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探討酵母菌核醣核酸蛋白 Rbp1p 之轉譯後修飾

Characterization of post-translational modification of RNA binding protein 1, Rbp1p, in Saccharomyces cerevisiae

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中文摘要

在真核細胞中，基因的表現在轉錄後會受到很多方面的調控，例如訊息核醣核酸的修飾、運送、降解，以及最後訊息核唐核酸要轉譯成蛋白質的過程，在這些調控的步驟中有許多可以和核醣核酸結合的蛋白質來協助調控的過程，這些蛋白質稱為核醣核酸結合蛋白。本實驗室在過去發現一個在酵母菌中可以與核醣核酸結合的蛋白質 Rbp1p，最初它被鑑定為抑制細胞生長的負調控因子，結構上它包含三個核醣核酸識別基序 RRM 及兩個富含麩氨酸區域，我們已知這個 Rbp1p 在酵母菌生長階段的晚期、葡萄糖剝奪、滲透壓力下可以作落到細胞質中特殊的細胞質聚集顆粒 P-bodies，並且經由二次元電泳的分析顯示 Rbp1p 分布在同一個分子量上但不同的等電點，暗示 Rbp1p 可能含有不同的轉譯後修飾，在本論文中再次利用二次元電泳的分析發現 Rbp1p 在生長階段的不同二維電泳上圖譜也會不同，我們推測外界的刺激會使 Rbp1p 被轉譯後修飾所調控，我們藉由質譜儀的分析找到了六個磷酸化的點，利用單點突變的技術將它們突變為丙氨酸，實驗證明同時突變其中的三個點 459、462、463 則 Rbp1p 負生長因子的能力下降，且突變型的 Rbp1p 在二維電泳上呈現的圖譜和野生型 Rbp1p 不同，但是這三個點的突變並不會影響 Rbp1p 坐落到 P-bodies，同時我們也研究一個 Rbp1p 可能的磷酸激酶 Ksp1p，我們發現剔除 Ksp1p 後 Rbp1p 負生長因子的能力也會下降，若挽回 Kspl 的表現則 Rbp1p 負生長因子的能力又回復了，剔除 Ksp1p 後也會影響到 Rbp1p 在二次元電泳圖譜上的分布，但是剔除 Ksp1p 後依然不會影響 Rbp1p 坐落到 P-bodies。這樣的結果說明了 Ksp1p 對於 Rbp1p 抑制生長的功能以及 Rbp1p 的轉譯後修飾是重要的。另外在本研究中我們藉由質譜儀的分析鑑定出 Rbp1p 在二次元電泳上每一個點的轉譯後修飾，並且也找到幾個可能與 Rbp1p 結合的蛋白質包括 Dhh1p、Hrp1p、Porin1p、Psp1p 以及 Pub1p。

Abstract

In eukaryotic cells gene expression subjected several level of posttranscriptional regulation included mRNA processing, transport, turnover, and tanslation regulation. Above regulation process involves various of RNA-binding proteins to support. Previously our lab found the *Saccharomyces cerevisiae* RNA-binding protein Rbp1p was first identified as a negative growth regulator, which contains three copies of an RNA recognition motif (RRM) and two glutamine-rich stretches. We have known that Rbp1p can localize to specific cytoplasm foci called P body when cell growth to stationary phase, glucose deprivation, and osmotic stress. Rbp1p revealed mutiple spot with the same molecular weight but different isoeletric points in 2-DE analysis. This result suggested that Rbp1p contained diverse post-translational modification. In this study using 2-DE analysis found under different growth stage, Rbp1p subjected to different post-translational modification. We speculated that when cell received external stimulus Rbp1p may regulated by post-translational modification. We also found six phosphorylation sites of Rbp1p by means of mass spectrometry. Using site-directed mutagenesis technique to produce phosphorylation sites mutants of Rbp1p. Data revealed simultaneously mutation on serine 459, 462, and 463 to alanine show partial growth inhibition ability lost. Compare to wild Rbp1p the mutant form show different post-translational modification pattern in 2-DE gel. However the mutant of Rbp1p had no effect to localize to P body. In addition we also studied a putative Rbp1p kinase Ksp1p, found that Rbp1p growth inhibition ability lost in Δ*ksp1* strain. Futhermore, in rescue experiment where *ADH* drove Myc-Ksp1p overexpression in Δ*ksp1* cells, Rbp1p restored its growth inhibition ability. However Ksp1p deletion had no effect on the localization of Rbp1p to P-body. These result suggested thatKsp1p is important for the function of Rbp1p, at least in growth phenotype and post-translational modification. Besides, in this study we determined each of the multiple post-translational modification spots of Rbp1p by mass spectrometry. Moreove we also identified several Rbp1p associated protein included Dhh1p, Hrp1p, Porin1p, Psp1p, and Pub1p.

Introduction

In Eukaryotic cells Gene expression is a complex process that involves several distinct stages : in the first gene transcription to produce primary mRNA; then primary mRNA undergo RNA processing include 5' cap , mRNA splicing, and 3' polyadenylation to produce mature mRNA. Mature mRNA will export from nuclear to cytoplasm than translation machinery will start out to produce protein. Finally nascent protein should be processed included cleavage or post-translational modification and protein sorting. Each of steps is subject to tight controls,the proper regulation of gene expression is important for all biological processes. Mis-regulation may cause many disease.

Although most analyses of gene expression focus on transcriptional regulation, but much of eukaryotic gene expression is regulated in post-transcription included mRNA processing, export, turnover and translation regulation. Post-transcriptional regulation of gene expression can involve the on/off regulation of particular gene products in a temporally and spatially regulated manner, allowing cells of different types or at different developmental stages to fine-tune their patterns of gene expression. All of above processes are mediated by numerous RNA-binding proteins and by small RNAs as stable ribonucleoprotein (RNP) complexes. RNA-binding proteins play central roles in the posttranscriptional regulation of gene expression. These proteins contain regions which function as RNA-binding domains, and auxiliary domains that mediate protein-protein interaction and subcellular targeting.

Changes in the environment require that cells continuously adapt in order to maintain their viability. Environmental stresses (oxidative stress, heat shock , UVradiation, osmotic shock, ER stress, and viral infection) are ancient stimuli that have shaped the evolution of all eukaryotic cells. In response to stress, cells continuously modify the repertoire of proteins that they synthesize. Especially mRNA turnover plays a key role in the control of gene expression both by setting the basal level of gene expression and as a site of regulatory responses.Recently, in yeast it was discovered that mRNA decay intermediates are found in specific cytoplasmic foci, termed P bodies (Sheth and Parker, 2003 ; Teixeira et al., 2005). These foci also accommodate proteins involved in mRNA decapping and 5'-to-3' exonucleolytic degradation (Sheth and Parker, 2003). Hence, P bodies likely represent sites wherein mRNAs are decapped and degraded. In mammalian cells, exposure to environmental stress results in the formation of cytoplasmic structures known as ‘‘stress granules,’’ which act as storage domains for ‘‘stalled’’ mRNAs (Kedersha and Anderson, 2002). Stress granules contain mRNAs, translation initiation factors, the

mRNAbinding proteins TIA and TIAR, and 40S ribosome subunits.

mRNA degradation

The level of mRNA expression is modulated through transcriptional and posttranscriptional control mechanisms. An important posttranscriptional control is exerted on mRNA turnover, which varies considerably from one mRNA species to another and can be modulated by extracellular stimuli. The turnover of mRNAs is mediated by the interplay between a number of different cis-acting sequences localized in the target substrate and the various trans-acting factors that interact with them. There are five mRNA decay pathways have been found . The general pathway involves deadenylation followed by decapping of the mRNA. In yeast, transcripts are primarily degraded in a 5' to 3'direction by the Xrn1 exonuclease (Muhlrad et al., 1995; Schwartz and Parker, 1999; Coller and Parker, 2004; Parker and Song, 2004), whereas in higher eukaryotes, mRNAs mostly are eliminated through second mammer that 3' to 5' degraded by the exosome after deadenylation (Chen et al., 2001; Hilleren et al., 2001; Wang and Kiledjian, 2001; Wilusz et al., 2001; Mukherjee et al., 2002; Tourriere et al.,2002). mRNA degradation also can occur through a third pathway that involves endonucleolytic cleavage of mRNAs (Schoenberg and Chernokalskaya, 1997), and in the fourth of known eukaryotic mRNA turnover pathways 3' to 5' decay nonstop decay (NSD), NSD targets mRNAs missing a proper stop codon. In the fifth is nonsense-mediated mRNA decay, referred to as NMD (reviewed in Czaplinski et al., 1999; Hilleren and Parker, 1999).

NMD is an RNA surveillance system that detects and destroys rapidly aberrant mRNAs containing premature termination codons and unspliced pre-mRNAs in the cytoplasm to produce proper full-length proteins to ensure quality control of gene expression.(He et al., 1993; Frischmeyer et al., 2002; van Hoofet al., 2002).

Both of NSD and NMD rely on the activity of the exosome, a multisubunit complex endowed with 3' to 5' exonuclease activity (Mitchell and Tollervey, 2000; Butler, 2002).

RNA binding proteins

ARE binding proteins

The stability of mRNA is determined in many cases by interactions between specific RNA-binding proteins and cis-acting sequences located in the 3' untranslated region (3' UTR) of the message (Guhaniyogi J and Brewer G. ,2001; Grzybowska et al., 2001). One of the best characterized cis-acting sequences is the adenylate/uridylate-rich element (ARE). AREs have been identified in numerous mRNAs (Frevel, M. A. E et al, 2003; Bakheet, T et al., 2001 Tebo, J et al., 2003),

including COX-2 and TNF-α. Several RNA-binding proteins have been identified which recognize ARE-containing sequences. For exampe T-cell internal vantigen 1 (TIA-1) has been best characterized as a suppressor of translation, as shown for the target ARE-bearing mRNAs encoding tumor necrosis factor alpha (TNF-α) and cyclooxygenase 2 (COX-2) (Dixon, 2003; Piecyk, 2000). Another ARE-binding protein is ELAV like protein HuR. Ecotopic overexpression HuR in the cytoplasm can specific inhibits the ARE-containing c-fos mRNA decay (Peng, S. S.-Y., 1998) .

Turn over regulatory protein

In yeast, a major pathway of mRNA degradation initiates with deadenylation of the mRNA poly(A) tail whose full length in yeast is usually 60–80 bases (Brown and Sachs, 1998; Mangus et al., 2003). When the length of the poly(A) tail reaches 10–12 bases or less, the mRNA 5' cap [m(7)GpppN] is removed (Decker and Parker, 1993) followed by an exonucleolytic digestion of the unprotected RNA. Deadenylation is carried out by Pan2p/Pan3p and the Ccr4p/Caf1p poly(A) nuclease complexes (Tucker et al., 2001), while decapping by Dcp1p/Dcp2p (Parker and Song, 2004). Decapping is probably a point of no return in the degradation process, as it exposes the 5' end of the mRNA to the major 5' to 3' exonuclease Xrn1p, and facilitates its activity. Several proteins regulate the decapping process, including Pat1p. Pat1p is considered to be the first decay factor recruited to mRNA, at a time when the mRNA is still associated with translation factors (Tharun and Parker, 2001). Subsequently, Pat1p recruits the hepta-heterodimer Lsm1–7 complex (Coller and Parker, 2004). The Lsm 1–7 complex,Pat1p, Dhh1p, Edc1p, Edc2, and Edc3 are positive regulators of decapping, characterized in yeast (Coller and Parker, 2004 ; Parker and Song, 2004), probably due to their influence on RNP architecture and on accessibility of the cap structure (Coller and Parker, 2004) and possibly by their direct effect on Dcp1/ Dcp2 activity (Zhang et al., 1999). When the processive 5' exoribonucleolytic mRNA decay pathway is blocked in yeast the 3' degradation pathway is easily observed. Cytoplasmic 3' mRNA degradation in yeast depends on the superkiller (Ski) proteins included SKI2, SKI3, SKI4, SKI6, SKI7, and SKI8 (Jacobs Anderson & Parker, 1998; van Hoof et al.,2000a, 2000b).

RNA granules

In both yeast and mammalian cells, the proteins involved in 5' to 3' mRNA decay are concentrated in cytoplasmic structures that have been designated mRNA ''processing bodies'' (P-bodies, also known as ''cytoplasmic foci'' and ''GW182 bodies'') (Eystathioy et al., 2003b; Sheth and Parker, 2003; Cougot et al., 2004). In addition to DCP1a/DCP2 and Xrn1, other proteins localize to P-bodies. These proteins include Sm-like proteins 1–7 (Lsm1–7), the DEAD box family

helicase Rck/p54, and the autoantigen GW182 (Bouveret et al., 2000; Coller et al., 2001; Eystathioy et al., 2003b; Cougot et al., 2004). Pbodies are sites of active mRNA degradation (Sheth and Parker, 2003; Cougot et al., 2004). P bodies are dynamic structures and can vary in size and number under different conditions.Treatment of cells with cyclohexamide, which inhibits translation elongation and traps mRNAs on polysomes, decreases the flow of mRNA to P-bodies and causes rapid loss of these structures. In contrast, when mRNAs are driven off polysomes by conditions reducing translation initiation, such as glucose deprivation or inhibition of Xrn1 blocks the 5' to 3' mRNA degradation step , P bodies rapidly increase in number and size. The observation that P-bodies are modified by changes in mRNA metabolism suggests that these structures are actively involved in mRNA decay. And propose mRNAs exist in two distinct functional states, one in polysomes and one sequestered into a nontranslating pool, which would be the pool present in P bodies and the direct substrate for mRNA decapping. Transitions between these two pools would be dictated by the translation status of the mRNA. P bodies show some remarkable similarities to another form of mRNA containing cytoplasmic particles, referred to as a stress granule. In mammalian cells, exposure to environmental stress results in the formation of cytoplasmic structures known as ‘‘stress granules’’ (SGs) (for review, see Kedersha and Anderson, 2002). SGs contain mRNAs, translation initiation factors, the mRNA-binding proteins TIA and TIAR, and 40S ribosome subunits. The accumulation and retention of the pre-stress or ‘‘housekeeping’’ pool of mRNAs in these structures permits mRNAs encoding stress and repair proteins to gain access to the cellular translation machinery. If the cell survives the environmental stress, SGs disappear and housekeeping mRNAs may return to active translation. The precise relationship between P-bodies and TIAcontaining SGs has not been determined.

Yeast RNA binding protein 1,Rbp1p

The Saccharomyces cerevisiae RNA-binding protein RBP1, also named NGR1, was initially identified as a negative growth regulator for S. cerevisiae (Lee and Moss, 1993). Rbp1p gene encodes a 672-amino acid, ~80-kD which contains three RRMs, two glutamine-rich sequences, and a C-terminal asparagine-methionine-proline-rich region. RBP1 is a non-essential gene,over-expression of Rbp1p in yeast yields a slow-growth phenotype, suggesting that Rbp1p is functionally critical in certain biochemical processes. Rbp1p is a similar in domain organization and amino acid sequence to two cytolytic lymphocyte proteins, TIA-1 and the closely related TIAR (Tian et al., 1991 ; Kawakami et al., 1992). TIA-1 and TIAR have three RRM domains near their N termini that confer high-affinity binding to uridine-rich motifs (Dember et al., 1996). Both TIA1 and TIAR can cause the general translational arrest

upon environmental stress. Following stress-induced phosphorylation of translation initiation factor eIF-2, TIA-1 and TIAR recruit selected cytoplasmic poly(A) RNAs to discrete foci known as stress granules.

Rbp1p was also reported to be a negative regulator of meiosis and spore formation (Deutschbauer, A. M., 2002). In the past our lab found that overexpression of Rbp1p impairs mitochondrial function and affects mitochondrial porin expression. rbp1 mutants showed increased stability of porin mRNA compared with wild-type cells, whereas cells overexpressing Rbp1p, but not N-terminal-deleted, or RRM1-, RRM2-, or RRM3-mutated Rbp1p, showed a decrease in the stability of porin mRNA. We further demonstrate that Rbp1p can accelerate porin mRNA turnover, possibly through binding to the 3'-UTR of porin mRNA. In addition, Rbp1p-mediated porin mRNA decay is elicited by Xrn1p, a 5' to 3' exonuclease.These data provide the first evidence that Rbp1p might be involved in post-transcriptional regulation of porin expression in intact cells. In recently our lab has demostrate Rbp1p in xrn1 mutant yeast localizes in specific cytoplasmic foci that are known as P-bodies. This localization is dependent on integrity of an Rbp1p complex through C-terminal self-interaction and the N-terminal and RNA recognition motif (RRM) 1 domains. In wildtype cells, Rbp1p can localize to P-bodies under glucose deprivation or treatment with KCl. P-bodies show some remarkable similarities to another form of mRNAcontaining cytoplasmic particles, referred to as stress granules. Stress granules form in response to decreased translation initiation and contain poly A mRNA, translation initiation factors, specific RNA-binding proteins TIA and TIA-R, and 40 S ribosomal subunits(Kedersha, N., and Anderson, P., 2002). After the stress induced phosphorylation of translation initiation factor eIF-2, TIA-1 and TIAR recruit most cytoplasmic mRNAs to discrete foci of stress granules (Kedersha, 1999), suggesting that the TIA-1/TIAR-dependent sequestration of these mRNAs prevents their translational initiation.

Stress granules and P-bodies are similar in their dynamics, because both are increased by blocking translation initiation, and both decline when mRNAs are driven into polysomes (Kedersha, 2000). At this time, however, stress granules and P-bodies appear to have distinct protein compositions and to differ physically. Hilleren and Parker (Hilleren and Parker, 1999) imply that the relative translational efficiency of a transcript is a major determinant of mRNA half-life. Not all mRNAs are routed into stress granules, indicating that the RNA composition of stress granules is selective.We propose that Rbp1p may be regulated by post-translational modification and function as a translational silencer, similar to TIA-1/TIAR, and therefore, interferes with or causes a lag in the association of ribosomal subunits with selective mRNAs resulting in inefficient translation and/or acceleration of mRNA decay.

Our lab previous study demostrated that Rbp1p show mutiple spot on the same molecular weight present in 2-DE gel (our lab, unpublished data). In this study we have demostrate that in differnet cellular environment Rbp1p will subject diverse post-translational modification. In order to determine which site in Rbp1p is important for growth inhibition ability. By the mass spectrometry result we find several phosphorylation site and generated site-direct mutagenesis mutant to investige the Rbp1p biological function,localization, and post-translational modification. Moreover by large-scale screening identified putative Rbp1p kinase Ksp1p, we studied how the Rbp1p gowth inhibition ability ,localization, and post-translational modification in Ksp1p deletion strain. And whether ectopic rescue Ksp1p protein expression in *ksp1* deletion strain the ability of Rbp1p to cause growth defect was restored.

Results

Part I. To demostrate under different growth stage, Rbp1p subjected to different post-translational modification.

The post-translational modification of Rbp1p is growth stage dependant .

Previously our lab found that HA-Rbp1p revealed multiple spots with the same molecular weight but different isoeletric points in two-dimensional electrophoresis (2-DE) western analysis. (Appendix 1 our lab, unpublished data).

2-DE is a good method to study protein post-translational modification status. In this method, proteins are first segregated by isoelectric point and then seperated by their molecular weights. This approach can reflect the existence of multiple phosphorylation or post-translational events.

Using 2-DE western analysis, we compared Rbp1p post-translational modification between different stages of cell growth. The recombinant HA-Rbp1p was overexpressed in YPH 499△*rbp1* Leu2 :: ADH HA-RBP1 strain. Cell growth started at OD 0.05, reached to the log phase at OD 0.75 after culturing for 8hr, and attained the stationary phase at OD 4.4 after culturing for 24hr (Fig. 1A). Using anti-HA antibody in 2-DE gel analysis, Rbp1p displayed different pattern in cell growth log phase and stationary phase (Fig. 1C). Simultaneously, we also found that the degradation form of Rbp1p in cell growth stationary phase increased notably when comparing with log phase in both SDS-PAGE and 2-DE gel (Fig. 1B,C). This result suggested that the post-translational of Rbp1p is growth stage dependant.

Part II. Functional characterization of the phosphorylation sites of Rbp1p

Rbp1p is a phosphorylated protein

We have also found that GST-Rbp1p has multiple post-translational modifications by two-dimensional electrophoresis (2-DE) analysis. Furthermore, alkaline phosphatase CIP treatment shifted the spots to the basic on the gel, suggesting that Rbp1p is a phosphorylated protein (Appendix 2 our lab, unpublished data).

The phosphorylation site of Rbp1p

Mass spectrometry is an analytical technique that identifies the chemical composition of a compound or sample on the basis of the mass-to-charge ratio of charged particles (Sparkman, 2000). The method employs chemical fragmentation of a sample into charged particles (ions) and measurements of two properties, charge and mass. The ratio of the two properties of the resulting particles is deduced by passing the particles through electric and magnetic fields in a mass spectrometer. The design of a mass spectrometer has three essential modules: an ion source, which transforms the molecules in a sample into ionized fragments; a mass analyzer, which sorts the ions by their masses by applying electric and magnetic fields; and a detector, which measures the value of some indicator quantity and thus provides data for calculating the abundances each ion fragment present. Mass spectrometry is an important method for the characterization of proteins. Proteins are enzymatically digested into smaller peptides using proteases such as trypsin or pepsin, either in solution or in gel after electrophoretic separation. The collection of peptide products are then introduced to the mass analyzer. When the characteristic pattern of peptides is used for the identification of the protein, the method is called peptide mass fingerprinting (PMF). If the identification is performed using the sequence data determined in tandem MS analysis, it is called de novo sequencing.

Previously our lab determined Rbp1p phosphorylation sites by mass spectrometry (LTJ), and found that Rbp1p bear S459,S462,S463,S524,S526, and T637 phosphorylation sites. In order to investigate whether phosphorylation in these sites is important for the function of Rbp1p, we generated mutant form Rbp1p T637A, S524A S526A, and S459A S462A S463A by site-directed mutagenesis, and then constructed into a 2μ plasmid (pVT101U) containing the influenza virus HA epitope tag fused at their N-terminus under the control of the constitutive *ADH1* promoter. The protein expression of these constructs was examined in Δ*rbp1* strain by Western blotting using anti-Rbp1p (Fig. 2C,3C).

Growth phenotype of Rbp1p mutants

To investigate whether Rbp1p mutant influence the function of Rbp1p, we investigated the growth of YPH499Δ*rbp1* and BY4741Δ*rbp1* strains, in which both strains contained construct vector alone, full-length, N-terminal truncated, or phosphorylation sites mutated. Cells were spotted onto plates and incubated at 30℃ for 3~4 days. In YPH499Δ*rbp1* strain, both of the mutant forms, HA-Rbp1p S524A S526A and HA-Rbp1p S459A S462A S463A, showed partial growth inhibition lost (Fig. 2B). The result was very similarity in BY4741Δ*rbp1* (Fig. 3B). In consistant, the

mutant form HA-Rbp1p T637A showed no effect on growth inhibition but both of HA-Rbp1p S524A S526A or HA-Rbp1p S459A S462A S463A showed partial growth inhibition lost in both YPH499Δ*rbp1* and BY4741Δ*rbp1* strains (Fig. 2B,3B). Unusually, cell growth was more rapid when overexpressing N-terminal truncated Rbp1p cell than phosphorylation sites mutated or even vector alone (Fig. 2B,3B).

Post-translational modification of Rbp1p mutant

In order to figure out whether the mutant form HA-Rbp1p S459A S462A S463A also influences the post-translational modification, protein was overexpressed in 2μ plasmid (pVT101U) under the control of the constitutive *ADH1* promoter.
Cell growth started at OD 600 0.1 after cultured for 12hr. We compared the 2-DE patterns between HA-Rbp1p and HA-Rbp1p S459A S462A S463A. The result revealed that mutantions in the phophorylation sites S459A S462A S463A actually affected the Rbp1p post-translational modification (Fig. 4).

Localization of Rbp1p mutant

We have already known that phophorylation sites mutant HA-Rbp1p S459A S462A S463A lose growth inhibition ability and has different post-translational modification. Previously our lab demonstrated that Rbp1p could localize to p-body (LTJ). Thus, we are very curious on whether mutant Rbp1p can localize to P-body. In order to monitor the Rbp1p localization, we constructed GFP-HA-Rbp1p and GFP-HA-Rbp1p S459A S462A S463A in pVT101U, and then transformed to 499rbp1 DHH1-mRFP strain, where Dhh1p is a P-body marker. Observation under fluorescent microscope showed that both GFP-HA-Rbp1p and GFP-HA-Rbp1p S459A S462A S463A could localize to p-body. Rbp1p bearing mutations in phosphorylation sites S459A S462A S463A did not influence the localization to P-body (Fig. 5).

Part III. Functional characterization of the putative Rbp1p kinase

Protein phosphorylation is a common regulatory manner that controls many basic cellular processes. Consequently protein kinase and phosphatase are the most direct factors that influence protein phosphorylation status. Finding the putative protein kinase may discover the protein regulatory pathway. In previously published paper, a Serine/Theronine kinase Ksp1p was found to be a putative kinase of Rbp1p (Ptacek *et al.*, 2005).

Growth phenotype of Ksp1p deletion strain

To investigate whether Ksp1p influence the function of Rbp1p, we compared the growth of BY4741Δ*rbp1* and BY4741Δ*rbp1*Δ*ksp1* strains, which contained construct vector alone or HA-Rbp1p, respectively. Cells were spotted onto plates and incubated at 30℃ for 3~4 days. The results revealed that the growth defect which was caused by overexpression of Rbp1p could be rescued in Δ*ksp1* strain, suggesting Rbp1p may lose partial function in the Δ*ksp1* strain (Fig. 6B). Moreover, we compared the growth of BY4741 wild type and Δ*ksp1* strains, which contained construct vector alone, HA-Rbp1p, Rbp1p, respectively. Both of HA-Rbp1p and Rbp1p could inhibit cell growth in BY4741 wild type strain (Fig. 7A). The growth defect which was caused by overexpression of HA-Rbp1p or Rbp1p could be rescued in Δ*ksp1* strain (Fig. 7A). The result in YPH499 was very similarity to BY4741 strain (Fig. 8A).

Futhermore, the ability of Rbp1p to cause growth defect was restored when using *ADH1* promoter to drive the overexpression of Myc-Ksp1p (Fig. 9A). This data suggested that Ksp1p may regulate the cellular function of Rbp1p.

Post-translational modification of Ksp1p deletion strain

We have known that Ksp1p deletion would affect the growth inhibition ability of Rbp1p. We also wanted to know whether Ksp1p deletion would impact on the post-translational modification. Therefore, we compared the 2D pattern of Δ*rbp1* and Δ*rbp1ksp1,* which contained Rbp1p, in BY4741 strain. The results revealed that Ksp1p deletion would affect the Rbp1p post-translational modification (Fig. 10).

Localization of Ksp1p deletion strain

We have known that Ksp1p deletion would influence on both the growth inhibition and the post-translational modification of Rbp1p. Moreover we wanted to know whether Ksp1p deletion would cause Rbp1p to localize to P-body. In order to monitor the Rbp1p localization, we constructed GFP-HA-Rbp1p in pVT101U and then transform to 499*rbp1* DHH1-mRFP strain and 499*rbp1ksp1* DHH1-mRFP strain. Dhh1p is a P-body marker. When observed by fluorescent microscope, both 499*rbp1* and 499*rbp1ksp1* GFP-HA-Rbp1p localized to p-body. Ksp1p deletion had no effect on the localization of Rbp1p to P-body (Fig. 11).

Part IV. To determine each of the multiple post-translational modification spots of Rbp1p by mass spectrometry

The recombinant GST-Rbp1p was overexpressed in YPH 499rbp1/GST-Rbp1/ pEGKT. Using glutathione beads to pull down GST-Rbp1and then precipitated with trichloroacetic acid method, GST-Rbp1was then separated by 2-DE gel followed by Coomassie blue staining. We observed about 8 spots in full-length GST-Rbp1p molecular weight (Fig. 12C). Subsequently, we gel off every spots and then carried out mass spectrometry to determine post-translational modification (Tab. 6). Besides we found pull-down GST-Rbp1p and western analysis revealed that about 60kDa show a major band but not Rbp1p (Fig. 12A). We also gel off these around 60kDa protein in 2-DE gel and identitfied them (Fig. 12C, Tab. 5).

Part V. To identify Rbp1p-interacting protein

To discover the Rbp1p-interacting proteins may help us to study protein regulatory mechanism. We constructed GST-Rbp1p in 2μ plasmid (pEGKT) under the control of the galactose inducible *GAL* promoter. In the presence of galactose, this pEGKT vector can induce GST protein by itself. Independently we pulled down GST alone and GST-Rbp1p protein by glutathione beads. Then we eluted the binding proteins, followed by precipitation with trichloroacetic acid and Western blot with several antibodys including Porin1, Dhh1p, Pub1p, Psp1p, Hrp1p. The result revealed Dhh1p, Porin1, and Pub1p show signification interact with GST-Rbp1p. The Hrp1p and Psp1p show weak interact with GST-Rbp1p (Fig. 13).

(1) *DHH1*

Dhh1p as a Member of the ATP-dependent DExD/H box helicase family. Required for the efficiency of decapping in vivo. Homologs across species are required for translational repression during mRNA storage events (Coller, J. and Parker., 2004). In yeast Dhh1, interacts with Dcp1 and enhances decapping in vitro (Coller et al., 2001; Fischer and Weis, 2002). In yeast Dhh1p can localize to P body (Sheth, U. and Parker, R. 2003).

(2) *HRP1*

Hrp1p acts as a DSE-binding factor that activates NMD. Hrp1p is also required for cleavage and polyadenylation of pre-mRNA 3' ends. Hrp1, a sequence-specific RNA-binding protein that shuttles between the nucleus and the cytoplasm, is required for mRNA 3' end formation in yeast (Kessler et al.,1997). Recently, Hrp1p have identified as a factor that specifically binds to the DSE and is required for the activity

of the NMD pathway. Hrp1p has been demonstrated to interact with the PGK1 DSE to provide a mark for PTC (Gonzalez et al., 2000).

(3)*PORIN1*

Porin1p is a essencial gene locate in mitochondrial. Recently in our lab published paper revealed in cells the porin mRNA is associated with Rbp1p RNP (ribonucleoprotein) complexes. In vitro binding assays showed that Rbp1p most likely interacts with a (C/G)U-rich element in the porin mRNA 3'UTR. *PORIN* mRNA level can be regulated by Rbp1p. Rbp1p enhanced *PORIN* mRNA turnover and the porin mRNA decay is elicited by Xrn1p, a 5' to 3' exonuclease. Our data also showed that porin mRNA could localize to P-bodies (Buu et al.,2004)

(4) *PSP1*

Asn and gln rich protein of unknown function. Null mutant is viable exhibits no apparent phenotype But overexpression results in growth inhibition.

(5) *Pub1*

The poly(U)-binding protein Pub1p is a yeast homologue of the mammalian ELAV-like proteins HuR and T-cell internal antigen 1 (TIA-1)/TIA-1-related protein. (Anderson et al., 1993 and Matunis et al., 1993). Pub1p has been recently implicated as a regulator of cellular mRNA decay (Ruiz-Echevarria et al., 2000 and Vasudevan et al., 2001). Pub1p can specifically bind to a chimeric yeast mRNA bearing the TNF-α ARE and stabilize this transcript (Vasudevan et al., 2001). Additionally, Pub1p has also been shown to selectively bind to a stabilizer element (STE) located in the 5' UTR of the upstream open reading frame (upstream ORF)- containing transcripts YAP1 and GCN4 and to prevent their turnover through the NMD pathway (Ruiz-Echevarria et al., 2000). These results demonstrate the Pub1p can bind to at least two classes of stability elements and modulate decay, based on cellular conditions.

Discussion

Rbp1p co-localizes with the P-body markers Dhh1p and Dcp2p when cells are grown to the stationary phase, but not in the early-log phase. Moreover, when subjecting to glucose deprivation or osmotic stress, Rbp1p localized to P-body. In addition, in a xrn1 strain we could easily observe that Rbp1p could localize to p-body even when cells were not grown to the stationary phase (LTJ). Based on these observations we purposed that Rbp1p is regulated by external stimulus. Furthermore, our previous finding that Rbp1p revealed multiple spots in the same molecular weight but different isoelectric point (Appendix 1) suggests that Rbp1p has multiple modifications. These observations raise the possibility that Rbp1p is regulated by post-translational modification. Post-translational modification of proteins is important for cells to respond to surrounding signaling. In order to study Rbp1p post-translational modification, we developed proteomic analyses system, especially focused on 2-DE analysis combined with mass spectrometry.

The post-translational modification of Rbp1p is growth stage dependant

We have observed that Rbp1p co-localizes with the P-body markers Dhh1p and Dcp2p when cells are grown to the stationary phase, but not in the early-log phase. The 2-DE result revealed different pattern when cells were grown to the log phase and the stationary phase (Fig. 1C). This result was consistent with our speculation that different environmental stimuli would subject Rbp1p to distinct post-translational modification. Recently our lab has shown that the RNA-binding protein Rbp1p, which appears in small punctate foci in the cytoplasm P body, is involved in *POR1* mRNA degradation. We speculated that when cells received external stimuli, Rbp1p may be regulated by post-translational modifications. Rbp1p bearing distinct modification may associate with different complex and localize to particular sites to carry out its cellular function. In the future our lab may monitor and compare Rbp1p post-translational modifications resulting from other stimuli including osmotic stress, glucose deprivation, or carbon source alteration.

Phosphorylation sites of Rbp1p

We have found that Rbp1p is a phosphorylation protein (Appendix 2) and bears some apparent phosphorylation sites by performing GST-Rbp1p pull-down assay following by SDS-PAGE and then mass spectrometry analysis of the sliced-out target band (LTJ). These sites included serine 459, 462, 463, 524, 526 and threonine 637, in

which the probability of 524 and 637 phosphorylation is very high. However, growth phenotype analysis revealed that T637A mutant had no obvious effect (Fig. 2B,3B). Interestingly, both of S459, 462, 463A and S524, 526A mutant had effect in YPH499 and BY4741 strain (Fig. 2B,3B). Although S459, 462, 463A mutant showed growth effect and post-translational modification differences, they did not affect the localization of Rbp1p to P body (Fig. 2B,3B,4,5). These results implied that the Rbp1p growth inhibition ability and the localization to P body may be independent events. An unusual observation was that the Rbp1p-dN form grew faster than vector alone, and the protein expression of Rbp1p-dN was more than others (Fig. 2B,3B). We don't know why Rbp1p-dN can accelerate growth, but it is possible that Rbp1p-dN overexpression may associate with and trap some growth inhibitor, causing their dysfunction.

In our attempt to determine the sites of Rbp1p post-translational modification by GST-Pull down, 2-DE gel separation, followed by mass spectrometry analysis of the gel-off spots, the mass spectrometry result did not show phosphorylation signal in serine 459,462, and 463 (Tab. 6). This result was incomprehensible, and may be due to experimental mistakes or the protein level was not enough to determine.

Putative kinase of Rbp1p

A Serine/Theronine kinase Ksp1p was found to be a putative kinase of Rbp1p (Ptacek *et al.*, 2005). Ksp1p was found as a high-copy-number suppressor of *prp20-10*, which is a temperature-sensitive mutant of guanine nucleotide exchange factor for the nuclear Ras-like Ran protein. Ksp1p is a nuclear protein that is not essential for vegetative growth of yeast. Prp20p is a phosphorylated protein, but no alteration of Prp20p phosphorylation was found in Δ*ksp1* cells, suggesting that the phosphorylation of Prp20p is not directly mediated by the Ksp1p kinase (Fleischmann *et al.*, 1996). Our data showed that overexpressing Rbp1p in Δ*ksp1* cells showed no growth defect as compared with cells expressing vector alone (Fig. 6,7,8,9). In rescue experiment where ADH drove Myc-Ksp1p overexpression in Δ*ksp1* cells, Rbp1p restored its growth inhibition ability (Fig. 9B). The post-translational modification of Rbp1p was also altered in Δ*ksp1* (Fig. 10). An unusual finding in Δ*ksp1* was that Rbp1p showed more signal in low PI (Fig. 10). This result suggested that Ksp1p may not be a direct Rbp1p kinase. However Rbp1p could localize to P body in Δ*ksp1* cells (Fig. 11). The above data raises the possibility that Ksp1p is important for the function of Rbp1p, at least in growth phenotype and post-translational modification.

Rbp1p and its associate protein

In this study we used two approaches to find out the Rbp1p associate protein. In the first approach, after GST-Rbp1p pull-down and Western check, we found a major band at roughly 60kDa, but it was not Rbp1p(Fig. 12A). Therefore we seperated GST-Rbp1p in 2-DE gel followed by CBR staining, and detected several spots at about 60kDa (Fig. 12B). Gel off of these spots followed by identification by mass spectrometry, the result revealed many carbon source metabolism regulated gene products (Tab. 5). However these proteins may not associate with Rbp1p in normal physiology condition because our GST-Rbp1 was overexpressed and induced by *GAL* promoter. The other approach was based on our previous lab study involving yeast two hybrid, in vitro binding assay, and in vivo binding assay, where we found some candidates of Rbp1p interacting protein.

(1) *DHH1*

Rbp1p can interact with Dhh1p. Previously our lab found that Rbp1p can interact with Dhh1p by yeast two hybrid assay. Moreover Rbp1p can interact with Dhh1p in an RNA-independant manner by in vivo pull down assay. In addition, when cells grow to stationary phase or under osmotic stress, Rbp1p can colocalize with Dhh1p in P body (LTJ).

(2) *HRP1*

Rbp1p can interact with Hrp1p. Previously our lab found that Rbp1p can interact with Hrp1p by in vito binding assay (LTJ).

(3)*PORIN1*

Rbp1p can interact with Porin1p. Previously our lab found that Rbp1p can regulate *POR1* mRNA (Buu and LTJ) and interact with Por1p(CYF) by yeast two hybrid assay.

(4) *PSP1*

Rbp1p can interact with Psp1p. Previously our lab found that similar to Rbp1p, V5-tagged Psp1p can localize to P body in *xrn1* mutant. Furthermore, subcellular localization of Psp1p and Rbp1p was similar in sucrose gradient fractionation assay (LTJ), and yeast two hybrid assay revealed that Rbp1p can interact with Psp1p (LTJ and CYW).

(5) *Pub1*

Rbp1p can interact with Pub1p. Pub1p was found to be associated with Nrp1p(Gavin et al, 2002). Previously our lab found that Pub1p, like Rbp1p, can

localize to P body in *xrn1* mutant. In addition, in vivo pull-down assay demonstrated that endogeneous GFP-tagged Nrp1p and Pub1p can interact with the overexpressed GST-Rbp1p but not GST alone.

In this study using GST-Rbp1p pull down method followed by Western check, we double confirmed that Hrp1p, Dhh1p, Porin1p, Psp1p, and Pub1p may indeed associate with Rbp1p (Fig. 13).

Materials and Methods

Yeast atrains, media and plasmids

All strains used in this study are list in Table 1. Yeast culture medium were prepared as described (Scherman et al., 1986). YPD contained 1% Bacto-yeast extract , 2 % Bacto-peptone, and 2 % glucose . Synthetic minimal medium contained 0.17 % Difco yeast nitrogen base(without any amino acid), 0.5% ammonium sulfate ,and 2% glucose. Nutrients essential for auxotrophic strains were supplied at specified concentrations(Sherman et al.,1986). Yeast strains were transformed by the lithium acetate method (Ito et al.,1983). Plasmids were constructed according to the standard protocols(Sambrook et al.,1989). The point-mutants were generated by site-directed mutagenesis and PCR cloning. The primers used in this study were listed in Table 2.

The E.coli vectors pSTBlue-1 were used for gene cloning. The yeast and E.coli shuttle vetor pVT101U (Vernet,et al.,1987), carrying the yeast URA3 gene, the replication origin of the yeast 2u plasmid, the replication origin of E.coli f1 plasmid and the E.coli gene for resistance to amplicillin, was replicated in E.coli and used to express proteins in yeast. All plasmids used in this study were listed in Table 3.

Yeast Transform

Yeast cells were grown in YPD at 30℃ overnight with shaking to OD_{600}>1.5. Enough overnight culture was transferred to fresh YPD medium to produce an OD_{600} 0.2~0.3 and incubated at 30℃ for 3 hrs with shaking. Cells were harvested at 600 g for 5 min after incubation, washed once in ddH_2O, and resuspended in 5 ml of freshly prepared, sterile 1X TE/LiOAc per 10ml of the starting culture. In a sterile 1.5 ml microcentrifuge tube, 100~200 ng of plasmid and 0.1 mg of carried DNA were added. 0.1 ml of competent cells were added to the DNA mixture, and mixed thoroughly before adding 0.5 ml of freshly prepared sterile PEG/TE/LiOAc. The transformation mixture was mixed by pipetting, incubated at 30℃ for 30 min with shaking at 100 rpm, and heat shocked for 15 min at 42℃. After chilling on ice for 2 min, the mixture was centrifuged at 600 g for 3 min at room temperature, washed once with ddH_2O, and the cell pellet was resuspended in 0.1 ml ddH_2O and plated onto an appropriate selective plate.

Yeast extract preparation and Western blot analysis

Optimal overnight yeast cells were harvested and suspended in 50 µl of ddH2O containing 4 µl of 100% TCA and half total-volume of glass beads. After a vigorous vortex for 3 min, 200 µl 5% TCA was added and the total proteins were collected by centrifugation at 15,500 g for 15 min. The protein pellet was washed once with cold ddH2O, and then resuspended in 1X sample buffer (50 mM Tris-HCl, pH6.8; 2 % β-mercaptoethanol; 2 % SDS; 10 % glycerol) and adjust to pH value by 2M Tris base, followed by incubation at 95℃ for 10 min, chilled on ice for a while, and then subjected to SDS-PAGE. For Western blot analysis, proteins were separated on SDS-PAGE and transferred to PVDF membranes (Millipore Corp.) by electro-semi-dry apparatus (Hoefer Semiphor, Hoefer Pharmacia Biotech Inc., Model TE70) (48 mM Tris-HCl, pH 8.3; 39 mM glycine; 0.037 % SDS; 15 % methanol). After protein transfer, the PVDF membrane was incubated in blocking solution containing 5 % non-fat dry milk (25 mM Tris-HCl; 0.8 % NaCl; 0.02 % KCl, pH 7.4; 0.1 % Tween 20)for membrane blocking. The primary antibody was diluted in blocking solution and incubated with membrane for 60 min at room temperature (Table 4). After four times of washing with TBST, the membrane was incubated with HRP-conjugated secondary antibody diluted by blocking solution at room temperature for 30 min (Table 4). After extensive wash, bound antibodies were detected with the ECL system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Yeast protein preparation for 2-DE analysis

Harvest the cells by centrifugation, discard the supernatant , resuspend the cells in PBS, and centrifuge again. Discard the supernatant, estimate the volume of the cell pellet. Add 3 volumes of ice-cold lysis buffer per volume of cell pellet. Keep the suspension ice. Add a volume of chilled glass beads equal to the total volume of the resuspended yeast cells. Vortex the suspension 30 seconds then on ice 30 seconds repeat 10 times. Centrifuge the suspension than carefully pour off the supernatant to a fresh tube. Add final concentration 15% TCA to supernatant react in -20^0C 3~4 hr. Centrifuge the supernatant wash the pellet with Cold Acetone(with 0.2% B-mercaptoethanol) than centrifuge to discard the supernatant repeat 5 times. Dry the pellet store in -80^0C

Lysis buffer (RIPA buffer)

Contents	Concentrate
NaCl	150mM
NP40	1%
Sodium deoxychoate	0.5%
SDS	0.1%
Tris-Cl pH7.2	50mM
Aprotinin	1 μg/ml
Leupeptin	1 μg/ml
Pepstatin	1 μg/ml
PMSF	50 μg/ml

Phosphate-buffered saline (PBS)

Contents	Concentrate
NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10.1 mM
KH_2PO_4	1.8 mM
pH 7.4	

2-DE Analysis

Isoelectrical focusing

Add appropriate volume 2D SB to protein pellet, than sonicate 1 minute on ice 1 minute repeat 5 times. Take out 100λ sample than add 150λ2D RB. Load the sample to the 2D strip holder than put 13 cm pH 3-10 2D strip. Cover the 2D strip gel with a thin layer of silicone oil to prevent it from drying out during IEF step. Perform IEF at 20 ^{0}C 50μA. IEF running program describe as follow. The total Voltage hours about 46000 Vhrs

2D Sampe buffer (store in -20 ^{0}C)

Contents	Concentrate
Urea	6M
Thiourea	2M
CHAPS	4%
DTT	1%
Carrier amplolytes (pH 3-10)	2%
Protease inhibitor cocktail	1X

2D Rehydration buffer (store in -20 ^{0}C)

Contents	Concentrate
Urea	8M
CHAPS	2%
DTT	2.8%

Step	Times
Rehydration	12 hr
30 Voltage	8 hr
500 Voltage	3 hr
1000 Voltage	3 hr
3000 Voltage	14hr
Total Voltage hr	≒46000

Equilibration

Dissolve 100mg of DTT in 10 ml of equilibration buffer. Make 10 ml per sample. Place the focused IPG gel strips into individual test tubes shack them 15 minutes than pour off the equilibration buffer. Dissolve 0.4 g of iodoacetamide in 10 ml of equilibration buffer and equilibrate for another 15 minutes. Pour off the equilibration buffer and transfer IPG gel strips to SDS-PAGE.

Equilibration buffer store in -20 ^{0}C

Contents	Concentrate
Urea	6M
Glycerol	30%
SDS	2%
Tris-Cl pH8.8	0.05M
Bromophenol blue	a trace

2D SDS-PAGE

Start SDS-PAGE with 10mA per SDS gel for 1hr. Continue with 30mA per SDS gel for 6-7 hr. Terminal the run when the bromophenol blue tracking dye has migrated off the lower end of the gel.

10% SDS-PAGE

Sol. A	30 ml
Sol. B	22.5 ml
ddH_2O	37.5 ml
100% TEMED	50 μl
10% APS	750 μl

- Sol. A: 30% Acrylamide + 0.8% Bisacrylamide
- Sol. B: 1.5 M Tris-HCl pH8.8 + 0.4% SDS
- APS: Ammonium persulfate solution

Agarose sealing solution

Contents	
Agarose	0.5g
5X SDS electrophoresis buffer	20 ml
Bromophenol Blue	a trace
ddH_2O	100ml

5X SDS electrophoresis buffer

Contents	Concentrate
Tris-base	125 mM
glycine	960 mM
SDS	0.5%
H_2O	to 1000 ml PH8.3

Growth phenotype analysis

Yeast strains were streaked on synthetic selection medium plates containing 2% glucose. After incubated at 30℃ for 3 days, cells were picked up and suspended in sterile ddH_2O. After adjusting the cell suspensions to OD_{600}=0.1, the serial 10-fold dilutions were carried out till $1x10^{-4}$. 5 μl of each diluted cell suspensions were on synthetic selection media plates, which contained 2% glucose. Cells were incubated at 30℃ for 4~5 days.

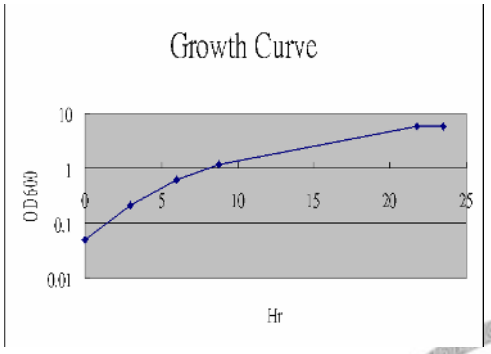
Pull-down Assay

Cell, in which the RBP1 gene was deleted, were transformed with plasmids carring GST-tagged RBP1. Cell were harvested by centrifugation, wash twice with PBS and dispersed in IP buffer. Cell were broken by tissue lyser with glass beads and debris was removed by centrifugation(1500 x g for 10 min). Than add Glutathione beads binding overnight at 4^0C. Wash twice with IP buffer contained 0.05% Tween 20. Eluted with elution buffer than add 100% TCA to final 20% concentration.

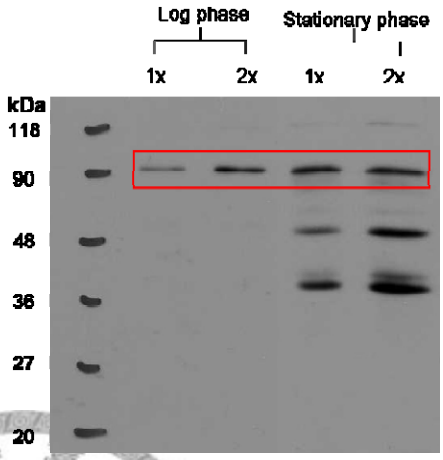
Figures

A.

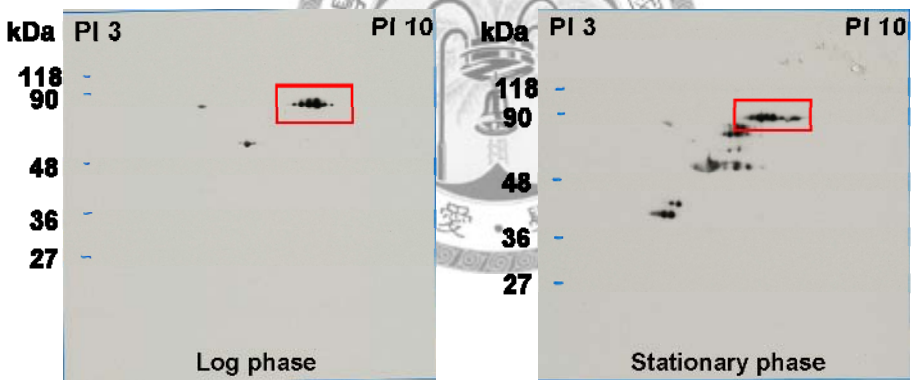
Time (Hr)	Log phase	Stationary phase
0	0.05	0.05
8	0.756	
24		4.459



B.



C.



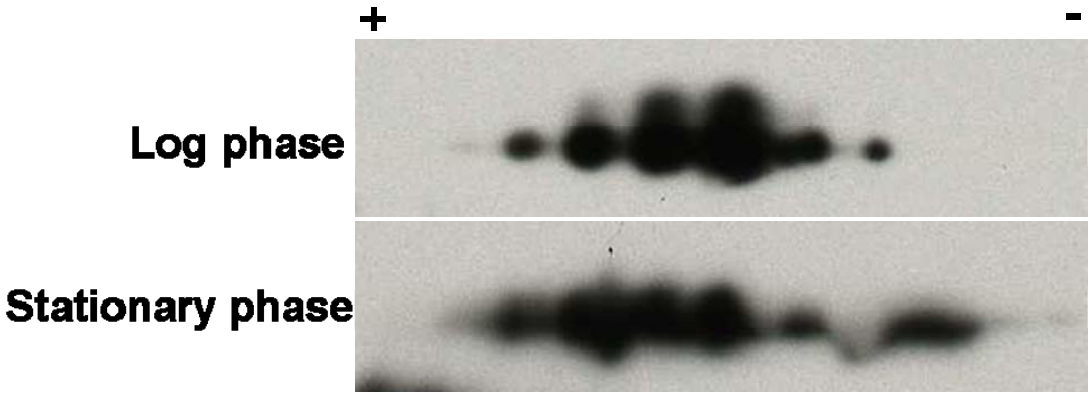


Figure 1. Rbp1p displayed different pattern in log phase and stationary phase of cell growth in 2-DE gel

1A. Growth cruve of yeast strain YPH 499△*rbp1* Leu2 :: ADH HA-RBP1

1B. Protein expression level of HA-Rbp1p in YPH 499△*rbp1* strain. Protein was seperated by SDS-PAGE and visualized by Western blot with anti-HA monoclonal antibody.

1C. HA-Rbp1p overexpression in YPH 499△*rbp1* Leu2 :: ADH HA-RBP1 strain. Protein was seperated by 2-DE, and spots were visualized by Western blot with anti-HA monoclonal antibody.

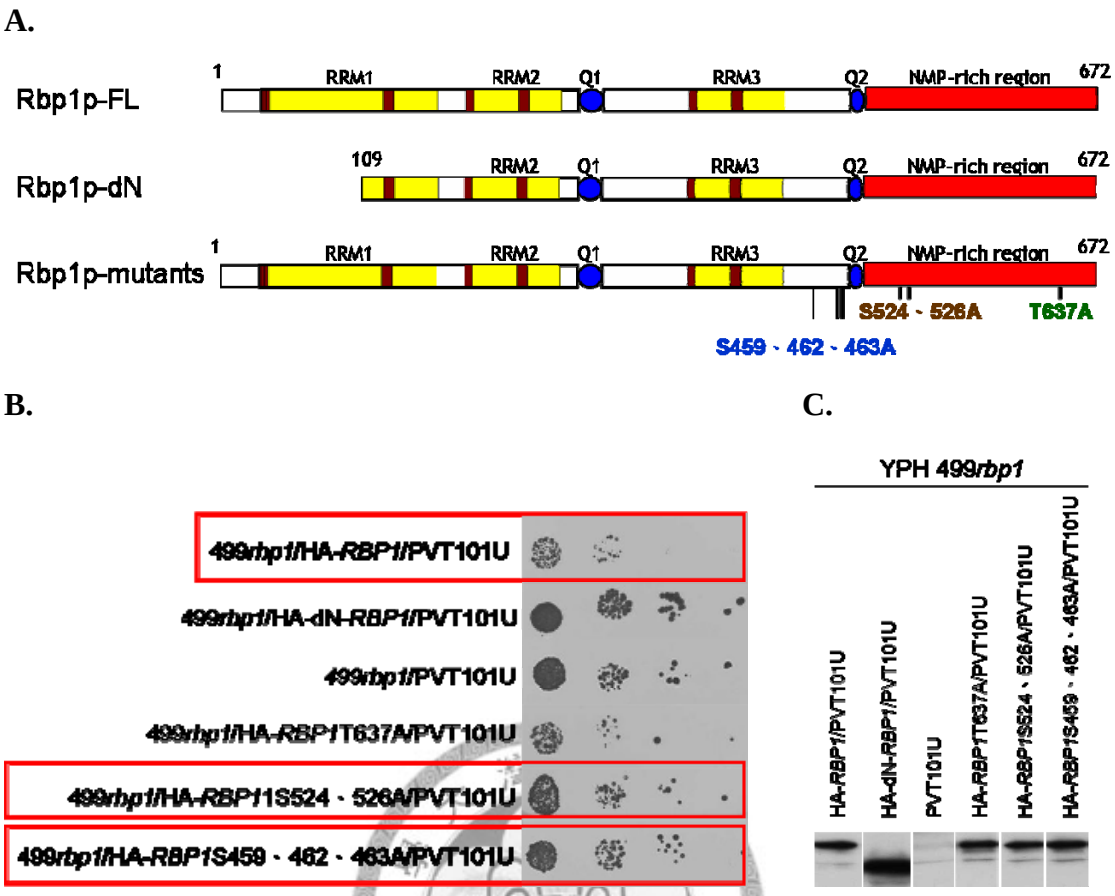


Figure 2. Growth phenotypes of cells expressing HA-Rbp1p, -dN or phosphorylation sites mutants in YPH499△*rbp1* strains.

2A.Schematic representation of Full length Rbp1p, Deletion N-terminal 108 Amino Acid, and phosphorylation sites mutants S459A,S462A,S463A, S524A,S526A, T637A. RRM: RNA-recognition motif; Q: glutamine-rich region; NMP: asparagines-methionine-proline-rich region.

2B. Every strains were grown overnight in glucose-containing medium. Subsequently, ten-fold serial dilutions were spotted onto synthetic media, and plates were incubated at 30℃ for 4~5 days.

2C. Protein expression of full-length Rbp1p, N-terminal Rbp1p, and Rbp1p mutants under control of the P_{ADH1} were expressed in the YPH499Δ*rbp1* strain. Cells were grown to mid-log phase and extracts were prepared by precipitation with trichloroacetic acid as described in Materials and Methods. Equal amounts of total protein were loaded in each lane as detected by antibodies against Rbp1p.

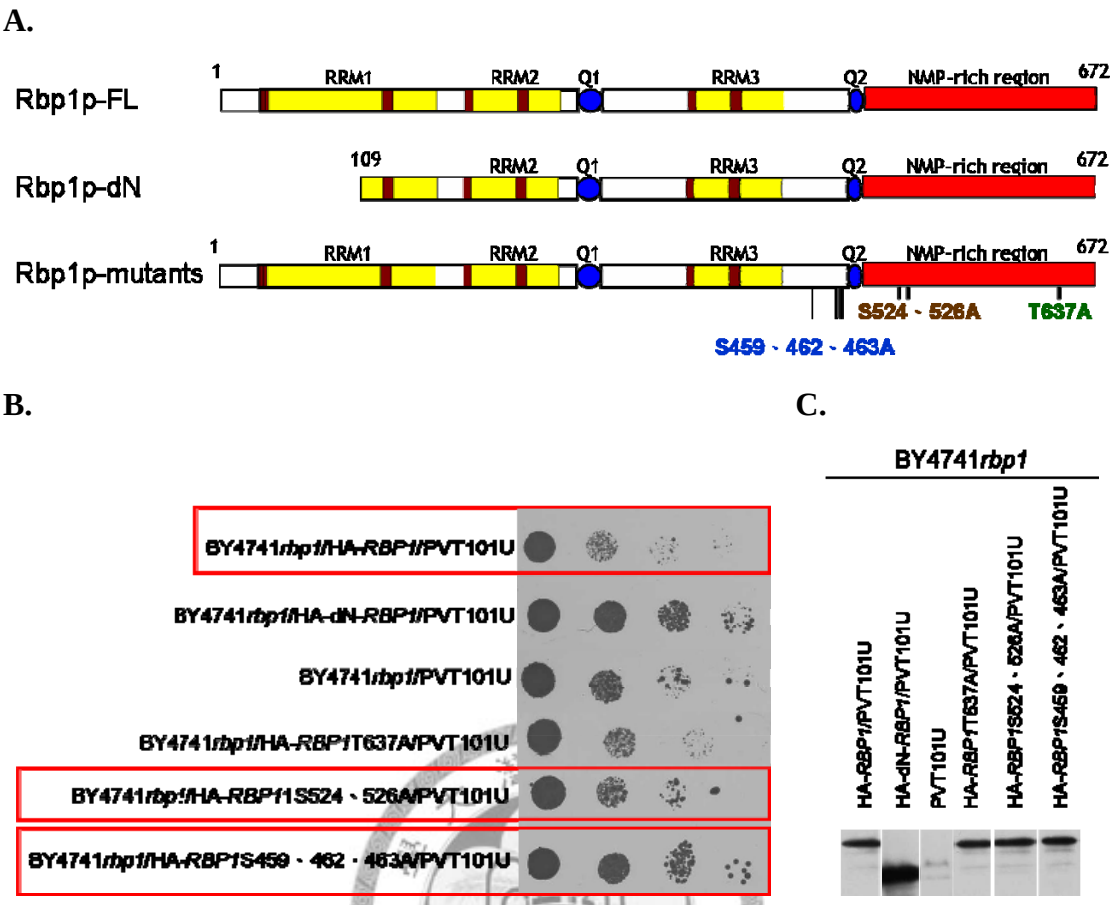


Figure 3. Growth phenotypes of cells expressing HA-Rbp1p, -dN or phosphorylation sites mutants in BY4741△*rbp1* strains.

3A. Schematic representation of Full length Rbp1p, Deletion N-terminal 108 Amino Acid, and phosphorylation sites mutants S459A,S462A,S463A, S524A,S526A, T637A. RRM: RNA-recognition motif; Q: glutamine-rich region; NMP: asparagines-methionine-proline-rich region.

3B. Every strains were grown overnight in glucose-containing medium. Subsequently, ten-fold serial dilutions were spotted onto synthetic media, and plates were incubated at 30℃ for 4~5 days.

3C. Protein expression of full-length Rbp1p, N-terminal Rbp1p, and Rbp1p mutants under control of the P_{ADH1} were expressed in the BY4741Δ*rbp1* strain. Cells were grown to mid-log phase and extracts were prepared by precipitation with trichloroacetic acid as described in Materials and Methods. Equal amounts of total protein were loaded in each lane as detected by antibodies against Rbp1p.

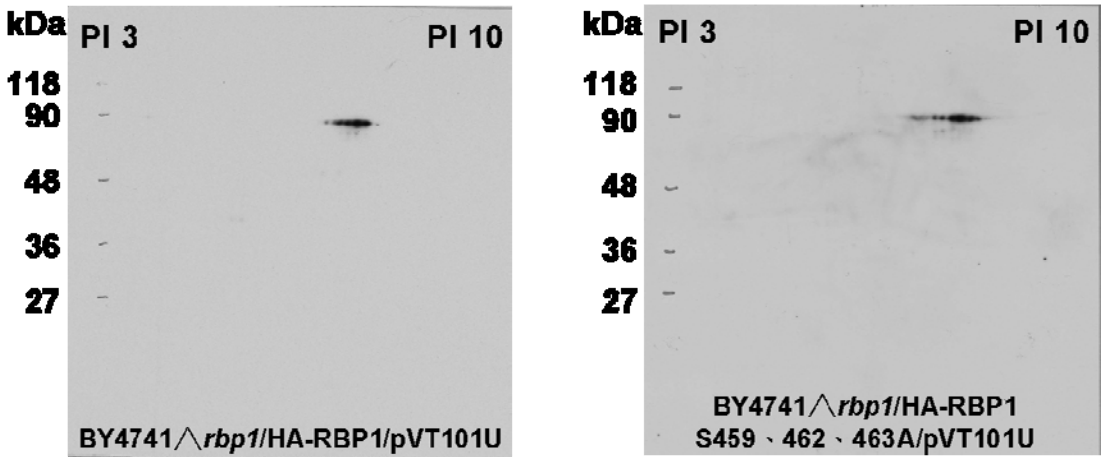


Figure 4. The post-translational modification of Rbp1p affected by mutation on Ser-459,462,463.

HA-Rbp1p overexpression in BY4741△*rbp1*. Cells goowth 12hr to log phase and extracted total protein. Protein was seperated by 2-DE, and spots were visualized by Western blot with anti-HA monoclonal antibody.

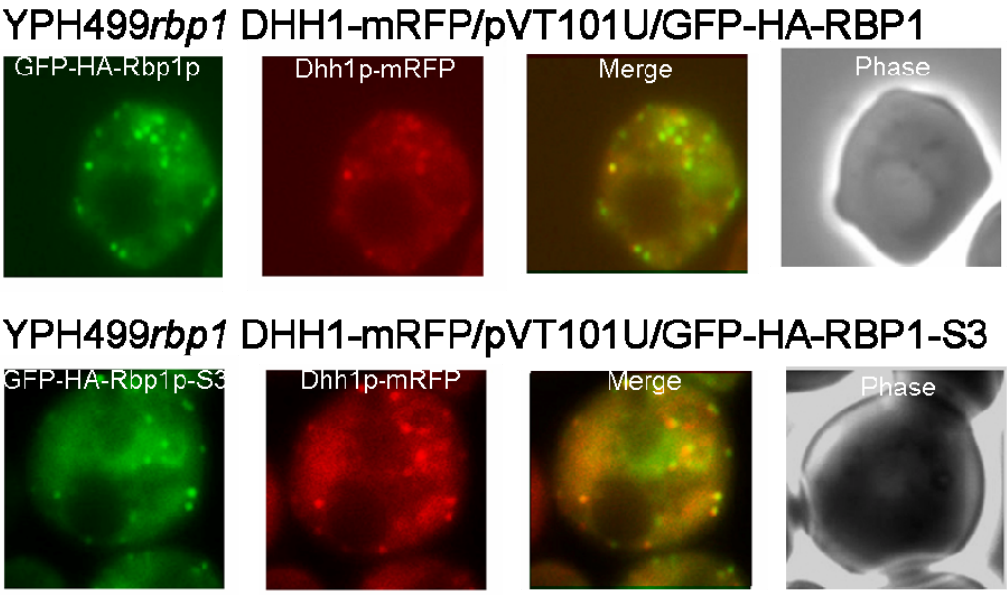


Figure 5. P-body localization of Rbp1p was not affected by mutation on Ser-459,462,463.

The YPH499Δ*rbp1* mutant strain with mRFP-tagged chromosomal *DHH1* gene was transformed with GFP-tagged HA-Rbp1p or HA-Rbp1p-S3. Both of HA-Rbp1p or HA-Rbp1p-S3 can colocalize with Dhh1p to P body.

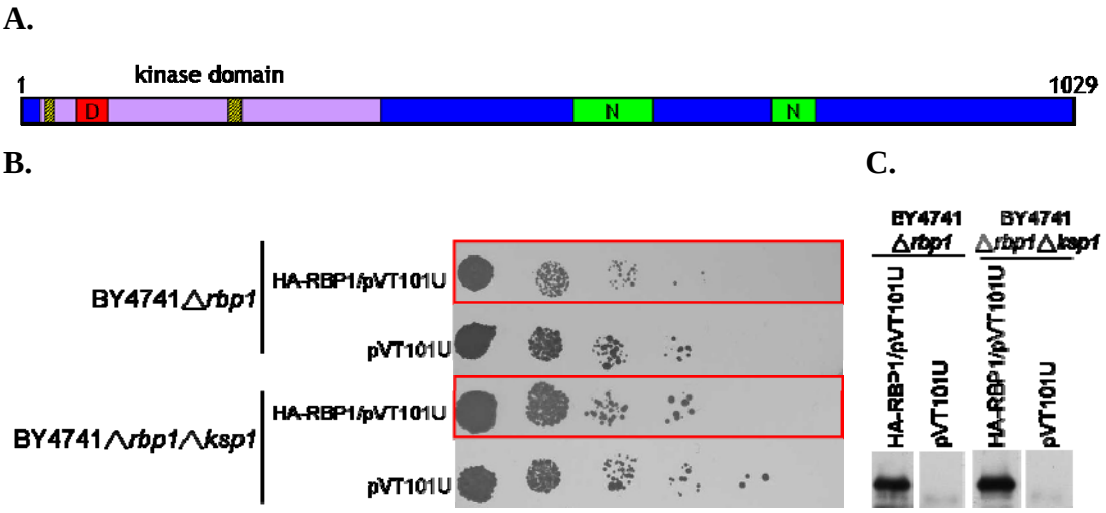


Figure 6. Growth phenotypes of cells expressing HA-Rbp1p in BY4741 *rbp1p* deletion or BY4741 *rbp1p* and *ksp1p* double deletion strains.

6A. Ksp1p is a Ser/Thr kinase belonging to the RAN kinase family. It contains an N-terminal kinase domain (yellow box); regions which are important for kinase activity are shaded. No other specialized functional domain was found in its primary sequence.

6B. Every strains were grown overnight in glucose-containing medium. Subsequently, ten-fold serial dilutions were spotted onto synthetic media, and plates were incubated at 30℃ for 4~5 days.

6C. Protein expression of full-length HA-Rbp1p. Cells were grown to mid-log phase and extracts were prepared by precipitation with trichloroacetic acid as described in Materials and Methods. Equal amounts of total protein were loaded in each lane as detected by antibodies against Rbp1p.

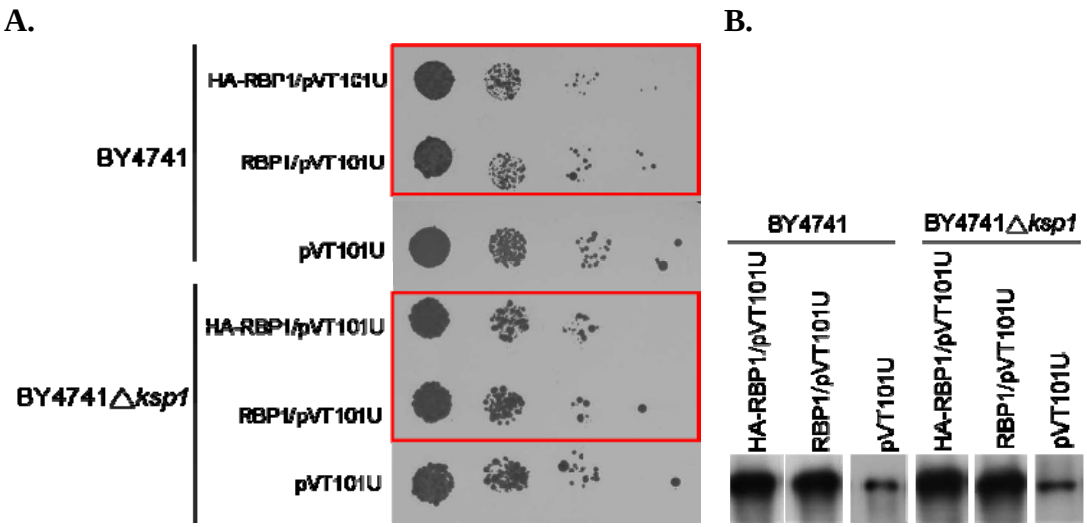


Figure 7. Growth phenotypes of cells expressing HA-Rbp1p or Rbp1p in BY4741 wild type or *ksp1p* deletion strains.

7A. Every strains were grown overnight in glucose-containing medium. Subsequently, ten-fold serial dilutions were spotted onto synthetic media, and plates were incubated at 30℃ for 4~5 days.

7B. Protein expression of full-length Rbp1p. Cells were grown to mid-log phase and extracts were prepared by precipitation with trichloroacetic acid as described in Materials and Methods. Equal amounts of total protein were loaded in each lane as detected by antibodies against Rbp1p.

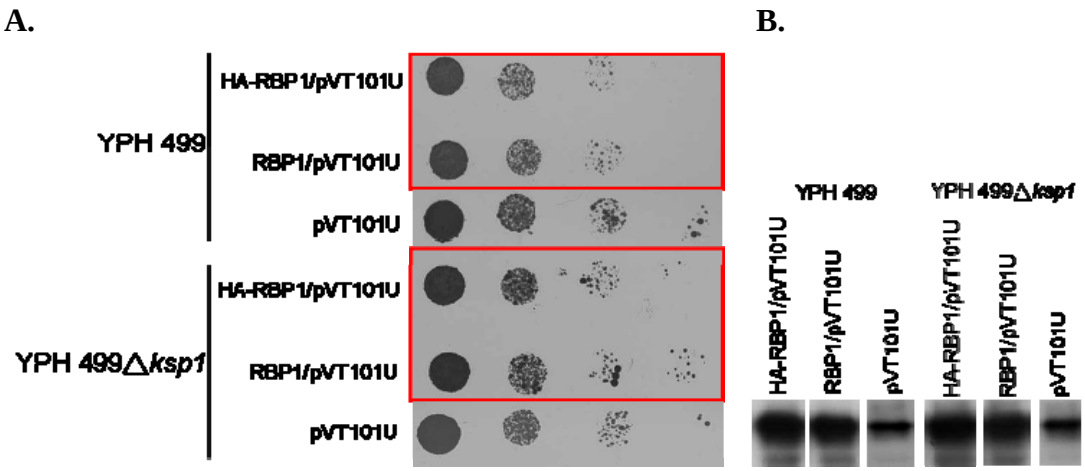


Figure 8. Growth phenotypes of cells expressing HA-Rbp1p or Rbp1p in YPH499 wild type or *ksp1p* deletion strains.

8A. Every strains were grown overnight in glucose-containing medium. Subsequently, ten-fold serial dilutions were spotted onto synthetic media, and plates were incubated at 30℃ for 4~5 days.

8B. Protein expression of full-length Rbp1p. Cells were grown to mid-log phase and extracts were prepared by precipitation with trichloroacetic acid as described in Materials and Methods. Equal amounts of total protein were loaded in each lane as detected by antibodies against Rbp1p.

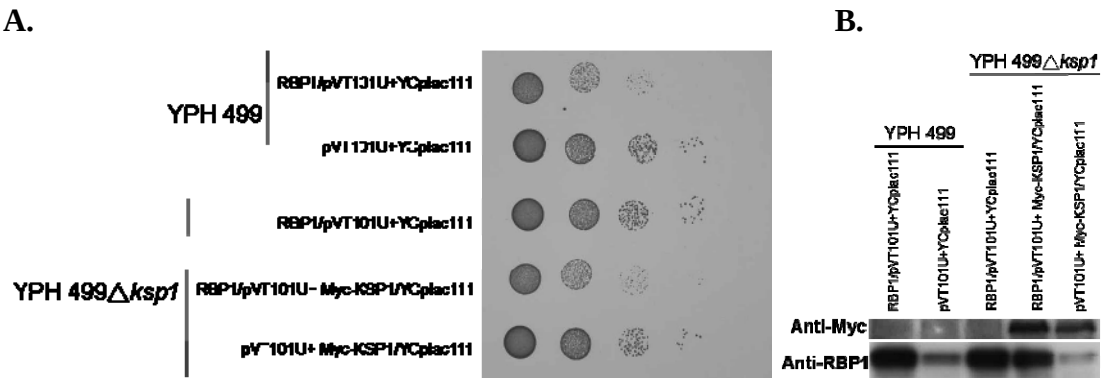


Figure 9. Growth phenotypes of cells expressing HA-Rbp1p or Myc-Ksp1p in YPH499 wild type or *ksp1p* deletion strains.

9A. Every strains were grown overnight in glucose-containing medium. Subsequently, ten-fold serial dilutions were spotted onto synthetic media, and plates were incubated at 30℃ for 4~5 days.

9B. Protein expression of full-length Rbp1p or Myc-Ksp1p. Cells were grown to mid-log phase and extracts were prepared by precipitation with trichloroacetic acid as described in Materials and Methods. Equal amounts of total protein were loaded in each lane as detected by antibodies against Rbp1p and Myc.

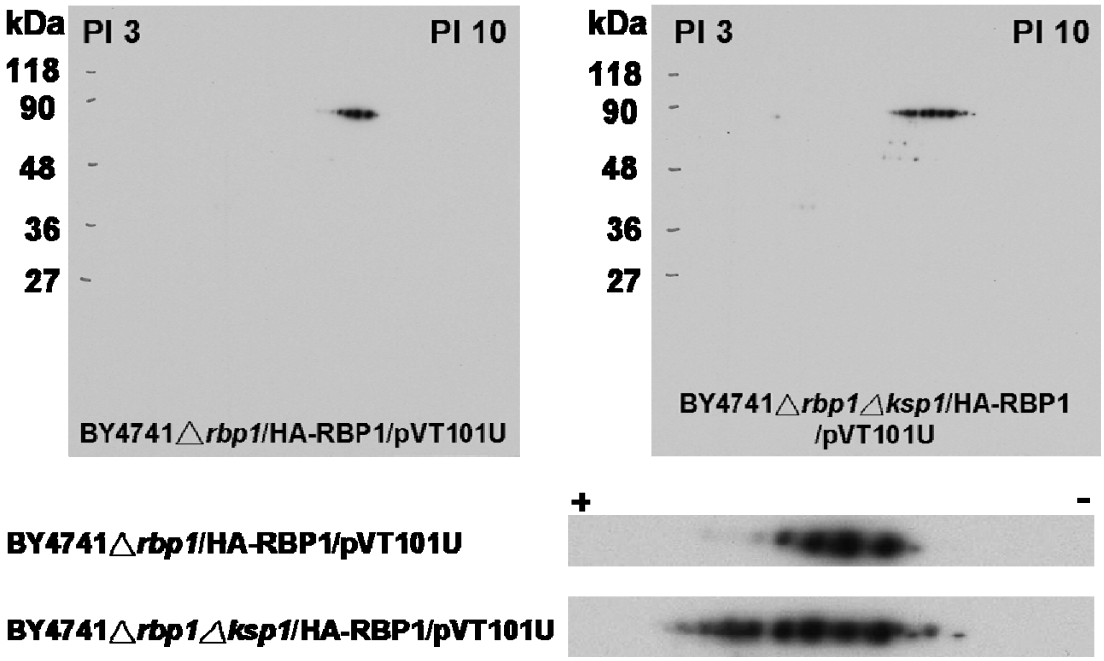


Figure 10 . The post-translational modification of Rbp1p affected by Ksp1p deletion. HA-Rbp1p overexpression in BY4741△*rbp1* or BY4741△*rbp1*△*ksp1* Cells goowth 12hr to log phase and extracted total protein. Protein was seperated by 2-DE, and spots were visualized by Western blot with anti-HA monoclonal antibody.

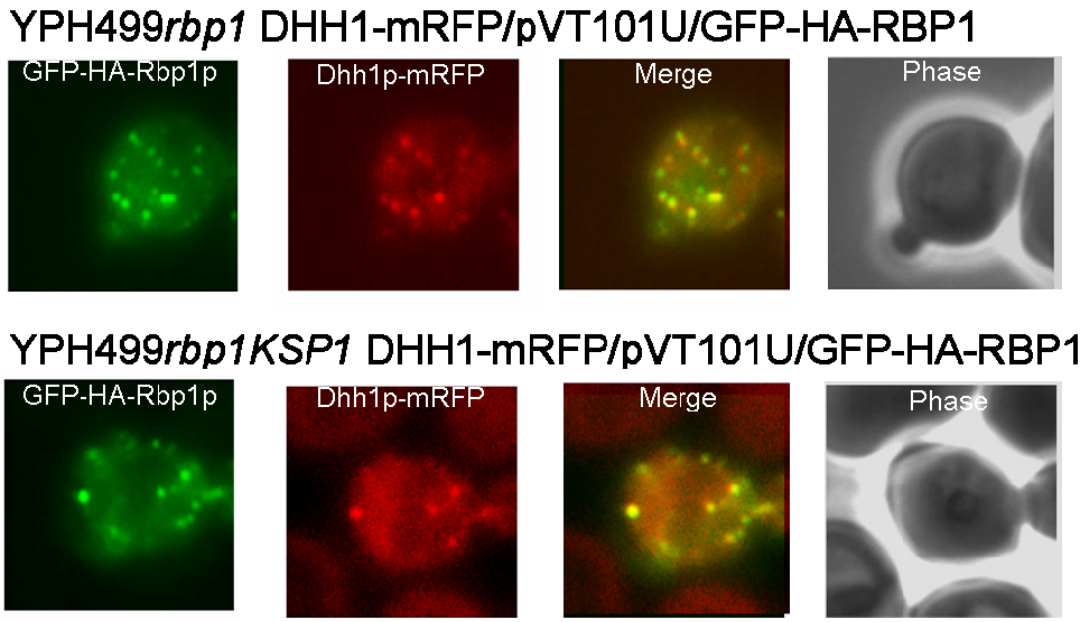


Figure 11 .P-body localization of Rbp1p is not affected by Ksp1p deletion.
The YPH499Δ*rbp1* and YPH499Δ*rbp1*Δ*ksp1* mutant strain with mRFP-tagged chromosomal *DHH1* gene was transformed with GFP-tagged HA-Rbp1p. Both of in YPH499Δ*rbp1* and YPH499Δ*rbp1*Δ*ksp1*HA-Rbp1p can colocalize with Dhh1p to P body.

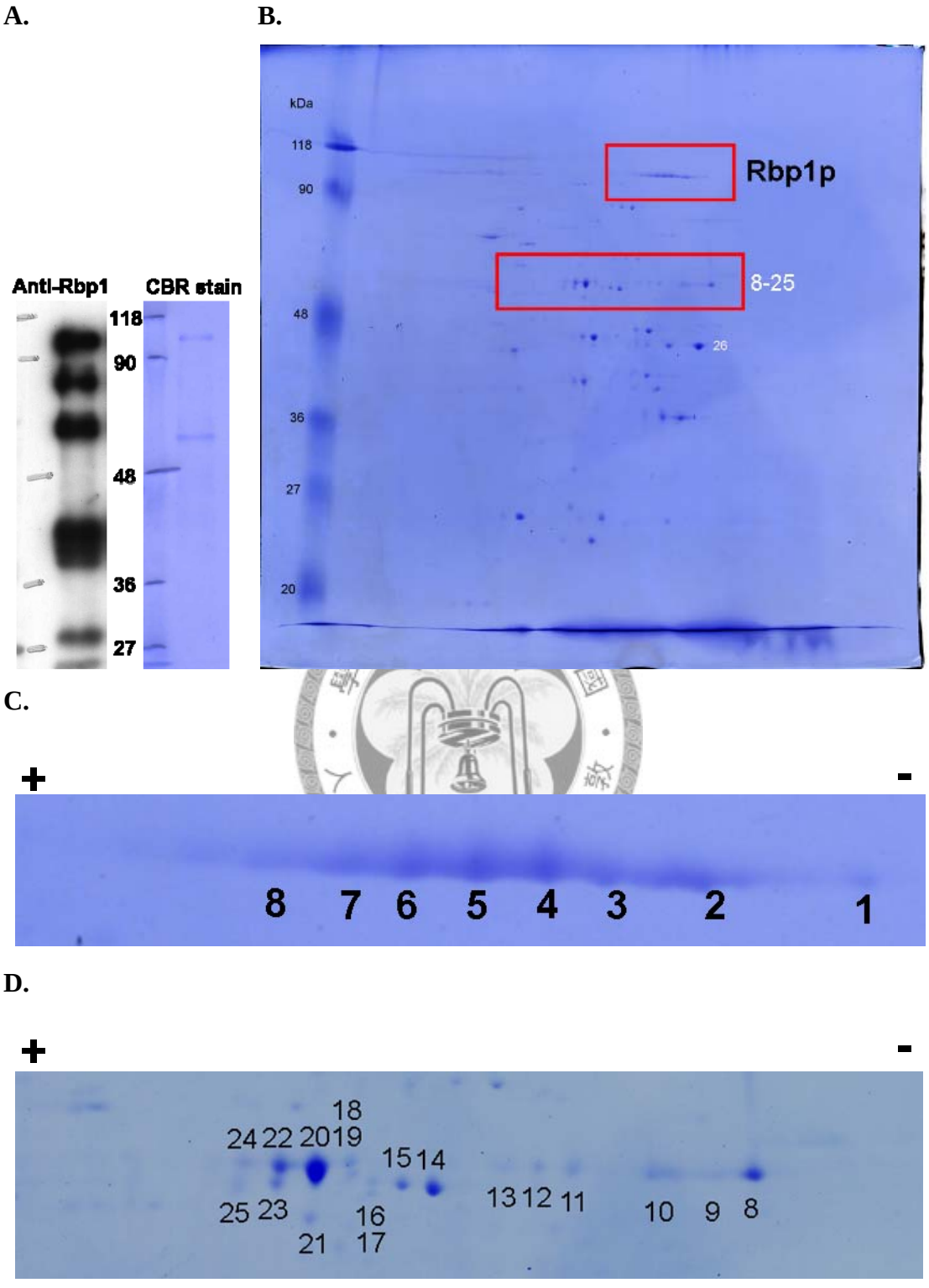


Figure 12 . Rbp1p and its associated proteins.

12A. Glutathione pull down GST-Rbp1p and western check by anti-Rbp1p antibodies. Right pannel is PVDF member CBR staining.

12B.GST-Rbp1p seperated in 2-DE gel and CBR staining.

12C.Full length GST-Rbp1p revealed about 8 spots.

12D.About 60 kDa displayed several non Rbp1p protein spots.

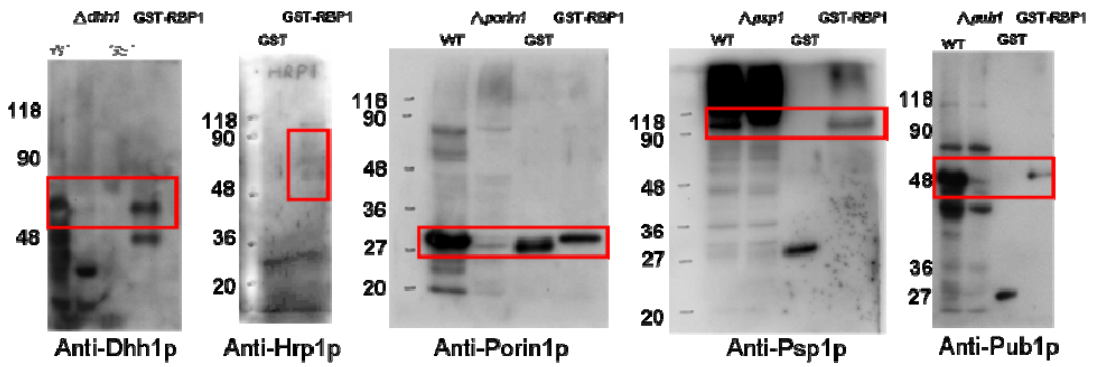


Figure 13 . Rbp1p associated proteins

GST only and GST-Rbp1p independant overexpression in YPH499Δ*rbp1* strain. Glutathione pull down GST only and GST-Rbp1p than Western check, using antibodies included anti-Dhh1p, anti-Hrp1p, anti-Porin1p, anti-Psp1p, and anti-Pub1p.

Tables

Table 1. Yeast strains used in this study

Strain	Genotype
YPH499	MATa *ura3*-52 *lys2*-801 *ade2*-101 *trp*-△63 *hus3*-△200 *leu2*-△1
YPH 499*rbp1*	YPH499 except *rbp1 :: Ura3*
YPH499*rbp1* HA-RBP1	YPH499 except *rbp1 :: Ura3* ADH HA-RBP1 :: Leu2
YPH499*rbp1* DHH1-mRFP	YPH499 except *rbp1 :: Ura3* DHH1-mRFP :: Leu2
BY4741	MARa *his3*△1 *leu2*△ *met15*△ *ura3*△
BY4741*rbp1*	BY4741 except *rbp1 :: Ura3*
BY4741*ksp1*	BY4741 except *ksp1 :: KanMX6*
BY4741*rbp1ksp1*	BY4741 except *rbp1 :: Ura3 ksp1 :: KanMX6*

*ade:*adenine-requiring
his: histidine-requiring
leu: leucine-requiring
lys: lyscine-requiring
trp: tryptophan-requiring
ura: uracil-requiring

Table 2. Primers used in this study

Gene	Primer[a]	Sequence (5’ to 3’)
Primer pairs for amplification of RBP1 and its mutants		
HA-*RBP1*	QyHA-1	5’ATTTCTCGAGATGTACCCATACGACGTCCCAGA CTACGCTATGTCTAACGTTGCTAACGCC
	QyHA-2	5’ATAATCTAGAGGACAAGATTAAAATTTTCTTT
HA-*RBP1*-dN	HAQdN[b]	5’ATTTCTCGAGATGTACCCATACGACGTCCCAGA CTACGCTTTAATATTAATGGA ATATCC
	QyHA-2	5’ATAATCTAGAGGACAAGATTAAAATTTTCTTT
HA-*RBP1*-S459-462-463A	Q1-459-462-463A-3’	5’CGACTGCAGCAGCCGCAGCTGGCGCTCTCAGG CCATTTGAGGA
	Q1-459-462-463A-5’	5’CCAGCTGCGGCTGCTGCAGTCGATAACTCCAAA CAAATTCTTG
HA-*RBP1*-S524-526A	Q1-524-526A-3’	5’GAGGTCGGCATGGGCGCCATTCTTGTTCCTGTG ATAATCG
	Q1-524-526A-5’	5’AGAATGGCGCCCATGCCGACCTCGTTAATCTGC AGAGATC
HA-*RBP1*-T637A	RBP1-T637A-3’	5’AAGGGAGGAGCCATCATCCTGGACACGTCATAC
	RBP1-T637A-5’	5’CAGGATGATGGCTCCTCCCTTAAATATAGCCCC

Table 3. A brief summary of plasmids used in this study

Plasmid name	Plasmid characteristic
pSTBlue-1 (T-vector)	Dual kanamycin/ampicillin resistance, dual opposed T7/SP6 promoters, f1 origin, blue/white screening a multiple cloning region.
pVT101U (Yeast expression vector)	An ampicillin-resistance gene, the *URA3* marker, the *E. coli* origin, the f1 origin, the yeast 2u origin, the *ADH1* gene promoter, and the *ADH1* 3' terminator.
YCplac111 (Yeast expression vector)	An ampicillin-resistance gene, the *LEU 2* marker, the *E. coli* origin, the pBR322 origin, the yeast ARS1-CEN4 origin.
pEG(KT) (Yeast expression vector)	An ampicillin-resistance gene, the *URA3* gene, the Leu2-d the *E. coli* origin, the f1 origin, the yeast 2u origin, the galactose-inducible *CYC1* promoter,and T7 promoter

Table 4. Antibodies used in this study

Name	Western blotting	Source
Primary antibodies		
monoclonal anti-HA	1:5000	Berkeley antibody company
monoclonal anti-myc	1:1000	Berkeley antibody company
polyclonal anti-Dhh1p	1:2500	FJL Lab
polyclonal anti-GST	1:2500	FJL Lab
polyclonal anti-Hrp1p	1:2500	FJL Lab
polyclonal anti-Porin1p	1:2500	FJL Lab
polyclonal anti-Psp1p	1:2500	FJL Lab
polyclonal anti-Pub1p	1:2500	FJL Lab
polyclonal anti-Rbp1p	1:5000	FJL Lab[a]
Secondary antibodies		
Goat HRP-conjugated anti-rabbit	1:5000	Amersham Pharmacia Biotech
Goat HRP-conjugated anti-mouse	1:5000	Amersham Pharmacia Biotech

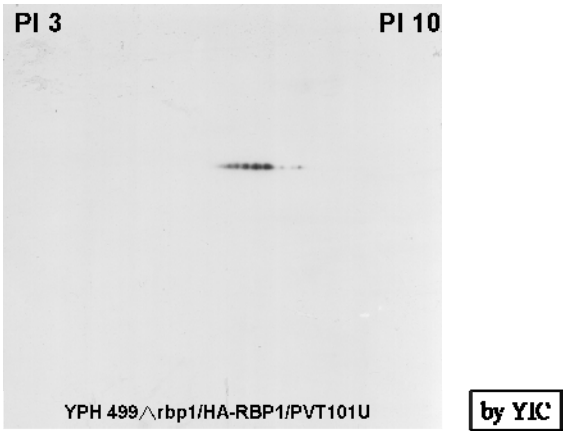
Table 5. Rbp1p associated proteins

Pos.	SwissProt	name	MW	PI	Score	Coverage
8	gi|6319279	Pyruvate kinase 1	55	7.5	220	52%
9						
11						
12						
13	gi|21450619	DASH complex subunit DAD3	11	5.3	61	46%
14	gi|6319673	Glucose-6-phosphate isomerase	61	6	172	60%
15	gi|6319673	Glucose-6-phosphate isomerase	61	6	118	42%
16						
17						
19	gi|21450619	DASH complex subunit DAD3	11	5.3	55	67%
20	gi|6323073	Pyruvate decarboxylase isozyme 1	61	5.8	192	56%
21	gi|6319809	Glucokinase GLK1	56	5.8	112	44%
22	gi|6323073	Pyruvate decarboxylase isozyme 1	62	5.8	200	65%
23	gi|7245976	Pyruvate decarboxylase isozyme 1	62	5.8	103	42%
24	gi|6323073	Pyruvate decarboxylase isozyme 1	62	5.8	78	33%
25						
26	gi|10383781	Phosphoglycerate kinase	45	7.1	292	77%

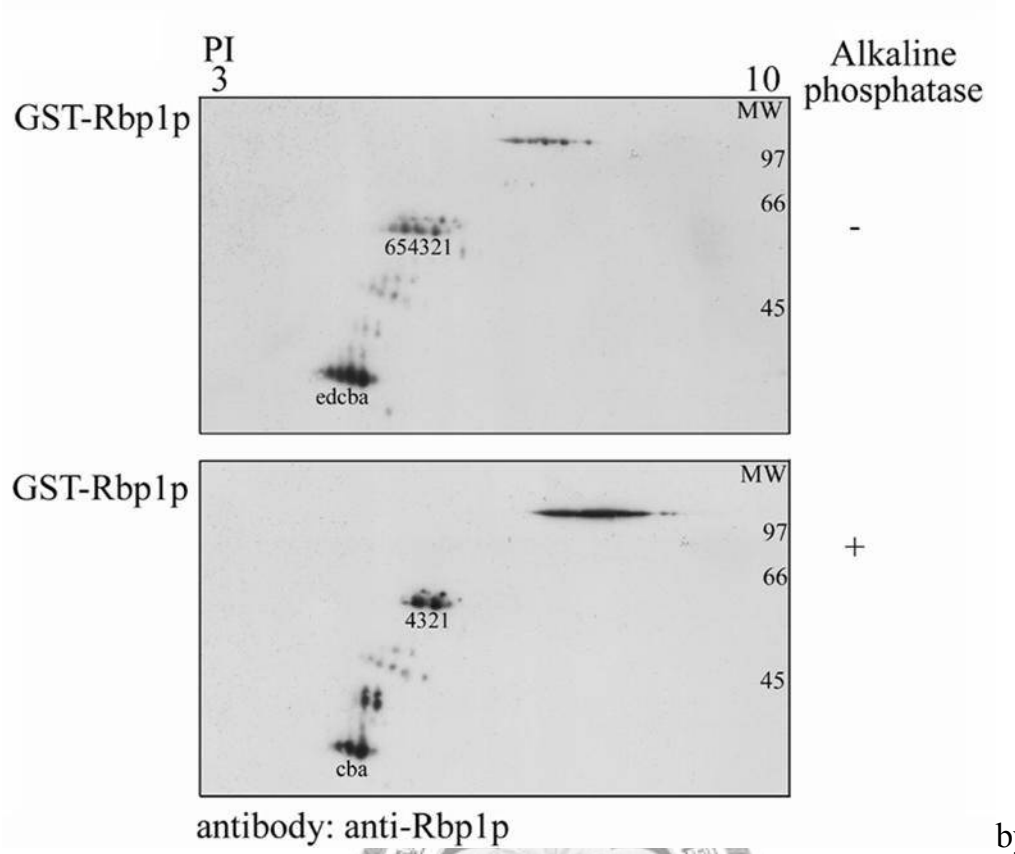
Table 6. Each of the multiple post-translational modification spots of Rbp1p

spot number	Oxidation	Dioxidation	Crboxymethylation	Methylation	Nitroxylation	Deamination	Phosphorylation
1	225、635、636	225、635			474	375、522	
2	225、236、444、450、635、636	635			474	469	524
3	225、236、444、450、635、636	225、635		226	474	375、440	524
4	225、236、254、268、444、450、635、636	225、268、635、639		245、246	268、474	469	524、526
5	225、236、429、444、450、635、636、678、679	225、635、639	235	373	474	375、440、448、522	524、526、637
6	225、444、450、635、636		235			469	524、637
7	225、429、450、474、635、636、638、639	225、635		373、476	474	440、448、522	524、526、637
8	225、635、636、641	225、635		226		375	524、526

Appendix



Appendix 1. HA-Rbp1p reveal mutiple spots with the same molecular weight but different isoeletric points in 2-DE western analysis.
2-D electrophoresis of HA-Rbp1p express in YPH499Δ*rbp1* strains. Protein spots were visualized by Western blot with anti-HA monoclonal antibody.



by YIC

Appendix 2. Rbp1p is a phosphorylated protein.
2-DE of GST-Rbp1p protein treated with alkaline phosphatase. GST-Rbp1p protein with (+) or without (-)alkaline phosphatasetreatment were separated b 2-DE , followed by Western blot with anti-Rbp1p antibody.

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