTACCTTGCCGCACCAGACA) [61] were used to analyze the mRNA levels of *LRG1* and *SCR1*. The fold changes in mRNA levels were calculated by using the delta delta Ct method and normalized to the *SCR1* reference gene. The statistical analysis was performed with Excel (Microsoft) using Tukey's test, and differences were considered significant when $p < 0.05$. The poly(A) tail length of *LRG1* mRNA was measured by using the poly(A) tail length assay kit (Affymetrix) according to the manufacturer's instruction. A fragment including *LRG1* poly(A) tail was amplified by using the forward primer anneals to *LRG1* 3'-UTR (CCAGTATGCTATGGAAATGG), *MCM2* 3'-UTR (CGCAATTTATACCTTGGGTCAC), *MCM7* 3'-UTR (GCCCAAGATTCTGATATCGATC), *ELM1* 3'-UTR (ATAATCGTATAGCCGATGTG), and the universal reverse primers included in the kit. The average length of poly(A) tail were determined by sequencing.

2.4. Protein extraction, western blotting analysis, and antibodies

The cells collected from indicated times were then treated with sodium hydroxide for protein extraction, as described previously [62]. Protein samples were loaded on to an 8% or 10% SDS-PAGE gel for protein electrophoresis and then transferred to a PDVF membrane (Millipore) for Western blot analysis. Anti-FLAG polyclonal antibody M2 (Sigma), anti-Mcm2 polyclonal antibody N-19 (Santa Cruz), anti-Mcm4 polyclonal antibody yC-19 (Santa Cruz), anti-Mcm7 polyclonal antibody yN-19 (Santa Cruz), and anti-Elm1 polyclonal antibody y-640 (Santa Cruz) were used to

detect 3Flag-Lrg1, Mcm2, Mcm4, Mcm7, and Elm1, respectively. The monoclonal anti-Pgk1 antibody 22C5D8 (Invitrogen) was used to detect Pgk1, as the loading control, since Pgk1 is reported to be a very stable protein based on its half-life [63]. Detection was carried out by using a LAS-4000 (Fuji Film) with Immobilon Western (Merck Millipore). Signal intensities were quantified by means of Image Quant (GE Healthcare).

2.5. Polysomes analysis

Cycloheximide was added to the cultures to the final concentration 100 μg/ml, and agitated for 15 min to stop the translation. The cells were harvested and resuspended in 0.5 ml lysis buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 30 mM $MgCl_2$, 100 μg/ml cycloheximide, 200 μg/ml heparin, 0.1% dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and then mixed with 0.5 ml glass beads. The cells were lysed by bead beating 4 times, each time for 30 s with 30 s interval on ice. After bead beating, 0.5 ml lysis buffer was added, and centrifuged at 14,000 rpm for 10 min at 4ºC to collect the supernatant. Twenty A260 nm units of the supernatant were loaded on top of sucrose gradients (10% - 50% w/v). Polysomes were fractionated by centrifugation at 27,000 rpm for 3 h at 4ºC with a SW28 Ti rotor (Beckman Coulter). The gradient was continuously collected from the Gradient Station (Biocomp), and the collection line was connected to a UV detector to monitor the 254 nm absorbance. Sixteen fractions (1.9 ml/fraction) were collected by a fraction collector. The RNA from polysomes fractions were precipitated by ethanol overnight at -30ºC and then purified by using RNeasy Mini kit (Qiagen). The cDNAs were generated from the same volume RNA samples using the Prime Script RT reagent Kit (Takara). The *LRG1* cDNA was amplified by Blend Taq (Toyobo) with specific primers (TCTCGATGATAAGGGCTATCAG and TAACACGCTGTTTCTCATCCTC).

Chapter 3. Results

3.1. Ccr4 negatively regulates poly(A) tail length and level of *LRG1* mRNA

Traven et al. have shown that, in the *ccr4*Δ mutant, mRNAs encoding regulators of septin assembly such as *CDC42*, *CDC24*, *RGA1*, and *ELM1*, harbor longer poly(A) tail; however, the levels of these mRNAs and proteins are not increased [28]. I examined poly(A) tail length, *LRG1* mRNA level, and Lrg1 protein level in WT and *ccr4*Δ mutant harboring the FLAG-LRG1 plasmid. This FLAG-LRG1 plasmid contains endogenous *LRG1* promoter, the coding sequence of *LRG1* gene fused with 3xFLAG tag at N terminal, and *LRG1* 3′-UTR. In this experiment, I cultured the cells in longer time, up to 120 h. The WT and *ccr4*Δ mutant reached saturated cell density after 60 h of culture (Fig. 7A). I harvested the cells at the time points including 4 h, 24 h and 48 h, and 72 h, corresponding to the early log phase, the late log phase, and the stationary phase, respectively.

In agreement with Traven's report [28], the *LRG1* poly(A) tail lengths in the *ccr4*Δ mutant were longer than those in WT (Fig. 7B, WT vs *ccr4*Δ, 4 h and 48 h). Consistent with the fact that poly(A) tail length is important for mRNA stability, *LRG1* mRNA levels in the *ccr4*Δ mutant were higher than those in WT through the time course (Fig. 8A, 4 h, 24 h, 48 h, 72 h). In WT cells, the *LRG1* mRNA level dramatically dropped throughout the time course (Fig. 8A, WT). In contrast, the *LRG1* mRNA level only slightly dropped throughout the time course in *ccr4*Δ mutant, and it remained relatively high level even at the 48 h and 72 h time points (Fig. 8A, *ccr4*Δ). These results suggest that Ccr4 negatively regulates the poly(A) tail length and the *LRG1* mRNA level, and that the longer poly(A) tail seems to be more important for the mRNA level at the later time points of cell growth.

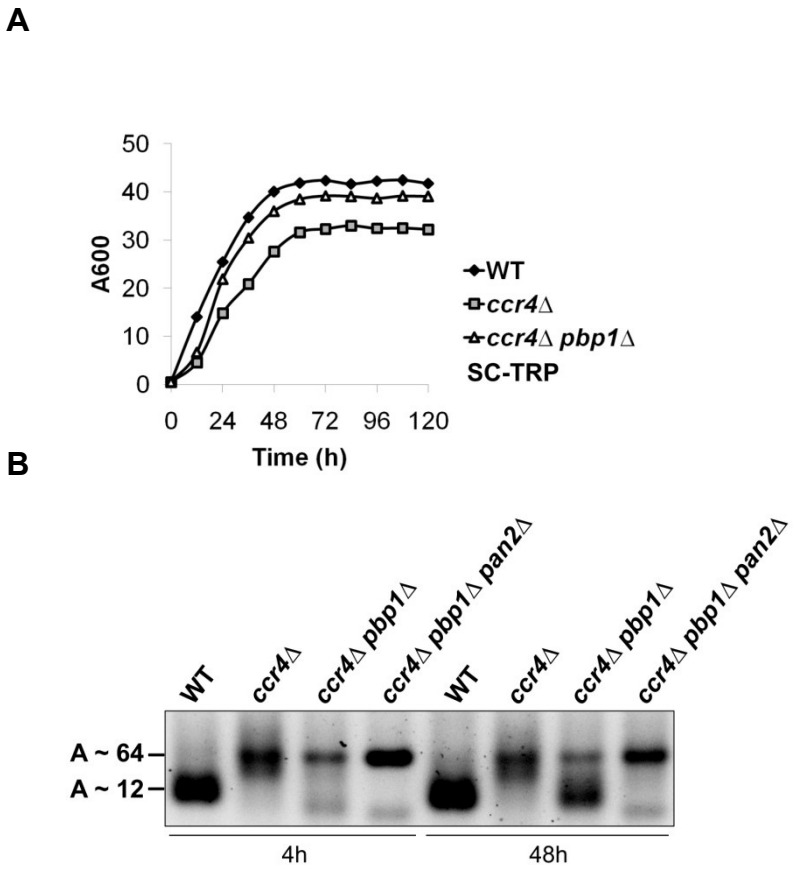

Figure 7. The *LRG1* poly(A) tail length in WT and mutant cells at the log phase and stationary phase. (A) The growth curves of WT, *ccr4*Δ, and *ccr4*Δ *pbp1*Δ cells in SC-Trp media. The strains harboring the plasmid pRS314-3FLAG-LRG1 were pre-cultured overnight and then transferred into fresh SC-Trp media to grow for 5 days at 28°C. The cell cultures were taken at the indicated times to measure A600 nm. (B) The *LRG1* poly(A) tail lengths in WT, *ccr4*Δ, *ccr4*Δ *pbp1*Δ, and *ccr4*Δ *pbp1*Δ *pan2*Δ mutant cells in the log phase (4 h) and the stationary phase (48 h). The strains were grown in YPD media from the log phase to the stationary phase at 28°C. The cells were collected at indicated time points for RNA isolation. The *LRG1* poly(A) tail was amplified using the poly(A) tail length kit. The average poly(A) tail lengths were determined by sequencing.

3.2. Lrg1 protein level is up-regulated in the stationary-phase *ccr4*Δ mutant cells

I then examined the Lrg1 protein levels in WT and *ccr4*Δ mutant (Fig. 8B). At the 4 h time point, Lrg1 protein level in *ccr4*Δ mutant was similar to that in WT, although the *LRG1* mRNA level in *ccr4*Δ mutant was slightly higher than that in WT (Figs. 8A and 8B, WT vs *ccr4*Δ, 4 h). This data also suggests that the longer poly(A) tail of *LRG1* mRNA has little effect on Lrg1 protein level at this 4 h time point. Correlated with the

observation that the *LRG1* mRNA level in WT dramatically dropped throughout the time course (Fig. 8A, WT), the Lrg1 protein level in WT also dramatically dropped throughout the time course (Fig. 8B, WT). In the *ccr4*Δ mutant, as the *LRG1* mRNA remained relatively high level even at the 24 h, 48 h, and 72 h time points (Fig. 8A, *ccr4*Δ), Lrg1 protein levels also remained relatively high level even at 24 h the 48 h and 72 h time points (Fig. 8B, *ccr4*Δ). The Lrg1 protein levels in the *ccr4*Δ mutant were continuously maintained higher than those in WT up to 120 h of the culture (data not shown). It is noted that, at 48 h time point, the *LRG1* mRNA level in *ccr4*Δ mutant was 2-fold higher than that in WT (Fig. 8A, WT vs *ccr4*Δ, 48 h), but Lrg1 protein level in *ccr4*Δ mutant was 8.9-fold higher than that in WT (Fig. 8B, WT vs *ccr4*Δ, 48 h). The relative Lrg1 protein level/ *LRG1* mRNA level ratios in WT and *ccr4*Δ mutant cells at this 48 h time point were 0.276 and 1.196, respectively. Thus, the effect of *ccr4*Δ mutation on Lrg1 protein level was dominant compared to that on *LRG1* mRNA level at the 48 h time point. In addition, the *LRG1* poly(A) tail length in the *ccr4*Δ mutant was also longer than that in WT at 48 h of the cultures (Fig. 7B, lane 5, 6). These data suggest that Ccr4 negatively regulates not only the *LRG1* mRNA level through the poly(A) shortening, but also the translation efficiency of *LRG1* mRNA.

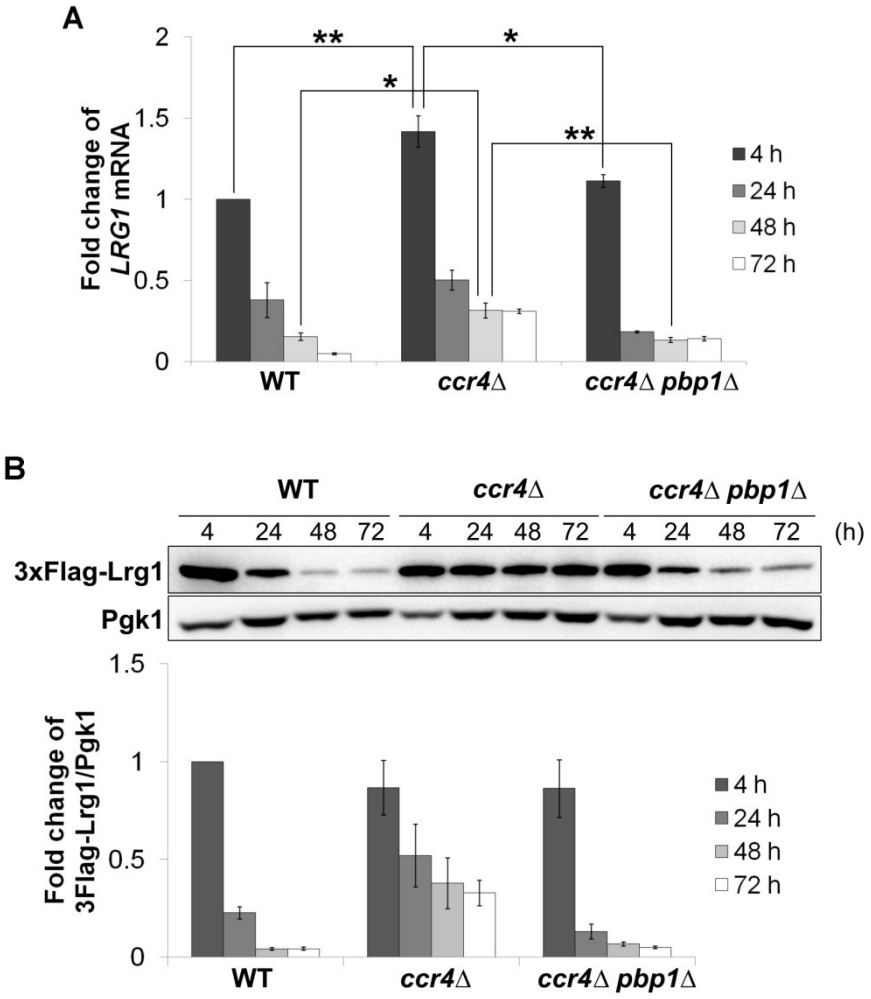

Figure 8. *LRG1* mRNA and protein levels were increased in the stationary-phase *ccr4*Δ mutant. (A) Expression of *LRG1* mRNA in WT, *ccr4*Δ*,* and *ccr4*Δ *pbp1*Δ mutants. The strains harboring the plasmid pRS314-3FLAG-LRG1 were grown at 28ºC from the log phase to the stationary phase in SC-Trp media. The cells were collected at the indicated times for RNA isolation. The *LRG1* mRNA levels were quantified by qRT-PCR analysis, and the relative mRNA levels were calculated using delta delta Ct method normalized to *SCR1* reference gene. The data show mean ± SEM (n = 4) of fold change of *LRG1* mRNA from WT cells at 4 h of culture. *P < 0.05, **P < 0.01 as determined by Tukey's test. (B) Expression of Lrg1 protein in WT, *ccr4*Δ*,* and *ccr4*Δ *pbp1*Δ mutants. The strains harboring the plasmid pRS314-3FLAG-LRG1 were grown at 28ºC from the log phase to the stationary phase in SC-Trp media. The cells were collected at the indicated times, and cell extracts were prepared for immunoblotting with anti-Flag (3xFlag-Lrg1) and anti-Pgk1 antibodies. The intensities of 3xFlag-Lrg1 signals were measured and normalized to the Pgk1 signals. The values are plotted as the fold change from WT cells at 4 h of culture. The data show mean ± SEM (n = 3).

To assess the role of the deadenylase activity of Ccr4 in the regulation of *LRG1* expression, the catalytic residue of Ccr4, Asp-713, which is required for *in vitro* deadenylase activity, was mutated to alanine [27]. While the wild-type *CCR4* gene could decrease the high Lrg1 protein level in the stationary-phase *ccr4*Δ mutant cell, the *CCR4-D713A* gene could not (Fig. 9). Consistently, the wild-type *CCR4* gene, but not *CCR4-D713A,* complemented the growth defect of *ccr4*Δ mutant (data not shown). Thus, the deadenylase activity of Ccr4 is required for the regulation of *LRG1* expression.

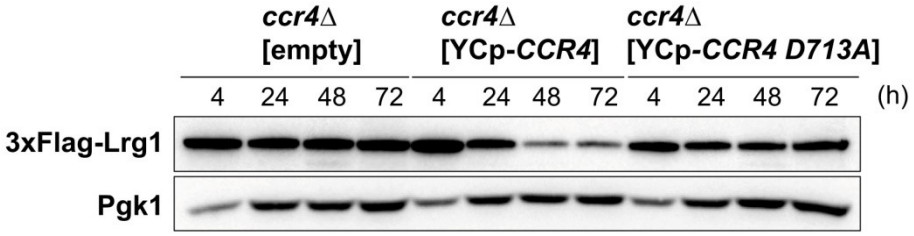

Figure 9. The deadenylase activity of Ccr4 is required for the regulation of *LRG1* expression. The plasmid YCplac33-CCR4 or plasmid YCplac33-CCR4-D713A or empty vector was transformed into the *ccr4*Δ mutant cells harboring plasmid pRS314-3FLAG-LRG1. Transformants were grown at 28ºC from the log phase to the stationary phase in SC-Trp-Ura media. The cells were collected at the indicated times, and cell extracts were prepared for immunoblotting with anti-Flag (3xFlag-Lrg1) and anti-Pgk1 antibodies. Pgk1 was used as the loading control.

3.3. Active translating polysomes are abundant in the stationary-phase *ccr4*Δ mutant cells

The Ccr4 deadenylase has been shown to associate with polysomes [25] and the Ccr4 ortholog in *Xenopus laevis* oocytes has been shown to have translational repression activity [64]. I therefore examined whether Ccr4 negatively regulates the translation in the later growth phase (i.e. 48 h or later time point in Fig. 7A). In this time, I cultured the cells not harboring the FLAG-LRG1 plasmid in YPD media (Fig. 10A), and determined the exact growth phases based on the glucose and ethanol levels [65]. The WT cells used up glucose and went into the post diauxic-shift after 12 h of culture, whereas the *ccr4*Δ mutant cells took 24 h (Fig. 10B). After glucose was exhausted in the media, the cells turned to utilize ethanol and went into the stationary phase after 48

h of culture, when the cell densities were saturated (Fig. 10A) and ethanol was depleted in the media (Fig. 10B).

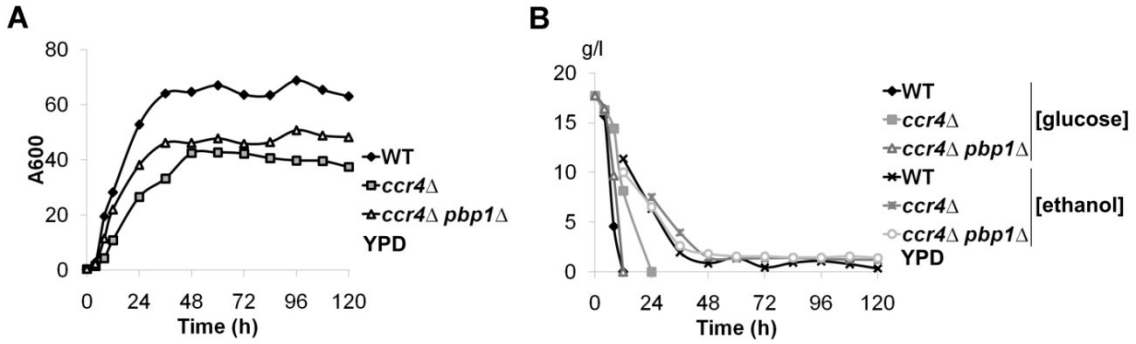

Figure 10. Identify growth phases of WT and mutants. (A) Growth curves of WT, *ccr4*Δ, and *ccr4*Δ *pbp1*Δ cells in YPD media. The strains were pre-cultured overnight and then transferred into fresh YPD media to grow for 5 days at 28°C. The cell cultures were taken at the indicated times to measure A600 nm. (B) The WT, *ccr4*Δ, and *ccr4*Δ *pbp1*Δ mutant cells went into the stationary phase after 48 h of culture in YPD media. The strains were pre-cultured overnight in YPD media and then transferred into fresh YPD media to grow for 5 days at 28°C. The cultures were taken at the indicated times to measure glucose concentration. The ethanol concentrations were measured after glucose in the media had been depleted.

I then performed polysome analysis of WT and *ccr4*Δ mutant at 4 h and 72 h of culture corresponding to the log phase and the stationary phase, respectively. Polysome profiles revealed that translation was active in both WT and *ccr4*Δ mutant at the 4 h time point when the carbon source was abundant (Fig. 11A). In this stage, the active translating polysomes were dominant compared with ribosome 80S, 60S, and 40S (Fig. 11A). It has been reported that, in the stationary phase when the carbon source is depleted, WT cells strongly reduce the protein synthesis and many other metabolic processes [65]. Consistently, the active polysomes were strongly decreased in WT cells at 72 h time point (Fig. 11B). In contrast, in the *ccr4*Δ mutant, the active polysomes were also decreased, but still remained more abundant than that in WT cells at 72 h time point (Fig. 11B). I obtained essentially the same data using SC-Trp media (Fig. 12). Although, in the culture using SC-Trp media, the active polysomes remained at low level in WT, the active polysomes remained much more abundant in the *ccr4*Δ mutant. These results indicate that Ccr4 indeed negatively regulates the translation in addition to the mRNA level. The active polysomes remained abundant even in the stationary phase, suggesting that Ccr4 seems to be required for global

translational repression in the stationary phase rather than the translation of specific mRNA, *LRG1* mRNA.

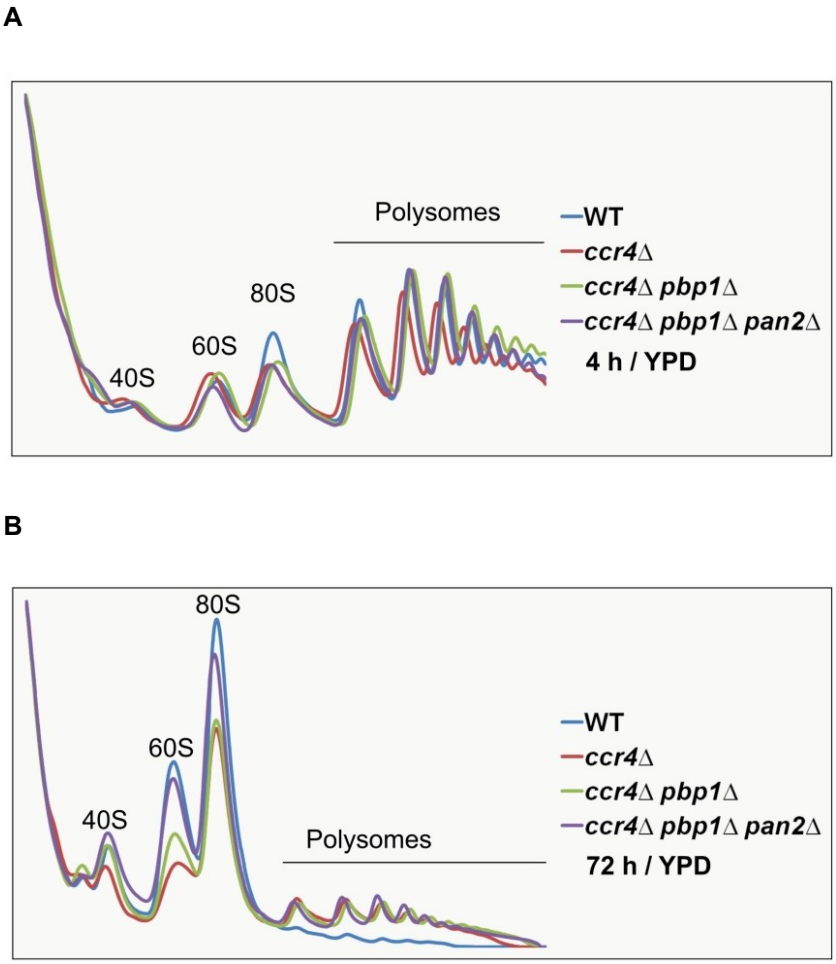

Figure 11. Active translating polysomes were still abundant in the stationary-phase *ccr4*Δ mutant. (A) Polysome analyses of WT, *ccr4*Δ, *ccr4*Δ *pbp1*Δ, and *ccr4*Δ *pbp1*Δ *pan2*Δ mutant cells in the log phase (4 h). The strains were pre-cultured overnight in YPD media and then transferred into fresh YPD media to grow for 4 h at 28°C. The cells were collected and cell lysates were prepared for polysome analysis as described in material and methods. (B) Polysome analyses of WT, *ccr4*Δ, *ccr4*Δ *pbp1*Δ, and *ccr4*Δ *pbp1*Δ *pan2*Δ mutant cells in the stationary phase (72 h) in YPD. The strains were pre-cultured overnight in YPD media and then transferred into fresh media to grow for 72 h at 28°C. The cells were collected and cell lysates were prepared for polysome analysis as described in material and methods.

To confirm whether translation of the *LRG1* mRNA was increased in the stationary-phase *ccr4*Δ mutant cells, I then examined the *LRG1* mRNA level in each

polysome fractions from WT and *ccr4*Δ mutant cells at 72 h of culture. The same volumes of purified mRNAs from each polysome fraction were subjected to RT-PCR reactions to generate cDNAs used as the template for *LRG1* amplification. As predicted, *LRG1* mRNA was more enriched in heavy polysome fractions in the *ccr4*Δ mutant than those in WT (Fig. 12). This result reveals that the translation of *LRG1* mRNA was increased in the stationary-phase *ccr4*Δ mutant cells, which lead to the increase in Lrg1 protein levels (Fig. 8B).

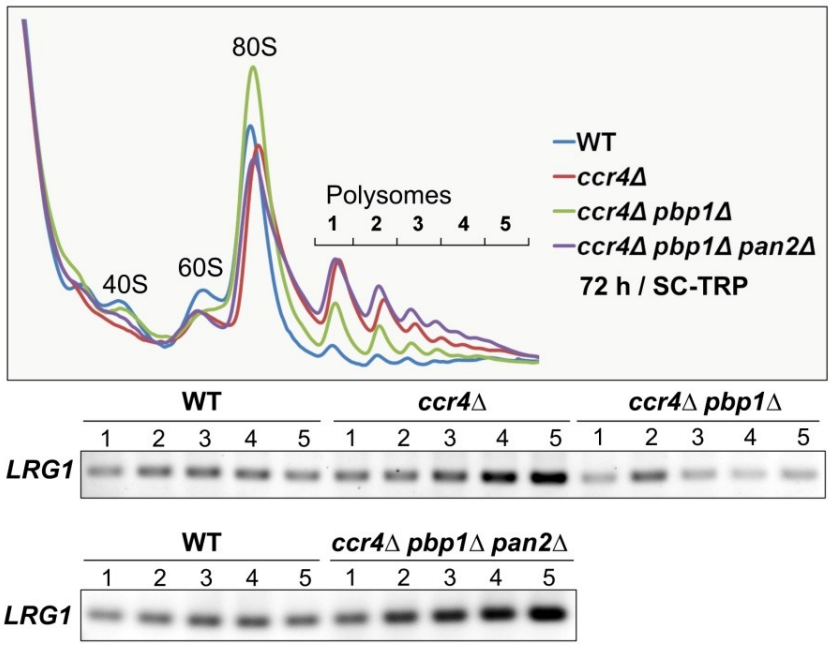

Figure 12. *LRG1* mRNA associates with polysomes in *ccr4*Δ but not in *ccr4*Δ *pbp1*Δ in the stationary phase. Polysome analyses and *LRG1* mRNA levels of WT, *ccr4*Δ, *ccr4*Δ *pbp1*Δ, and *ccr4*Δ *pbp1*Δ *pan2*Δ mutant cells in the stationary phase (72 h) in SC-Trp media. The strains were pre-cultured overnight in SC-Trp media and then transferred into fresh media to grow for 72 h at 28^{o}C. The cells were collected and cell lysates were prepared for polysome analysis as described in material and methods. The same volumes of RNA isolated from each of polysome fractions were subjected to RT-PCR to synthesize cDNAs. The *LRG1* cDNA was amplified using Taq polymerase. The data show the relative amounts of *LRG1* cDNA from the polysome fractions of the strains. I obtained similar results in two independent experiments and show a representative.

3.4. Loss of *PBP1* reduces Lrg1 level in the stationary-phase *ccr4*Δ mutant cells

My laboratory has previously reported that deletion of *PBP1* suppressed the slow growth defect and temperature-sensitive growth defect of the *ccr4*Δ single and the

*ccr4*Δ *khd1*Δ double mutants, and that the *pbp1*Δ mutation did not suppress the increased *LRG1* mRNA level of the *ccr4*Δ *khd1*Δ mutant [32]. Since in previous experiment, the *LRG1* mRNA and protein levels were measured only in the log phase culture, I re-examined the *LRG1* mRNA and protein levels in WT, *ccr4*Δ, and *ccr4*Δ *pbp1*Δ mutants in the longer time course (Figs. 7A and 10A). As shown in figures 7A and 10A, the *ccr4*Δ *pbp1*Δ mutant showed better growth than the *ccr4*Δ mutant in both SC-Trp and YPD media. The *ccr4*Δ *pbp1*Δ mutant reached the stationary phase after 60 h of culture in SC-Trp media (Fig. 7A) and 48 h of culture in YPD media (Figs. 10A and 10B).

Then I examined the poly(A) tail length of *LRG1* mRNA, *LRG1* mRNA level, and Lrg1 protein level in the *ccr4*Δ *pbp1*Δ mutant harboring the FLAG-LRG1 plasmid. It has been reported that Pbp1 is involved in the regulation of poly(A) tail length [44]. In addition, the cell extract of the *pbp1*Δ mutant in the stationary phase has shown stronger deadenylase activity *in vitro* compared to that in the log phase [42]. At the 4 h time point, *LRG1* poly(A) tail length in the *ccr4*Δ *pbp1*Δ mutant as well as that in the *ccr4*Δ mutant was longer than that in WT (Fig. 7B, lanes 1, 2, 3). However, at the 48 h time point, a large portion of the *LRG1* mRNAs in the *ccr4*Δ *pbp1*Δ mutant harbored shorter poly(A) tail than those in the *ccr4*Δ mutant (Fig. 7B, lane 6, 7). The *LRG1* mRNA levels in the *ccr4*Δ *pbp1*Δ mutant were decreased compared to those in the *ccr4*Δ mutant throughout the time course (Fig. 8A, *ccr4*Δ and *ccr4*Δ *pbp1*Δ). Interestingly, although the Lrg1 protein levels in the *ccr4*Δ *pbp1*Δ mutant were also decreased compared to those in the *ccr4*Δ mutant throughout the time course (Fig. 8B), the decrease in Lrg1 protein level was more evident than the decrease in the mRNA level. While the *LRG1* mRNA levels in the *ccr4*Δ *pbp1*Δ mutant was 2-fold lower than those in the *ccr4*Δ mutant at 48 h and 72 h time points (Fig. 8A), the Lrg1 protein levels in the *ccr4*Δ *pbp1*Δ mutant were decreased 5.7-fold and 6.7-fold compared to those in the *ccr4*Δ mutant at 48 h and 72 h time points, respectively (Fig. 8B). These data suggest that the *pbp1*Δ mutation not only down-regulates the increased *LRG1* mRNA level but also abandons the translation of *LRG1* in the *ccr4*Δ mutant. Since the *ccr4*Δ *pbp1*Δ mutant had the shorter poly(A) tail of the *LRG1* mRNA than that in the *ccr4*Δ mutant at the 48 h time point (Fig. 7B, lane 6, 7), the decrease in *LRG1* poly(A) tail length may account for the reduction of Lrg1 protein level in the *ccr4*Δ *pbp1*Δ mutant (Fig. 8B). It should be noted that the Lrg1 protein levels in WT, *ccr4*Δ, and *ccr4*Δ *pbp1*Δ mutants were similar at the 4 h time point (Figs. 8A and 8B), and that the effects on

Lrg1 protein levels by the *ccr4*Δ and *pbp1*Δ mutations were found in the later growth phase such as 48 h and 72 h time points. I also examined the Lrg1 protein level in the stationary-phase *pbp1*Δ single mutant, but I could not find any difference compared to that in WT (data not shown), suggesting that the *pbp1*Δ mutation may only affect the translation of the mRNAs harboring longer poly(A) tail in the *ccr4*Δ mutant.

3.5. Deletion of *PBP1* does not reduce aberrant active polysomes in the stationary-phase *ccr4*Δ mutant cells.

Because the *pbp1*Δ mutation reduced *LRG1* poly(A) tail length, *LRG1* mRNA level, and Lrg1 protein level in the *ccr4*Δ mutant in the later growth phase, I performed polysome analysis of *ccr4*Δ *pbp1*Δ mutant (Figs. 11A, 11B, and 12). Polysome profiles revealed that translation was similarly active in WT, *ccr4*Δ, and *ccr4*Δ *pbp1*Δ mutant at the 4 h time point (Fig. 11A). Surprisingly, although the Lrg1 protein level in the *ccr4*Δ *pbp1*Δ mutant was much lower than that in the *ccr4*Δ mutant at the 72 h time point (Fig. 8B), the active polysomes still remained abundant in the *ccr4*Δ *pbp1*Δ mutant similar to that in the *ccr4*Δ mutant (Fig. 11B). The active polysomes also remained more abundant in both *ccr4*Δ and *ccr4*Δ *pbp1*Δ mutants than in WT at the 72 h time point in SC-Trp media (Fig. 12). These results indicate that deletion of *PBP1* does not reduce aberrant active polysomes in the stationary-phase *ccr4*Δ mutant, although the *pbp1*Δ mutation affects the translation of the *LRG1* mRNA.

Since the Lrg1 protein level in the *ccr4*Δ *pbp1*Δ mutant was much lower than that in the *ccr4*Δ mutant at the 72 h time point (Fig. 8B), I next examined the *LRG1* mRNA level in each of polysome fractions from the *ccr4*Δ *pbp1*Δ mutant at 72 h time point (Fig. 12). Consistent with the decrease in Lrg1 protein level in the *ccr4*Δ *pbp1*Δ mutant at 72 h time point, *LRG1* mRNA was less enriched at heavy polysome fractions in the *ccr4*Δ *pbp1*Δ mutant than those in the *ccr4*Δ mutant (Fig. 12). Thus, Pbp1 may promote the association of *LRG1* mRNA to polysomes to enhance the translation in the absence of Ccr4.

3.6. Regulation of *LRG1* expression by Ccr4 and Pbp1 is important for proper cell growth

The *LRG1* gene encoding for a GAP protein of the small GTPase Rho1, the key regulator of the CWI pathway, and high level of Lrg1 protein inhibits the cell growth at high temperature [50]. To confirm whether the regulation of Lrg1 protein expression by

Ccr4 and Pbp1 is important for growth control, I transformed a multi-copy plasmid carrying *LRG1* gene into WT, *ccr4*Δ, and *ccr4*Δ *pbp1*Δ mutant cells. As shown in figure 10, overexpression of *LRG1* is more toxic to the *ccr4*Δ mutant, but less toxic to WT and *ccr4*Δ *pbp1*Δ mutants at 37°C. These data are consistent with that the increased Lrg1 protein level in the stationary-phase *ccr4*Δ mutant contributed to its slow growth, and that the decreased Lrg1 protein level by the *pbp1*Δ mutation also contributed to the suppression of the slow growth of the *ccr4*Δ mutant. Thus, Ccr4 and Pbp1 regulate the expression of *LRG1* gene together, and this regulation is important for proper cell growth.

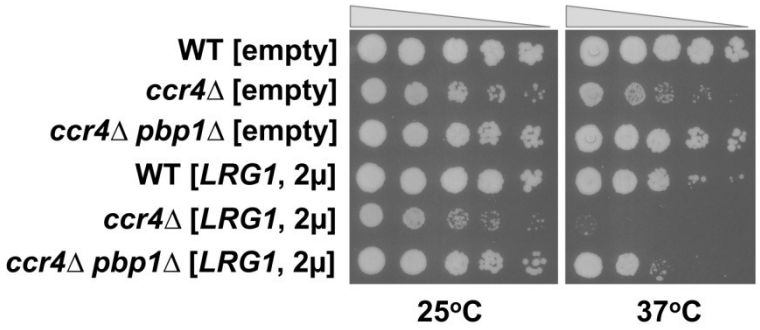

Figure 13. Overexpression of *LRG1* was toxic to the *ccr4*Δ mutant but not to the *ccr4*Δ *pbp1*Δ mutant at high temperature. The WT, *ccr4Δ*, and *ccr4Δ pbp1Δ* mutant strains harboring the plasmid YEplac195-LRG1 were grown at 28ºC to the mid log phase. The same optical densities of cells were spotted onto SC-Ura plates and then incubated at 25°C or 37°C for 3 days.

3.7. Pbp1 negatively regulates Pan2 activity in the absence of Ccr4 in the stationary phase

Mangus et al. have reported that Pbp1 negatively regulates mRNA poly(A) tail length through negative regulation of the Pan2 deadenylase activity [44]. I also reported that suppression of the *ccr4*Δ mutation by the *pbp1*Δ mutation is partly dependent on *PAN2* [32]. If Pan2 activity is inhibited by Pbp1, the *LRG1* poly(A) tail length in the *ccr4*Δ *pbp1*Δ *pan2*Δ triple mutant would be longer than that in the *ccr4*Δ *pbp1*Δ double mutant in the stationary phase. As predicted, whereas the poly(A) tail length of *LRG1* mRNA in the *ccr4*Δ *pbp1*Δ double mutant was decreased at the 48 h time point than that in the *ccr4*Δ mutant, the poly(A) tail length of *LRG1* mRNA in the *ccr4*Δ *pbp1*Δ *pan2*Δ mutant was not decreased (Fig. 7B). The poly(A) tail length of

LRG1 mRNA in the *ccr4*Δ *pbp1*Δ *pan2*Δ mutant was around 64 nucleotides that was similar to those in the *ccr4*Δ mutant (Fig. 7B). These data suggest that the shortening of poly(A) tail length by Pbp1 is dependent on Pan2 activity in the stationary-phase *ccr4*Δ mutant.

Then I examined the Lrg1 protein level in the *ccr4*Δ *pbp1*Δ *pan2*Δ mutant. Unexpectedly, the increase in *LRG1* poly(A) tail length did not result in the increase in Lrg1 level in the *ccr4*Δ *pbp1*Δ *pan2*Δ mutant in the stationary phase (Fig. 14). Thus, the translation of *LRG1* mRNA seems to require Pbp1 even in the absence of Pan2. I then performed polysome analysis of the *ccr4*Δ *pbp1*Δ *pan2*Δ mutant and found that the active polysomes still remained abundant in the *ccr4*Δ *pbp1*Δ *pan2*Δ mutant similar to that in the *ccr4*Δ and *ccr4*Δ *pbp1*Δ mutants (Figs. 11A, 11B, and 12). I also examined the *LRG1* mRNA level in each of polysome fractions from the *ccr4*Δ *pbp1*Δ *pan2*Δ mutant at 72 h time point (Fig. 12). While Lrg1 protein level was decreased in the *ccr4*Δ *pbp1*Δ *pan2*Δ mutant at 72 h time point (Fig. 14), *LRG1* mRNA was still enriched at heavy polysome fractions in the *ccr4*Δ *pbp1*Δ *pan2*Δ mutant (Fig. 12). Thus, Pbp1 may enhance the translation in the absence of Ccr4 and Pan2 in an independent manner of the association of *LRG1* mRNA to polysomes.

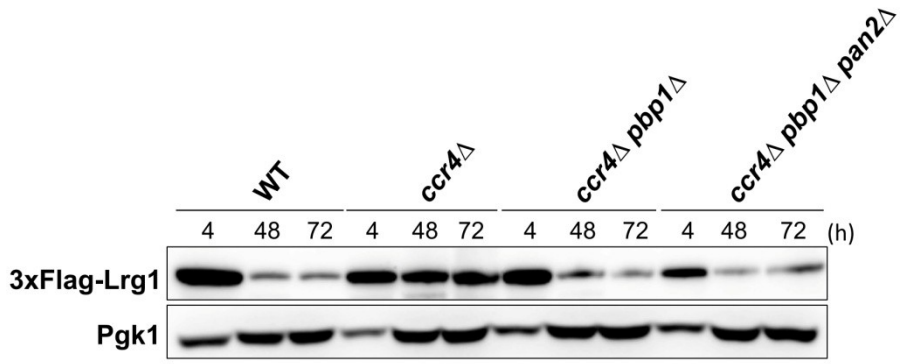

Figure 14. Effect of *PAN2* deletion on the expression of Lrg1 protein in *ccr4*Δ *pbp1*Δ mutant. The WT, *ccr4*Δ, *ccr4*Δ *pbp1*Δ, and *ccr4*Δ *pbp1*Δ *pan2*Δ mutant strains harboring the plasmid pRS314-3FLAG-LRG1 were grown at 28ºC from the log phase to the stationary phase in SC-Trp media. The cells were collected at the indicated times, and cell extracts were prepared for immunoblotting with anti-Flag (3xFlag-Lrg1) and anti-Pgk1 antibodies. Pgk1 was used as the loading control.

To confirm the involvement of *LRG1* poly(A) tail length in the regulation of *LRG1* mRNA translation, I overexpressed *PAN2* in the *ccr4*Δ mutant and then examined Lrg1 protein level. My laboratory has previously reported that *PAN2* overexpression from the

multi-copy plasmid suppresses the growth defect of the *ccr4*Δ *khd1*Δ mutant [32]. *PAN2* overexpression partially decreased *LRG1* poly(A) tail length (Fig. 15B, lane 3). However, the overexpression of *PAN2* did not reduce Lrg1 level in the *ccr4*Δ background in the stationary phase (Fig. 15A). It may be more Pbp1 loaded on long *LRG1* poly(A) tail and inhibit the access of Pan2. I also examined the expression of Lrg1 protein in the *ccr4*Δ *pan2*Δ double mutant from the log phase to the stationary phase. At 48 h time point, the Lrg1 protein in the *ccr4*Δ *pan2*Δ double mutant was maintained at high level similar to that in *ccr4*Δ mutant (Fig. 15C). However, at 72 h and 96 h time points, Lrg1 protein levels in the *ccr4*Δ *pan2*Δ double mutant were decreased compared to those in *ccr4*Δ mutant. The *LRG1* poly(A) tail length in *ccr4*Δ *pan2*Δ mutant was more longer than that in *ccr4*Δ mutant at 72 h time point (Fig. 15B, lane 4), suggesting that the longer poly(A) tail may interfere the translation of *LRG1* mRNA in the late stationary phase. Alternatively, since the *ccr4*Δ *pan2*Δ double mutant shows more severe growth defect than the *ccr4*Δ single mutant, the decreased protein levels may be caused by the growth defect.

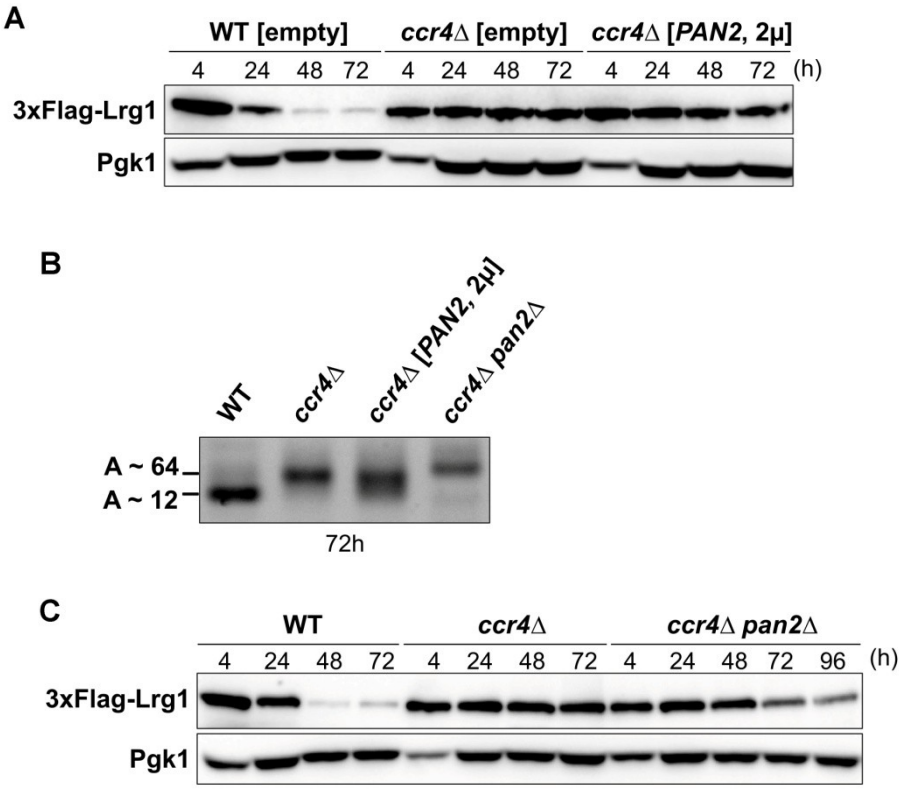

Figure 15. Effects of *PAN2* deletion, and *PAN2* overexpression on the expression of Lrg1 protein in *ccr4*Δ mutant. (A) Effect of *PAN2* overexpression on the expression of Lrg1 protein. The multi-copy plasmid YEplac195-PAN2 or empty vector was transformed into WT and *ccr4*Δ mutant cells harboring plasmid pRS314-3FLAG-LRG1. Transformants were grown at 28ºC from exponential phase to the stationary phase in SC-Trp-Ura media. The cells were collected at the indicated times, and cell extracts were prepared for immunoblotting with anti-Flag (3xFlag-Lrg1) and anti-Pgk1 antibodies. Pgk1 was used as the loading control. (B) Effect of *PAN2* deletion and *PAN2* overexpression on *LRG1* poly(A) tail length in the stationary-phase *ccr4*Δ mutant cells. The strains were grown in SC-Trp-Ura media from the log phase to the stationary phase at 28^{o}C. The cells were collected at 72 h time point for RNA isolation. The *LRG1* poly(A) tail was amplified using the poly(A) tail length kit. (C) Effect of *ccr4*Δ *pan2*Δ mutation on the expression of Lrg1 protein. The WT, *ccr4*Δ, and *ccr4*Δ *pan2*Δ mutant strains harboring plasmid pRS314-3FLAG-LRG1 were grown at 28ºC from the log phase to the stationary phase in SC-Trp media. The cells were collected at the indicated times, and cell extracts were prepared for immunoblotting with anti-Flag (3xFlag-Lrg1) and anti-Pgk1 antibodies. Pgk1 was used as the loading control.

3.8. *MCM2, MCM4, MCM7*, and *ELM1* show the expression pattern similar to that of *LRG1* in the stationary phase

Previous report has shown that the suppression of the *ccr4*Δ mutation by the *pbp1*Δ mutation was not identical to that by the *lrg1*Δ mutation [32]. Whereas the *pbp1*Δ mutation suppressed both the slow growth phenotype at room temperature and the growth defect at 37ºC of the *ccr4*Δ *khd1*Δ double mutant, the *lrg1*Δ mutation suppressed only the growth defect at 37ºC, but not the slow growth phenotype at room temperature [52]. Thus, deletion of *PBP1* can suppress the growth defect of the *ccr4*Δ mutant by decreasing the expression probably not only of Lrg1 protein but also of other proteins, in the stationary phase. I then searched for the other potential candidates similar to *LRG1* gene. The *LRG1* mRNA is one of the targets of Puf5/Mpt5, an RNA binding protein [53, 54, 66]. Puf5 binds to specific site in 3'-UTR of its target mRNAs and recruits Ccr4-Not complex for deadenylation [55, 56]. Among the targets of Puf5 [53, 54], I investigated the protein levels of *MCM2*, *MCM4*, *MCM7*, and *ELM1* genes in WT, *ccr4*Δ, and *ccr4*Δ *pbp1*Δ mutant strains in the longer culture, because the antibodies for these proteins were commercially available. The data showed that the poly(A) tail lengths of these mRNAs were also increased in the *ccr4*Δ mutant and decreased in the *ccr4*Δ *pbp1*Δ mutant at 48 h of culture (Fig. 16A). The protein levels of these genes were strongly decreased in WT but slightly decreased in the *ccr4*Δ mutant after 48 h of culture (Fig. 16B, WT and *ccr4*Δ, 48 h and 72 h). Similar to the results of Lrg1 protein, deletion of *PBP1* also reduced these protein levels in the stationary-phase *ccr4*Δ mutant cells (Fig. 16B, *ccr4*Δ *pbp1*Δ, 48 h and 72 h). These data suggest that Ccr4 is required for translational repression not only of *LRG1* mRNA but also of other Puf5 target mRNAs in the stationary phase.

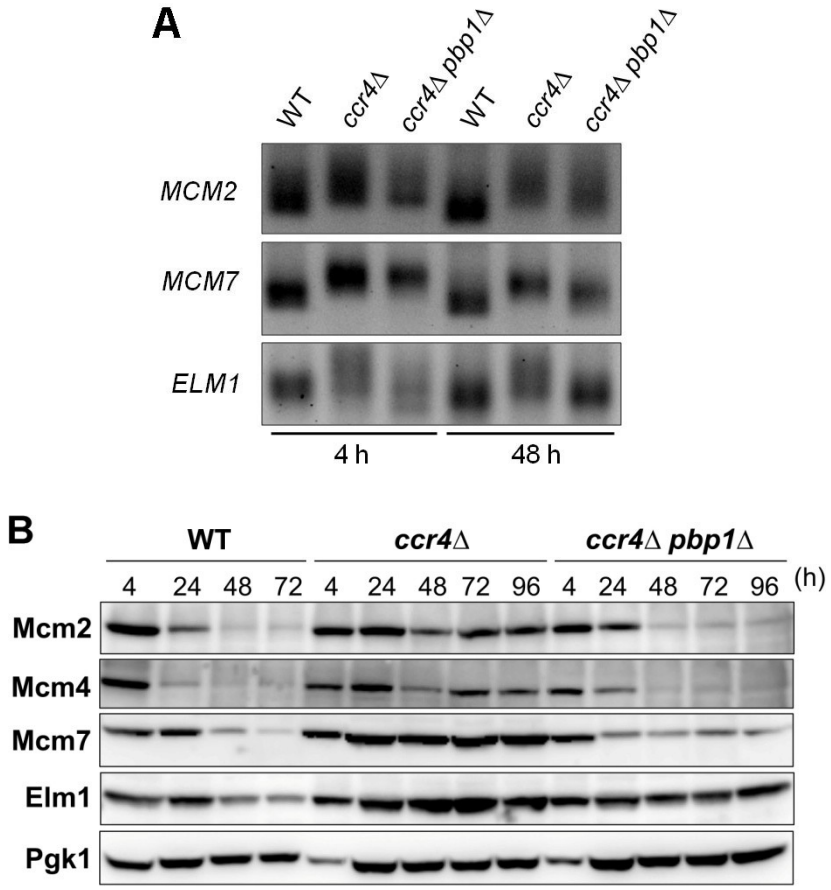

Figure 16. The other target mRNAs of Puf5 including *MCM2*, *MCM4*, *MCM7*, and *ELM1* showed the expression patterns similar to that of *LRG1*. (A) The *MCM2, MCM*, and *ELM1* poly(A) tail lengths in WT, *ccr4*Δ*,* and *ccr4*Δ *pbp1*Δ mutant cells in the log phase (4 h) and the stationary phase (48 h). The strains were grown in YPD media from the log phase to the stationary phase at 28°C. The cells were collected at indicated time points for RNA isolation. The *LRG1* poly(A) tail was amplified using the poly(A) tail length kit. (B) Protein expressions for products of Puf5 target mRNAs in WT, *ccr4*Δ*,* and *ccr4*Δ *pbp1*Δ mutant cells. The WT, *ccr4*Δ*,* and *ccr4*Δ *pbp1*Δ mutant cells harboring the plasmid pRS314-3FLAG-LRG1 were grown at 28ºC from the log phase to the stationary phase in SC-Trp media. The cells were collected at the indicated times, and cell extracts were prepared for immunoblotting with anti-Flag (3xFlag-Lrg1), anti-Mcm2, anti Mcm4, anti-Mcm7, anti-Elm1, and anti-Pgk1 antibodies. Pgk1 was used as the loading control.

I also addressed to the question whether Puf5 is required for the regulation of *LRG1* mRNA. At 48 h and 72 h of *puf5*Δ mutant culture, Lrg1 protein level was decreased but still remained higher than those in WT cells (Fig. 17), suggesting that Puf5 as well as Ccr4 is required for the down-regulation of Lrg1 in the stationary phase.

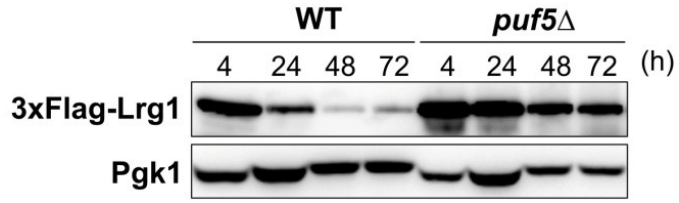

Figure 17. The Lrg1 protein level in WT, *puf5*Δ mutant in the stationary phase. WT and *puf5*Δ mutant strains harboring the plasmid pRS314-3FLAG-LRG1 were grown at 28ºC from the log phase to the stationary phase in SC-Trp media. The cells were collected at the indicated times, and cell extracts were prepared for immunoblotting with anti-Flag (3xFlag-Lrg1) and anti-Pgk1 antibodies. Pgk1 was used as the loading control.

Chapter 4. Discussion

4.1. The *LRG1* poly(A) tail length positively correlated to *LRG1* mRNA and protein levels in the stationary phase

The increase in poly(A) tail length is supposed to inhibit mRNA degradation and enhance translation *in vivo* [3, 4, 19, 21, 64]. In contrast, Traven et al. have reported that long poly(A) tails of the mRNAs encoding regulators of septin assembly do not affect their mRNA and protein levels in the *ccr4*Δ mutant [28]. In addition, in a genome-wide analysis, Subtelny et al. have shown that the poly(A) tail length positively correlates to translation efficiency only in early zebrafish and frog embryo, and deadenylation primarily enhances mRNA decay [16]. The poly(A) tail length, however, does not affect translation in yeast [16]. Therefore, it is still ambiguous about the relationship between poly(A) tail length and translational control. In this study, for the first time, I have provided the evidences that poly(A) tail length positively correlates to the level and translational efficiency of *LRG1* mRNA in the stationary phase, but not in the log phase. Consistent with the report of Traven et al. [28], the longer *LRG1* poly(A) tail in the *ccr4*Δ mutant did not affect Lrg1 protein level in the log phase. It is likely that poly(A) tail length is not important to translational control of *LRG1* mRNA in the log phase. The regulation of mRNA stability and translational efficiency in the log phase may involve other factors rather than poly(A) tail. Interestingly, when the cells reached saturated cell density, deletion of *CCR4* has stronger effect on Lrg1 protein level rather than on *LRG1* mRNA level. The aberrant *LRG1* mRNA and protein levels in the *ccr4*Δ mutant were correlated to the long *LRG1* poly(A) tail length, suggesting that the down-regulation of *LRG1* in the stationary phase requires the deadenylation of mRNA that is mediated by Ccr4. The longer poly(A) tail length, where more Pab1 may bind to and facilitate the formation of mRNP loop structure, inhibits mRNA degradation and facilitates the translation, and vice versa. The *pbp1*Δ mutation decreased the *LRG1* poly(A) tail length to the similar extend in WT in the stationary-phase *ccr4*Δ mutant cells, and then decreased the aberrant *LRG1* mRNA and protein levels. Thus, the poly(A) tail length and Ccr4 deadenylase seems to play an important role in regulation of *LRG1* mRNA and protein levels in the stationary phase rather than that in the log phase. Since deletion of *PBP1* reduced the *LRG1* poly(A) tail length in the stationary-phase *ccr4*Δ mutant, it comes to the question how Pbp1 contributes to the regulation of *LRG1* poly(A) tail? Mangus et al. have shown that Pbp1 negatively regulates Pan2

deadenylase by disturbing the Pab1-Pan2 interaction, and that the cell extract from *pbp1*Δ single mutant in the stationary phase has stronger deadenylase activity than that in WT *in vitro* [42, 44]. Consistently, I found that the shortening of the *LRG1* poly(A) tail length in the *ccr4*Δ *pbp1*Δ mutant required Pan2 deadenylase *in vivo*, and that Pbp1 negatively inhibited Pan2 activity only in the stationary phase but not in the log phase. It is thought that the Pan2-Pan3 complex act as primary deadenylase [38]; however, here I found that this complex could also act as secondary cytoplasmic deadenylase in the absence of both Ccr4 and Pbp1 in the stationary phase. Although *LRG1* mRNA harbored longer poly(A) tail in the stationary-phase *ccr4*Δ *pbp1*Δ *pan2*Δ triple mutant cells, Lrg1 protein level was not increased in the cells, suggesting that the translation of *LRG1* mRNA still requires Pbp1. On the other hand, overexpression of *PAN2* had little effect on *LRG1* poly(A) tail length and did not reduce Lrg1 protein level in the *ccr4*Δ mutant. It may be explained by the unusual Pbp1 loading onto long *LRG1* poly(A) tail, resulted in blocking of the Pan2 access to the *LRG1* poly(A) tail. Taken together, I first described here that Pbp1 together with the Pan2-Pan3 complex contributes to the regulation of poly(A) tail length in the stationary phase *in vivo* through a particular example, *LRG1* poly(A) tail. Further analysis should be needed to elucidate the physiological role of Pan2 inhibition by Pbp1 in the stationary phase.

4.2. Ccr4 is required not only for translational repression of *LRG1* mRNA but also for global translational repression in the stationary phase

The yeast cells enter into the stationary phase when the carbon source is depleted in the media. To adapt to this environmental signal, cells reduce cellular activities including protein synthesis and other metabolic processes to save energy for long-term survival and turn into quiescent state [65, 67]. There are several reports that translational repression required the mRNA regulatory factors upon nutrient depletion. For example, Coller et al. have shown that the decapping activators Dhh1 and Pat1 are required for general translational repression in the glucose starvation condition [68]. In addition, Preissler et al. have revealed that Not4, a component of Ccr4-Not complex, is also required for translational repression in response to nutrient withdrawal [25]. In this study, I have shown that the translation of *LRG1* mRNA is repressed prior to the decrease in *LRG1* mRNA level upon the stationary phase, and this translational repression requires the Ccr4 deadenylase. Intriguingly, the active translating ribosomes were decreased in the stationary-phase WT cells but still remained abundant in the

stationary-phase *ccr4*Δ mutant cells, suggesting that Ccr4 is required not only for translational repression of *LRG1* mRNA but also for global translational repression. Taken together with previous observations [25, 68], translational repression is tightly coupled with mRNA decay, and requires mRNA degradation machinery such as the Ccr4-Not complex and the decapping activators.

How does Ccr4 repress the global translation in the stationary phase? One of the possibilities is that Ccr4 shortens the poly(A) tail length in order to decrease mRNA stability and translation efficiency through disrupting mRNP loop structure. The mRNAs harboring shortened poly(A) tail would avoid the aberrant translations. In case of the *LRG1* mRNA, the *pbp1*Δ mutation suppressed the longer poly(A) tail caused by the *ccr4*Δ mutation, and then reduced the *LRG1* mRNA and Lrg1 protein levels in the stationary phase. However, the *pbp1*Δ mutation did not suppress the aberrant translating polysomes of the stationary-phase *ccr4*Δ mutant cells. Thus, Pbp1 may regulate the translation in a gene specific manner rather than a general consequence through the interaction with ribosomes. Since Caf1, a deadenylase catalytic component of Ccr4-Not complex, has been reported to repress the translation independent of its deadenylation in *Xenopus laevis* oocytes [64], Ccr4 may have a translational repression function independent of its deadenylase activity. However, the deadenylase-dead *CCR4 (D713A)* mutant could not decrease high Lrg1 protein level in the stationary-phase *ccr4*Δ mutant cell, suggesting that translation repression role of Ccr4 required its deadenylase activity. As to the regulation of translational repression by Ccr4 in the stationary phase, there are several lines of evidence that support the relationship between Ccr4 and protein kinase A (PKA) pathway. PKA pathway is known to be inactivated in the stationary phase. Lenssen et al. suggested that Ccr4 acts as downstream activator of PKA pathway in the regulation of Msn2/Msn4 dependent transcription [69, 70]. However, translational activity was still abundant in the absence of Ccr4 in the stationary phase, and constitutively activated PKA pathway also maintained high Lrg1 protein level (data not shown), implicating that PKA pathway might be the downstream effector of Ccr4 instead. Perhaps the defect in the inactivation of PKA activity in the stationary-phase *ccr4*Δ mutant cells could cause high translational activity, and further analysis need to be done to clarify this involvement. Taken together, I found here that Ccr4 deadenylase is required for global translational repression including translational repression of *LRG1* mRNA in the stationary phase.

4.3. Puf5 contributes to the down-regulation of its target mRNAs in the stationary phase

Beside *LRG1* mRNA, I have also found that the other target mRNAs of Puf5 including *MCM2*, *MCM4*, *MCM7*, and *ELM1* are also up-regulated in a manner dependent on Pbp1 in the stationary-phase *ccr4*Δ mutant cells. Previous report showed that Pbp1 also affects the translation of *HO* mRNA [46], another target of Puf5, raising the possibility of the involvement of Pbp1 specifically in the translational regulation of Puf5 target mRNAs. Recent finding revealed that ataxin-2, the human ortholog of Pbp1, stabilizes mRNAs by binding to specific site within 3'-UTR and enhance translation [49]. Likewise, the 3'-UTR of Puf5 target mRNAs may contain the specific binding site where Pbp1 binds to and ensures the translation. Moreover, the longer poly(A) tail found in the *ccr4*Δ mutant would provide the opportunity for the binding of numerous Pbp1 to the specific sites and facilitate the translation. On the other hand, Puf5 recruits Ccr4-Not complex for deadenylation by binding to the specific site in the 3'-UTR of its target mRNAs [55, 56]. I have also found that Lrg1 protein level in the *puf5*Δ mutant is higher than that in WT in the stationary phase, indicating that Puf5 contributes to the down regulation of its target mRNAs in the stationary phase. Furthermore, Puf5 contains phosphorylation motif of PKA [71], implicating the possibility that Puf5 would become more active and would repress the translation of their target mRNAs, together with Ccr4, in the stationary phase, when the PKA activity is very low.

Chapter 5. Conclusions and perspectives

In conclusions, the results presented in this study demonstrate that *LRG1* poly(A) tail length is important to *LRG1* mRNA and protein levels in the stationary phase. Although the role of poly(A) tail has been discussed in a number of studies, I identified here the first evidence in which poly(A) tail length positively correlates with translational efficiency in the stationary phase in yeast. In term of low energy state and the shortage of resources, translation is favored to those mRNAs harboring long poly(A) tail. In addition, I found that global translational repression that happens in the stationary phase requires Ccr4 deadenylase. It is likely that Ccr4 plays an important role in proper cellular homeostasis upon the stationary phase by inhibiting aberrant translation of Puf5 target mRNAs which is facilitated by Pbp1. Furthermore, I found that Pbp1 together with the Pan2-Pan3 complex regulates *LRG1* poly(A) tail *in vivo*. The working model is illustrated in figure 18.

In perspectives, from my study, there are several issues need to be further analyzed including:

- Poly(A) tail length may be important to translation control in specific situations, for example in stress condition.

- How cytoplasmic deadenylase Ccr4 repress the global translation upon the stationary phase?

- Is PKA pathway regulated by Ccr4 in the stationary phase?

- How Pan2-Pan3 complex activity is up-regulated in the stationary phase?

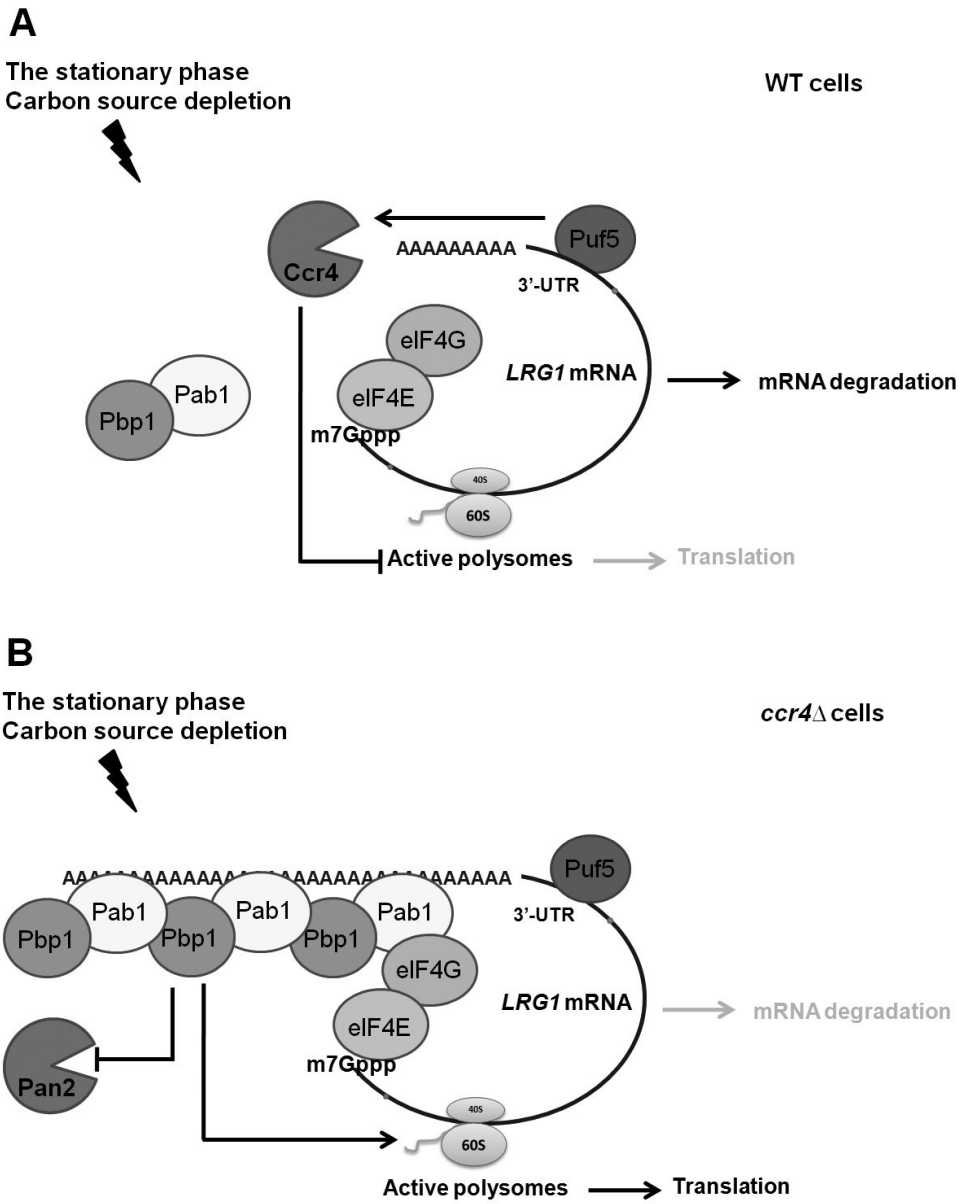

Figure 18. Depicted model illustrates the role of Ccr4 deadenylase in translational repression of *LRG1* mRNA upon the stationary phase. (A) In the stationary phase when the carbon source is depleted in the media, the WT cells reduce the translational activity to save energy for long term survival through deadenylation activity. Puf5 recruits Ccr4 to mRNA targets and shortens the poly(A) tail, leads to disruption of mRNP loop structure, which results in inhibiting translation and enhancing mRNA decay. (B) In the stationary phase, in the absence of Ccr4, Puf5 mRNA targets harboring long poly(A) tail results in maintaining the mRNP loop structure, which inhibit mRNA decay and enhance the translation facilitated by Pbp1. In addition, Pbp1 also protects the tail by inhibiting Pan2-Pan3 activity.

Tables

Table 1. Strains used in this study

Strains	Genotype	Reference
10B	*MATα ade2 trp1 can1 leu2 his3 ura3 GAL psi+ HOp-ADE2-HO 3' UTR*	[72]
10BD	*MATa/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3*	[72]
*ccr4*Δ	*MATa ade2 trp1 can1 leu2 his3 ura3 ccr4Δ::CgLEU2*	[31]
*ccr4*Δ *pbp1*Δ	*MATa ade2 trp1 can1 leu2 his3 ura3 ccr4Δ::CgLEU2 pbp1Δ::CgHIS3*	[32]
*ccr4*Δ *pbp1*Δ *pan2*Δ	*MATa ade2 trp1 can1 leu2 his3 ura3 ccr4Δ::CgLEU2 pbp1Δ::CgURA3 pan2Δ::CgHIS3*	[32]
*puf5*Δ	*MATa ade2 trp1 can1 leu2 his3 ura3 puf5Δ::CgHIS3*	[73]

Table 2. Plasmids used in this study

Plasmids	Relevant markers	Reference
pRS314	*TRP1, CEN-ARS*	[74]
YEplac195	*URA3, 2μ*	[75]
YCplac33	*URA3, CEN-ARS*	[75]
pRS314-3FLAG-LRG1	*TRP1, CEN-ARS, pLRG1-3FLAG-LRG1-LRG1 3'-UTR*	This study
YEplac195-LRG1	*URA3, 2μ, pLRG1-LRG-LRG1 3'-UTR*	This study
YEplac195-PAN2	*URA3, 2μ, pPAN2-PAN2-PAN2 3'-UTR*	[32]
YCplac33-CCR4	*URA3, CEN-ARS, pCCR4-CCR4-CCR4 3'-UTR*	This study
YCplac33-CCR4-D713A	*URA3, CEN-ARS, pCCR4-CCR4-D713A-CCR4 3'-UTR*	This study
pCgLEU2	*C. glabrata LEU2* in pUC19	[60]
pCgHIS3	*C. glabrata HIS3* in pUC19	[60]
pCgTRP1	*C. glabrata TRP1* in pUC19	[60]

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Acknowledgements

I sincerely express my gratitude to my advisor, professor **Kenji Irie**, for the kind support during my Doctoral course in University of Tsukuba. I appreciate the time and patience you gave me. From the beginning time of my study till now, you have been given me much valuable advice and motivation in doing research.

I would like to thank to associate professor of University of Science, **Dang Thi Phuong Thao**, who supports me kindly since I was an undergraduate student.

To fulfill this dissertation, I would like to thank to the thesis committee members, professor **Koji Hisatake**, associate professor **Takashi Matsuzaka**, associate professor **Atsushi Kawaguchi**, and assistant professor **Yuji Funakoshi** for the kind comments and important advice.

I would like to thank to assistant professor **Yasuyuki Suda**, assistant professor **Tomoaki Mizuno** and assistant professor **Yuichi Kimura** for your kind support during my experiments and publication. I would like to thank to **Pham Thi Kim Lien**, **Yuto Masuda**, **Tetsuro Ohmori**, **Ryosuke Takagi** for your kind support during my stay. I greatly appreciate to **all members in Prof. Irie's Laboratory** for your friendship and kind supports.

I would like to appreciate to the **Japanese Government** for providing me the **MEXT Scholarship** to support my study and life in Tsukuba. I would like to thank the members of the **International office**, **Medical department office**, and **Academic Service Office** for the Medical Science Area in University of Tsukuba for the help during my school years. I also would like to thank **Vuong Cat Khanh**, **Nguyen Thi Le Thuy**, and my **Vietnamese and International friends** who are beside and encourage me during the time staying here.

Finally, I always appreciate my families who are always beside and loving and supporting me.

University of Tsukuba, April 27th 2018

Duong Long Duy