이학박사학위논문

분열성 효모에서 포도당 고갈에 대응하는 전사인자 **Rsv1**의 역할과 조절

Role and Regulation of Transcription Factor Rsv1 under Glucose Starvation in *Schizosaccharomyces pombe*

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Role and Regulation of Transcription Factor Rsv1 under Glucose Starvation in *Schizosaccharomyces pombe*

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ABSTRACT

Cells encounter many stress conditions during their lifetime, and nutrient starvation is one of the main stresses that cells experience. Glucose is the preferred carbon source for most organisms, so the perception and regulation of intracellular sugar level are critical for cell survival. The absence of glucose triggers the reprogramming of cell metabolism and the adaptive mechanisms for nutrient stresses. In *Schizosaccharomyces pombe*, Rsv1 (required for stationary phase viability) was first reported to be the zinc finger transcription factor which is important for maintaining cell viability upon glucose depletion. Cells deleted with *rsv1* gene showed sensitivity to ethanol, heat, and zymolase stresses at stationary phase. Also, it was revealed that cAMP-PKA pathway is involved in the expression of *rsv1* at stationary phase. But the comprehensive mechanisms for inducing *rsv1* expression specifically under glucose starvation has not been revealed. And more importantly, the function of Rsv1 as a transcription factor including its target genes is in need to be revealed.

To investigate the regulatory mechanisms for *rsv1* expression under glucose depletion, the involvement of signaling pathways which are activated under the starvation condition was examined. MAPK pathway and PKA pathway are required for responding to glucose signal, and for reprogramming the transcription of target genes. When the *sty1* MAPK gene was deleted, the expression of *rsv1* gene was not fully induced. Deletion of *atf1* gene, encoding the main effector protein of Sty1 MAPK, also showed the decreased induction of *rsv1* gene with similar extent to the *Δsty1* mutant. These results indicate that MAPK pathway is involved in the transcriptional activation of the *rsv1* under glucose starvation. Involvement of cAMP-PKA pathway was examined by observing the phenotype of *Δcgs1* mutant, with the constitutively active Pka1. The expression of *rsv1* decreased in *Δcgs1* mutant, indicating that the PKA

pathway is involved in regulating *rsv1* expression.

To find out factors that bind to the *rsv1* gene to control transcription, representative effector proteins, such as Atf1 of MAPK pathway and Rst2 of PKA pathway, involved in glucose signaling pathways were examined. Chromatin immunoprecipitation analysis revealed that Atf1 and Rst2 became more enriched to the *rsv1* upstream region under glucose starvation, and the binding of these proteins decreased upon glucose up-shift. In addition, another zinc finger protein Scr1, which is known to recognize the same binding motif as Rst2, was found to bind to the *rsv1* upstream region under glucose-rich condition, and the binding decreased under glucose starvation. Thus, Atf1 and Rst2 seem to serve as direct activators for *rsv1* expression, whereas Scr1 seems to act as a repressor for *rsv1* expression.

To investigate the direct target genes whose transcriptions depend on Rsv1, genome-wide ChIP-seq and RNA-seq analyses were conducted. In ChIP-seq, 71 peaks of Rsv1-enriched sites were isolated, and the motif analysis revealed the presence of a consensus sequence 'CCCCNC', which is likely to be the binding motif of Rsv1. Parallel analysis of Rsv1-dependent transcriptome by RNA-seq revealed 232 genes that were differentially expressed in *Δrsv1* cells. And among them, 21 genes had the ChIP-seq peak of Rsv1, suggesting that these are the direct targets of Rsv1. Most of these genes encode proteins that are predicted to be involved in the process of carbon metabolism. Among the target genes, *gcd1* gene encoding a glucose dehydrogenase showed the most dramatic regulation by Rsv1, being repressed under glucose starvation. Rsv1 enrichment was the greatest in the *gcd1* upstream region, and the *gcd1* expression was highly derepressed in the *Δrsv1* mutant. Introduction of *Δgcd1* mutation recovered the viability defect of the *Δrsv1* cells in the stationary phase, suggesting that repressing Gcd1 is critical to maintain viability under glucose starvation. The regulation by Rsv1 of *gcd1*, *gnd1* encoding a phosphogluconate

dehydrogenase, *gut2* encoding a mitochondrial glycerol 3-phosphate dehydrogenase, and *fbp1* encoding a fructose bisphosphatase suggests the role of Rsv1 in reprogramming glucose metabolism to achieve survival by minimizing pentose phosphate pathway and activating glycerol-3-phosphate pathway for energy generation.

Key Words:

Fission yeast, Cell survival, Glucose starvation, Rsv1, MAPK pathway, Sty1, Atf1, PKA pathway, Cgs1, Rst2, Scr1, ChIP-seq, RNA-seq, Carbon metabolism, Gcd1, Gluconate shunt.

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ABBREVIATIONS

AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
cAMP	cyclic AMP
CBF	CCAAT-binding factor
cDNA	complementary DNA
CDS	coding sequence
ChIP	chromatin immunoprecipitation
Co-IP	co-immunoprecipitation
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
EGFP	enhanced green fluorescence protein
EMM	Edinburgh minimal medium
IP	immunoprecipitation
Kb	Kilo base pair
kDa	Kilo Dalton
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PKA	protein kinase A
PMSF	phenylmethylsulfonyl fluoride
MAPK	mitogen-activated protein kinase
qPCR	quantitative PCR
SAGA	Spt-Ada-Gcn5-acetyltransferase
SDS	sodium dodecyl sulfate
seq	sequencing
TDW	triple distilled water
TOR	target of rapamycin
WT	wild-type

CHAPTER I.
INTRODUCTION

I. 1. Biology of *Schizosaccharomyces pombe*

I. 1. 1. Fission yeast as a model organism

Schizosaccharomyces pombe, also called "fission yeast", is one of the important model organisms for cell biology. Fission yeast was first isolated in 1893 from East African millet beer, and developed as an experimental model in 1950s. Paul Nurse, *et al*. have received the Nobel Prize for their study on cell cycle regulation with fission yeast (Fantes and Hoffman, 2016). Fission yeast is a single cell eukaryote which has a short life cycle and can be grown easily in the lab. This single cell is also easy to manipulate genetically, and the genes of fission yeast are highly conserved in higher eukaryotes, which makes fission yeast as an important model for biomedical researches. Fission yeast has only three chromosomes, making *S. pombe* a good organism for the genetic study. Different from *S. cerevisiae*, *S. pombe* has a shelterin-like telomere complex and RNAi machinery genes like the vertebrates. And *S. pombe* also has the large, repetitive centromeres similar to the mammalian centromeres.

There is a well-established database for fission yeast, PomBase (pombase.org), which provides a hub for the researchers. In PomBase, the various data like GO analysis, phenotype, target information, protein features, gene expression information, physical interaction, genetic interaction, and genome-wide information are accessible (McDowall et al., 2014). Moreover, there is a genome-wide deletion mutant library for fission yeast (Kim et al., 2010). A diploid deletion mutant library covers about 98% of *S. pombe* genome, and 95% genome coverage is available for a haploid deletion mutant library.

I. 1. 2. Life cycle of *S. pombe*

Life cycle of *S. pombe* is described in Fig. I-1. Fission yeast can be maintained at either a diploid or a haploid, but is usually a haploid cell. But under the unfavorable conditions, usually nitrogen deficiency, the haploid cells with

opposite mating types (P and M) can conjugate to form a diploid. A diploid zygote enters meiosis to produce four haploid spores. When the environmental condition becomes favorable, the spores can germinate to generate proliferating haploid cells. The proliferating haploid cell is rod-shaped, and has a diameter of approximately 3 to 4 μm. It grows to 8~12 μm in length at G2 phase, and to 14 μm at M phase. Before the cytokinesis, the fission yeast cell is 16 μm in length (Zhu et al., 2015).

The cell cycle of *S. pombe* typically consists of G1 (gap1), S (synthesis), G2 (gap2), and M (mitotic) phases. In mitotic cell division, fission yeast cells replicate DNA during the S phase of cell cycle, and the cells divide into two cells during M phase. S phase and M phase can be separated by two gap phases, G1 phase and G2 phase. These two phases are carefully regulated to check the cell cycle, and many proteins act to regulate these checkpoints. These check points are important for repairing DNA damages or for the exact DNA segregation. During G1 phase, the cells check their external environment and their intracellular state before a new cycle. During G2 phase, the cell checks whether DNA synthesis and repair are completed, and whether the appropriate cell size is made. Besides, cells can enter the G0 phase from G1 phase checkpoint, called start point (yeast). This usually occurs in response to lack of growth factors or nutrients, and the cells stop dividing and enter the quiescent state. And when they are faced to the environment for proliferation, they re-start the cell cycle (Oliva et al., 2005).

I. 1. 3. Genome of *S. pombe*

S. pombe is the sixth eukaryote whose genome was fully sequenced as the part of the Genome Project (Wood et al., 2002). It has the genome of approximately 14 Mb, and its mitochondria has 20 Kb. *S. pombe* has only 3 chromosomes when compared with 16 chromosomes of *S. cerevisiae*. Genome is distributed to

chromosome I (5.7 Mb), II (4.6 Mb), and III (3.5 Mb). There are about 5120 protein-coding genes, 47 rRNAs, 171 tRNAs, and 1522 non-coding RNAs in fission yeast. Mitochondrial genome has 11 protein-coding genes, with 2 rRNAs and 25 tRNAs. Fission yeast genome GC content is 36.06%, with mitochondria of 30.09% GC contents.

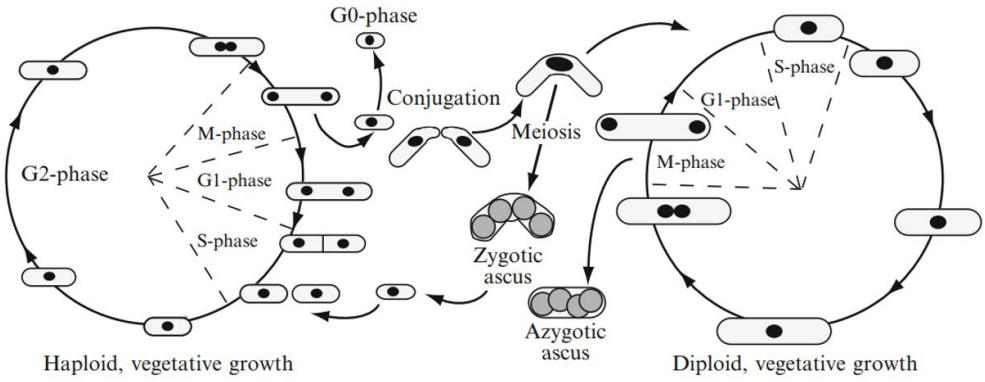


Figure I-1. Life cycle of *Schizosaccharomyces pombe*.

S. pombe cell stays mainly in a haploid state, h- or h+ according to mating type, and forms a diploid reluctantly under nitrogen-starved condition. Haploid cells are maintained at G2 phase (70%), and stay at other phases shortly; M phase (10%), G1 phase (10%), and S phase (10%). After meiosis is triggered by nutrient starvation, the zygotic ascus is formed with four spores. When environmental nutrient becomes rich, the spores can germinate to be the haploid cells for vegetative growth. (Sabatinos and Forsburg, 2010)

I. 2. Lifespan of Fission Yeast

The lifespan of *S. pombe* can be categorized to replicative lifespan (RLS) and chronological lifespan (CLS). In *S. cerevisiae*, replicative lifespan means the capacity of mother cell to reproduce daughter cells before senescence. So, replicative lifespan is defined by the number of daughter cells that mother cell can produce before death. In this replicative lifespan model, the age-associated damage is asymmetrically distributed from mother cell to daughter cell, resulting in the alleviated damages in the daughter cell (Steinkraus et al., 2008). In contrast to *S. cerevisiae*, *S. pombe* grows by linear extension and divides into two cells by medial fission. The two sibling cells are connected to each other through the old-pole tip, and the new tip grows at the site of septation. And the new growth occurs at the old-pole of the cell. As replicative lifespan assay requires to remove the daughter cell after divisions, it was difficult to measure the replicative lifespan of *S. pombe* on agar plates. Recently, the microfluidic device, the multiplexed fission yeast lifespan microdissector, makes it easy to capture the individual *S. pombe* cells (Spivey et al., 2017). Fission yeast cells continued to replicate for over 75 generations, and cells died without the aging phenotypes reported. Their deaths seemed to be random, and the chance of cell death did not increase as the cells grew older (Spivey et al., 2017).

Chronological lifespan measures the viability of cells after cells enter stationary phase or plateau phase. Cells are grown to the stationary phase where cells enter non-dividing state because of glucose depletion in media, and the ability to resume the cell cycle is observed. In *S. pombe*, two related nutrient signaling pathways, Pka1 pathway and Sck2 pathway, are involved in regulation of chronological lifespan (Roux et al., 2006). Like in *S. cerevisiae*, *S. pombe* cAMP/PKA pathway controls the chronological lifespan. And of the two homologues of *SCH9* in *S. cerevisiae*, only *sck2* gene was necessary for regulating aging, and this pathway seemed to be independent on the

cAMP/PKA pathway. The double deletion of *pka1* and *sck2* displays an additive prolonged lifespan, and this phenomenon is reported to correlate with the increased resistance to oxidative stress and heat stress (Roux et al., 2006). There is also a report of the gene which can extend the lifespan of *S. pombe*. The gene *ecl1* was identified to enhance the viability of wild-type cells and *Δsty1* cells when *ecl1* gene is overexpressed (Ohtsuka et al., 2008). And it is assumed that the effect of *ecl1* overexpression may affect to Pka1 pathway negatively.

I. 3. Rsv1 as a C_2H_2-Type Zinc Finger Protein

I. 3. 1. Isolation and fundamentals of Rsv1

Rsv1 (required for stationary phase viability) was first isolated as a multicopy suppressor of the temperature-sensitive *pat1-114* mutant (Hao et al., 1997). Rsv1 has 428 amino acids, and it contains two C_2H_2-type zinc fingers in its N-terminus. There are other zinc finger proteins which are homologous to Rsv1 protein (Fig. I-2). These proteins contain the highly conserved cysteine and histidine sequences for the zinc finger domain in their N-terminus, and they also have the conserved amino acid sequences that are important for target DNA recognition. In *S. pombe*, Rst2 and Scr1 are the homologous to Rsv1, and in *S. cerevisiae*, Mig1 is found to be homologous to Rsv1. Also, Rsv1 homologue proteins from other organisms like *Schizosaccharomyces japonicas*, *Schizosaccharomyces octosporus*, and *Candida albicans* (called cas5p) were found to be functionally homologous to Rsv1 of *S. pombe* (Pataki et al., 2017).

After being isolated, Rsv1 was reported to be important for maintaining cell viability at stationary phase (Hao et al., 1997). When cells are deleted with *rsv1* gene, cells lost their viability after entering stationary phase. Also, *Δrsv1* cells showed the increased sensitivities to ethanol stress, heat stress, and zymolase stress. The expression of *rsv1* gene increased under various starvation conditions like glucose starvation, nitrogen starvation, and phosphate

starvation. But *Δrsv1* deletion cells showed the shortened lifespan only under glucose starvation. Moreover, the induction of *rsv1* gene was found to be regulated by cAMP-Pka1 pathway. But still, the detailed mechanisms for regulation of *rsv1* expression are not investigated. Also, the function of Rsv1 as a transcription factor and the direct target genes of Rsv1 are not explored.

I. 3. 2. C_2H_2-type zinc finger proteins homologous to Rsv1

Mig1 is involved in repressing a variety of target genes for utilization of other carbon sources in *S. cerevisiae*. Mig1 functions as a repressor in association with Hxk2 protein in the nucleus, regulating target genes like SUC2, respiratory genes, and gluconeogenesis genes. Hxk2 associates with Mig1 under glucose-rich conditions and is supposed to inhibit the phosphorylation of Mig1 by Snf1. When glucose becomes scarce, Snf1-Gal83 complex enters the nucleus to phosphorylate target protein, Mig1 (Conrad et al., 2014). In fission yeast, there are two other zinc finger proteins homologous to Rsv1; Rst2 and Scr1. These three proteins are also homologous to Mig1 of budding yeast. As presented in Figure I-2, Mig1, Rsv1, Rst2, and Scr1 have the two conserved C_2H_2 zinc fingers in their N-terminus (lined boxes). Rst2 shows the longest N-terminal sequences and Rsv1 has very short N-terminal amino acid sequences. Also, they have the conserved amino acid sequences for DNA recognition (Fig. I-2, dashed boxes). For zinc finger proteins, DNA recognition codes of amino acids which can recognize the nucleotide sequences are well-established (Siggers et al., 2014). Rst2 has 'REHR' in -1, 2, 3, 6 positions for the first zinc finger, and 'RDLR' for the second zinc finger. For Rsv1, there are 'REHR' in -1, 2, 3, 6 recognition sites for the first zinc finger and 'RDER' for the second zinc finger. Scr1 and Mig1 also have 'REHR' and 'RDER' sequences like Rsv1. And these amino acids are known to recognize the same DNA sequence, 'GGGGcG'.

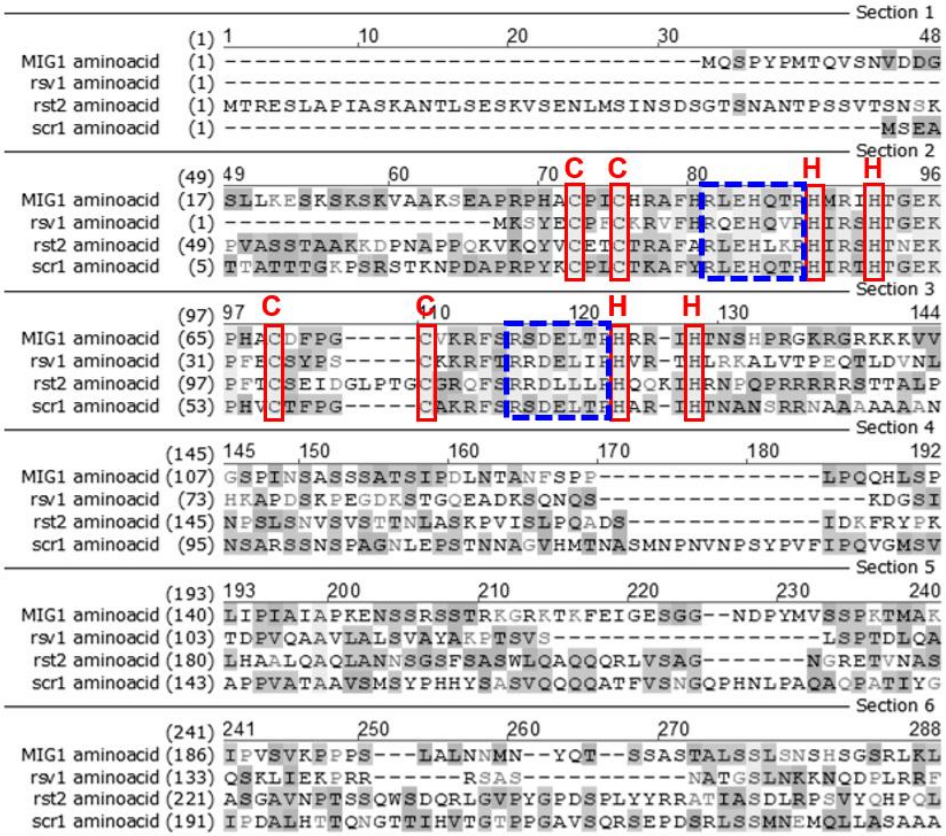


Figure I-2. Alignment of amino acid sequences of zinc finger proteins.

Parts of amino acid sequences from *MIG1* gene of *S. cerevisiae* and *rsv1*, *rst2*, and *scr1* genes of *S. pombe* were aligned. Lined boxes mean the conserved cysteine and histidine amino acids of the zinc finger proteins. And dashed boxes indicate the conserved sequences which are involved in recognition of DNA substrate ('REHR' for the first zinc finger and 'RD(E/L)R' for the second zinc finger).

I. 4. Glucose Repression and Derepression

I. 4. 1. Glucose repression

Cells can modulate transcription of many genes in response to environmental signals. Especially, when glucose is available, cells turn off the transcription of genes required for utilization of other carbon sources. This phenomenon is called glucose repression or catabolite repression (Magasanik, 1961). In fission yeast, the *fbp1* gene, encoding fructose-1,6-bisphosphatase, is an important target of glucose repression (Hoffman and Winston, 1990). The *fbp1* is constitutively expressed when *git2* (*cyr1*) encoding adenylate cyclase is mutated. And the addition of cAMP suppresses the transcriptional defect of *git2* mutant cells, repressing the transcription of *fbp1* gene (Hoffman and Winston, 1991). So, glucose can trigger activation of adenylated cyclase, resulting in the activation of PKA (protein kinase A) to repress *fbp1* transcription. Other signaling pathways are also important for the transcriptional regulation of *fbp1*. MAPK pathway can be stimulated by glucose starvation, leading to derepression of *fbp1* gene (Stettler et al., 1996). There is a cis-acting element of *fbp1* gene, UAS1, which is the binding site of Atf1-Pcr1 heterodimer, and MAPK pathway is important for activation of this heterodimer. Binding of these proteins is stimulated by glucose starvation and is dependent on Sty1-MAPK pathway (Neely and Hoffman, 2000).

I. 4. 2. Glucose derepression

Glucose derepression occurs under glucose-starved condition, leading to the transcriptional changes of cells for utilization of other carbon sources. In *S. cerevisiae*, Snf1 protein kinase-dependent signaling pathway plays an important role for glucose derepression of many genes. When cells are deprived of glucose, the Snf1 kinase becomes activated by upstream kinases (Tos3, Sak1, and Elm1), and imported into the nucleus to phosphorylate the

target proteins like Mig1. Phosphorylated Mig1 is dissociated from the *SUC2* gene promoter, resulting in derepression of the gene (Santangelo, 2006). In *S. pombe*, Ssp2 kinase plays the same role as Snf1 kinase. Ssp2 can phosphorylate Scr1 protein, leading to re-localization of the protein out of the nucleus. As Scr1 represses the transcription of *inv1* (encoding invertase) and *gld1* (encoding glycerol dehydrogenase) under glucose-rich condition, the movement of Scr1 to the cytosol results in the derepression of these genes (Matsuzawa et al., 2012). Also, it was revealed that Rst2 and Scr1 can regulate the target genes by reciprocal nuclear shuttling (Hirota et al., 2006). Under glucose-starved condition, where Scr1 localizes to the cytosol, Rst2 can be imported to the nucleus and occupy the UAS2 region of *fbp1*, which is formally occupied with Scr1, activating the transcription of *fbp1*.

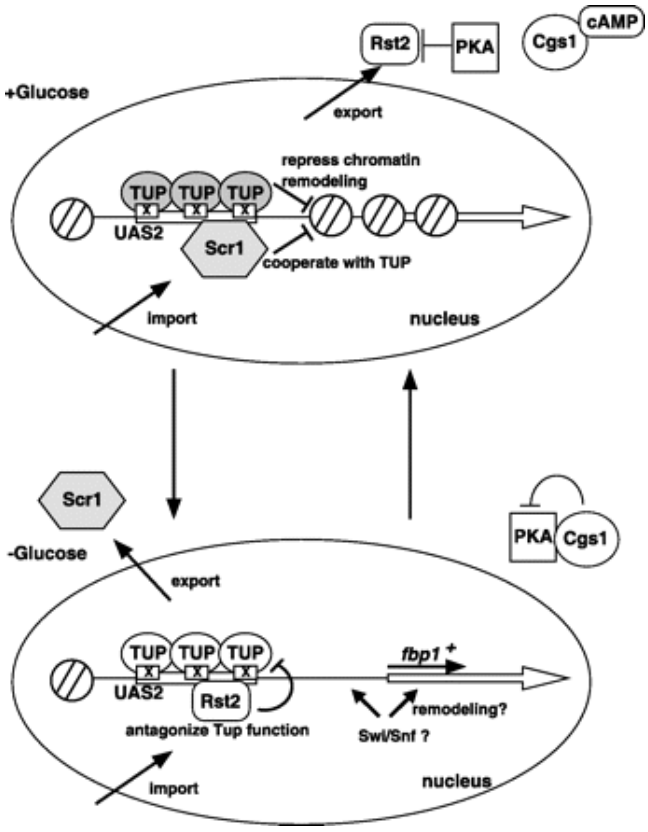


Figure I-3. Proteins involved in glucose repression and derepression.

In fission yeast, Scr1 and Rst2 play reciprocally to regulate the expression of *fbp1* gene. Under glucose-rich condition, Scr1 resides in the nucleus to repress the *fbp1* gene, while it is exported to the cytosol when glucose becomes depleted. In contrast, Rst2 exists in the nucleus upon glucose starvation, activating the transcription of *fbp1* gene (Hirota et al., 2006).

I. 5. Nutrient Signaling Pathways

I. 5. 1. PKA pathway

cAMP-Pka1 pathway is the main pathway for sensing glucose and nitrogen in *S. pombe*. Both in *S. cerevisiae* and *S. pombe*, sensing of glucose results in the rise of cAMP levels, activating cAMP-dependent protein kinase A (PKA) (Byrne and Hoffman, 1993). For glucose detection, many factors are necessary; a seven-transmembrane GPCR (G-protein coupled receptor) Git3 and a heterotrimeric G-protein (Gpa2 Gα, Git5 Gβ and the Git11 Gγ) (Hoffman, 2005). Git3 is important for glucose repression of *fbp1* gene, and Git3 receptor is activated by glucose and transduces the glucose signal to Gpa2. Gpa2 in turn activates Git2 (Cyr1) adenylyl cyclase. So when glucose level is high, cAMP is produced, and releases the Pka1 kinase from Cgs1 regulatory subunit, leading to translocation of Pka1 to the nucleus. As a result, the transcriptions of *ste11* for regulation of mating genes and *fbp1* for gluconeogenesis are repressed.

I. 5. 2. MAPK pathway

In fission yeast, the Sty1 MAP kinase plays an important role in responding to diverse environmental stresses for cell survival. Upon stresses, Sty1 is phosphorylated by MAPKK and accumulates in the nucleus, facilitating the transcription of target genes partially dependent on Atf1 (Shiozaki and Russell, 1996; Wilkinson et al., 1996). Atf1 is phosphorylated directly by Sty1, which is caused by Sty1 activation by stress conditions. Atf1 is a basic leucine zipper (bZIP) transcription factor which can form a heterodimer with another bZIP protein Pcr1. Although their function as a heterodimer is well-studied, they also have the distinct roles in regulating target genes (Lawrence et al., 2007; Sansó et al., 2008). There are 11 putative (S/T)P MAPK phosphorylation sites in Atf1, and 6 residues (S152, S172, T204, T216, S226, and T249) are essential for the activity of Atf1. And 5 residues out of the 6 residues are located in the trans-

activation domain (Salat-Canela et al., 2017). The role of Atf1 phosphorylation by Sty1 can be explained with two different modes (Salat-Canela et al., 2017; Sánchez-Mir et al., 2017). In the first case, non-phosphorylated Atf1 resides in the promoters of genes like *gpd1* and *hsp9* prior to stress. When Sty1 is activated by oxidative stress, pre-bound Atf1 can be phosphorylated by Sty1, which promotes the recruitment of the transcriptional machinery. In the second case, non-phosphorylated Atf1 of basal level is bound to the target gene promoters like *ctt1* and *srx1*. Especially, for this case, another transcription factor Pap1 is oxidized and located to AP-1 sites of gene promoters in an Atf1-independent manner under stress condition. As a result, more Atf1 proteins can be recruited to the CRE site, and phosphorylated Atf1 by Sty1 affects the recruitment of transcriptional machinery.

I. 5. 3. AMPK pathway

AMPK (the serine/threonine AMP-activated protein kinase) is a major pathway for sensing and regulating the intracellular energy. AMPK is a heterotrimeric protein kinase complex, which is composed of α, β, and γ subunits; α is a catalytic subunit Ssp2, βγ are regulatory subunits Amk2 and Cbs2 (Townley and Shapiro, 2007). The binding site of ATP or ADP/AMP resides in the γ subunit. When cellular ATP level is high, γ subunit binds to ATP resulting in the inactive AMPK. But in low ATP level, γ subunit binds to AMP contributing structural change of the heterotrimer, which can induce the phosphorylation of the catalytic subunit (Rubenstein et al., 2008). Therefore, AMPK can sense the energy status of a cell by detecting the ratio of ATP and AMP/ADP.

In fission yeast, Ssp2 kinase is responsible for glucose derepression process, like assimilation of sucrose and glycerol (Matsuzawa et al., 2012). And interestingly, Scr1, a homologous protein of Rsv1, was shown to be the direct

target of Ssp2. Under glucose-rich condition, Scr1 localizes in the nucleus, while Scr1 localizes in the cytosol when glucose is scarce. But when *ssp2* gene is deleted, Scr1 resides in the nucleus even under glucose-starved condition. And Ssp2 is responsible for phosphorylation of Scr1 under glucose-starved condition (Matsuzawa et al., 2012). Therefore, Ssp2 plays an important role for regulating the phosphorylation status and the localization of Scr1 in response to glucose, which is necessary for derepression of genes like *inv1*, *fbp1*, and *gld1* under glucose deprivation.

I. 5. 4. TOR pathway

TOR (target of rapamycin) is a highly conserved serine/threonine kinase which plays a critical role for regulating cell growth in response to environmental changes. TOR plays an important role in coupling nutrients to cell growth, integrating extracellular and intracellular signals from nutrients, growth factors, and various stresses. Also, TOR is involved in autophagy, endoplasmic reticulum stress, and metabolism (Kapahi et al., 2010). It has been identified that two TOR complexes, TORC1 and TORC2, exist in both mammalian cells and yeast cells. In fission yeast, there are two different TOR kinases; Tor1 and Tor2. Tor1 kinase is involved in TORC2, and Tor2 kinase in TORC1. Fission yeast TORC1 contains Mip1, Wat1/Pop3, Tco89, Toc1, and Tor2, while TORC2 contains Ste20, Wat1/Pop3, Sin1, Bit61, and Tor1 (Otsubo and Yamamato, 2008).

In *S. pombe*, Tor1 is required for the growth under stress conditions and for amino acid uptakes. When cells are deleted with *tor1*, cells cannot arrest in G1 phase under nutrient-starved condition, and cells show the sterile phenotype. They had an abnormally elongated morphology, and showed high sensitivity to various stresses like high/low temperature, osmotic stress, pH stress, and oxidative stress (Kawai et al., 2001; Weisman and Choder, 2001). In contrast to

Tor1, Tor2 shows lethality when deleted, and is essential for vegetative cell growth. So Tor2 is supposed to regulate cell growth positively. Tor2 is also shown to negatively regulate G1 arrest, sexual development, and amino acid uptake (Kawai et al., 2001).

The AGC-family (protein kinase A, G, C family) kinase Gad8 is the central downstream target of TORC2 in fission yeast, and cells deleted with *gad8* gene phenocopy the TORC2-defective cells (Ikeda et al., 2008). Gad8 is phosphorylated by TORC2 to have the enhanced kinase activity critical for its functions. And TORC2 has been found to be regulated by upstream Rab small GTPase Ryh1 (Tatebe et al., 2010). The active Ryh1, the GTP-bound form, can interact with TORC2 and promote the phosphorylation of Gad8 by TORC2. It was reported that TORC2 can response to glucose, and TORC2-Gad8 is essential for cell growth under glucose starved condition (Hatano et al., 2015). Under glucose-rich condition, Ryh1 can induce TORC2-dependent phosphorylation of Gad8. But during glucose starvation, Ryh1 becomes inactive, resulting in inactive TORC2-Gad8. But Gad8 phosphorylation was found to gradually recover independently of Ryh1, implying that there is an additional pathway for activation of TORC2 (Hatano et al., 2015). Meanwhile, there was a report that TOR2-Gad8 pathway and cell integrity MAPK pathway can cross-talk to adapt to the stress conditions like salt stress, cell wall stress, and glucose starvation (Madrid et al., 2016).

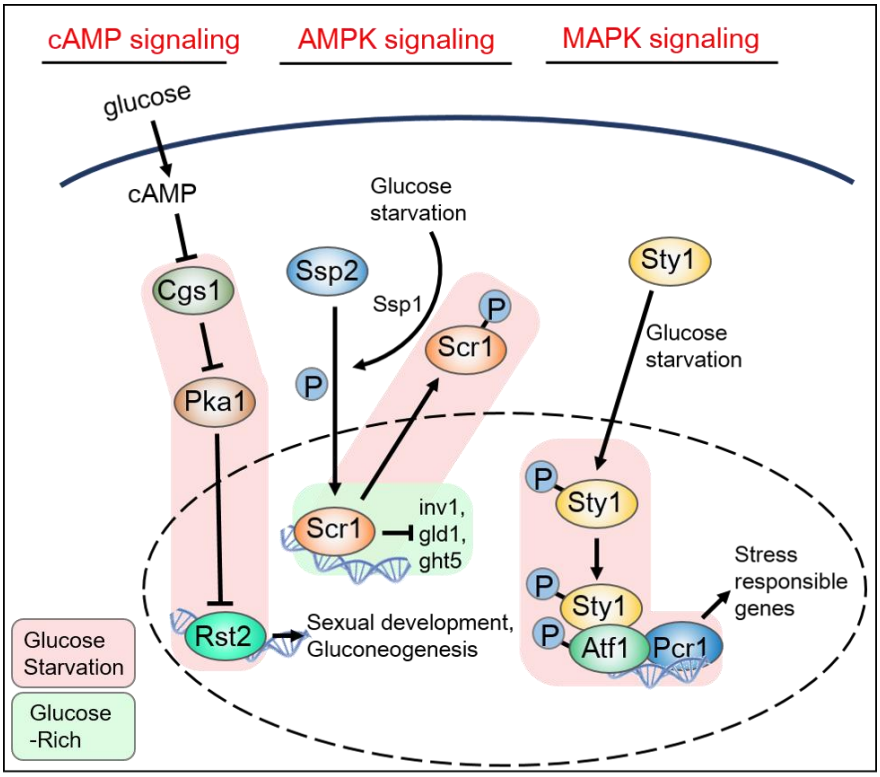


Figure I-4. Signaling pathways sensing glucose starvation.

Three representative pathways which are important for responding to glucose signal and regulating downstream target genes are depicted; PKA pathway, AMPK pathway, and MAPK pathway. The pathway activated upon glucose starvation is shaded with red, and the pathway activated under glucose-rich condition is shaded with green.

I. 6. Regulation of Gene Expression

I. 6. 1. Tup11 and Tup12 corepressors

Corepressors play an important role in regulation of gene expression. They are recruited to specific genes by binding to transcription factors that can respond to various stimuli. In *Saccharomyces cerevisiae*, Ssn6-Tup1 corepressor is responsible for modulating diverse cellular processes like glucose repression, stress responses, and DNA damage repairs (Nehlin et al., 1991; Proft and Struhl, 2002; Wahi et al., 1998). It has been suggested that the repressive activity comes from Tup1 protein, and Ssn6 may be necessary for interaction between Tup1 and DNA-bound proteins (Fagerström-Billai et al., 2007; Tzamarias and Struhl, 1994; Zhang and Reese, 2004). Tup1 can repress gene expression via distinct mechanisms. Tup1 has been proposed to have genetic interactions as well as physical interactions with the components of RNA polymerase II holoenzyme (Gromöller and Lehming, 2000; Lee et al., 2000; Papamichos-Chronakis et al., 2000), and to cover the activation domain of transcription activator (Wong and Struhl, 2011). Also, Tup1 can modulate the chromatin organization through histone acetylation and nucleosome positioning (Chen et al., 2012; Cooper et al., 1994). Ssn6-Tup1 complex binds physically to class I histone deacetylases (HDACs), like Rpd3 and Hos2 (Watson et al., 2000). Recruited HDACs by interaction with Ssn6-Tup1 induce the acetylation of the histone tails, which further facilitates the interaction between Ssn6-Tup1 complex and the histones (Davie et al., 2002). So chromatin structure reorganization occurs, and the interaction between Ssn6-Tup1 complex and DNA becomes more stable.

Meanwhile, there are several reports that Ssn6-Tup1 complex remains to target regions even under derepression condition (Mennella et al., 2003; Papamichos-Chronakis et al., 2002; Proft and Struhl, 2002), which contributes to recruitment of the SAGA and SWI/SNF complexes. It has also been proposed that Ssn6-Tup1 blocks the activation domain of DNA-binding

protein under repressing condition. And upon derepressing condition, the DNA-binding protein is modified, leading to the change of interaction with Ssn6-Tup1 complex. As a result, the activation domain becomes exposed to recruit the SAGA and SWI/SNF complexes (Wong and Struhl, 2011).

In *S. pombe*, there are two Tup1-like proteins, Tup11 and Tup12. It has been shown that Tup11/12 proteins are also involved in regulation of transcription, repressing chromatin remodeling (Hirota et al., 2003). In fission yeast, the mechanisms by which Tup11/12 proteins control the transcription of *fbp1* gene are well studied (Asada et al., 2014; Hirota et al., 2006; Hirota et al., 2003; Janoo et al., 2001).

I. 6. 2. CCAAT-binding factors

In budding yeast, 'CCAAT' sequence is sensed by HAP complex, which consists of Hap2/Hap3/Hap5 proteins with DNA-binding capacity. And the fourth subunit Hap4 acts as an activator through its activation domain (Forsburg and Guarente, 1989; McNabb et al., 1995). Yeast *HAP4* gene transcription is repressed in glucose-rich media, while it is derepressed in non-fermentable carbon source media. And this *HAP4* expression mainly regulates the transcriptional activation capacity of HAP complex (Forsburg and Guarente, 1989). In fission yeast, Php2/Php3/Php4/Php5 (CBP complex) have the conserved short regions with budding yeast counterparts, and they can also recognize the 'CCAAT' motif (Mcnabb et al., 1997; Mercier et al., 2006).

I. 6. 3. Activators, corepressors, and CBF: Lessons from *fbp1* gene

As eukaryotic chromosome is highly organized structure composed of DNA tightly condensed with histones, modulation of chromatin structure is important for the transcriptional processes. The *fbp1* gene encodes fructose-1,6-bisphosphatase, and its transcription is tightly regulated by Atf1, Rst2,

Tup11/12, and Php5 (Fig. I-5) (Asada et al., 2014; Hirota et al., 2006; Hirota et al., 2003; Janoo et al., 2001). Under glucose-rich condition, Tup11/12 proteins are bound to DNA inhibiting the activator recruitment. And when glucose becomes depleted, Atf1 is recruited to UAS1 (upstream activation sequence 1) region, suppressing the repression of Tup corepressors. CBF can bind to DNA, further reorganizing chromatin structure around TATA box. So, around TATA box, Rst2 can suppress the Tup11/12 corepressors to enhance transcription of *fbp1*, presumably by stabilizing the transcriptional machinery.

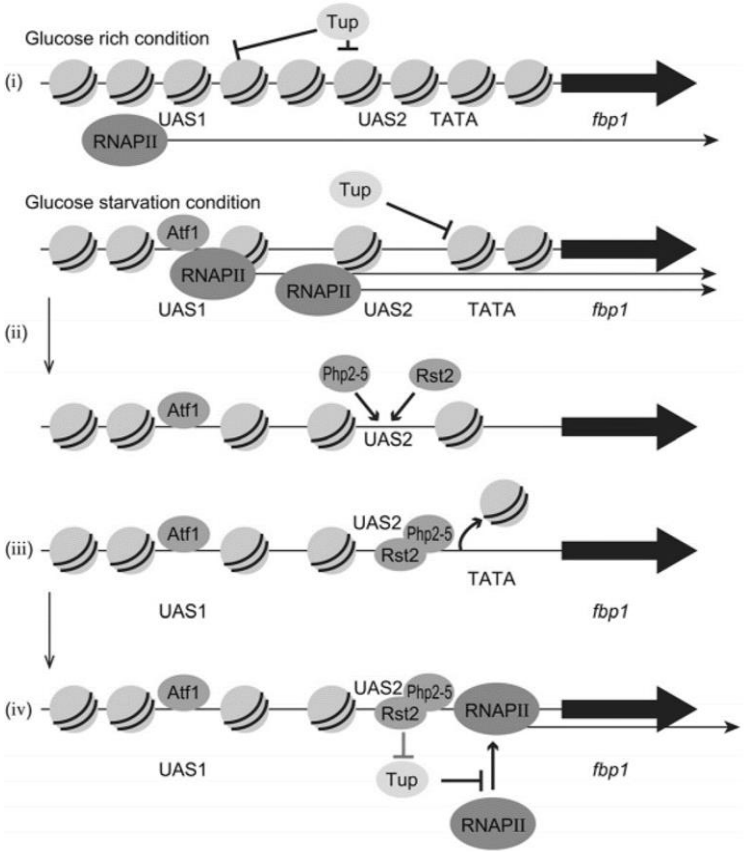


Figure I-5. Target gene regulation of Atf1 and Rst2 upon starvation.

Atf1 and Rst2 are involved in activation of *fbp1* gene under glucose starvation. Atf1 can bind to UAS1 region and Rst2 to UAS2 region, respectively. By binding to UAS regions, Atf1 and Rst2 can suppress the Tup11/12 protein, leading to activation of transcription of *fbp1* gene (Asada et al., 2014).

I. 7. Gluconate Shunt

Glycolysis is the process of degrading glucose for generation of energy, and there are several pathways of glycolysis like EMP (Embden–Meyerhof–Parnas) pathway, ED (Entner-Doudoroff) pathway, PPP (pentose phosphate pathway), and phosphoketolase pathway (Flamholz et al., 2013). The general scheme of degrading glucose is quite similar in EMP and ED pathways; glucose is phosphorylated, and broken down to three-carbon units to make ATP. PPP is also an important pathway which is conserved in all domains of organisms. PPP can be divided into two different phases; oxidative phase and non-oxidative phase. In non-reversible oxidative phase, NADP+ is reduced to NADPH while converting glucose-6-phosphate to ribulose-5-phosphate. In non-oxidative phase, which is a reversible branch, pentose phosphates are connected to the intermediates of glycolysis (Wamelink et al., 2008).

Gluconate shunt is a less-studied metabolic pathway which can degrade glucose like glycolysis pathway and pentose phosphate pathway (Fig. I-6) (Peekhaus and Conway, 1998). In gluconate shunt, glucose is oxidized to gluconate by a glucose dehydrogenase, and the gluconate is phosphorylated to make 6-phosphogluconate by a gluconate kinase. As the 6-phosphogluconate is also the intermediate of ED pathway, the role of gluconate shunt is established in plants, algae, and some bacteria, which have the ED pathway for degrading glucose or gluconate (Chen et al., 2016). Although ED pathway is not investigated in other organisms, the activities of a glucose dehydrogenase and a gluconate kinase were detected in mammals, flies, and fission yeast, who lack the key enzymes of ED pathway (Corkins et al., 2017). Besides the intermediates of ED pathway, gluconate shunt also provides the intermediates of PPP. In gluconate shunt, 6-phosphogluconate is generated and directed to PPP as the second intermediate. And like this, 6-phosphogluconate can be introduced to PPP bypassing the rate limiting enzyme G6PD (glucose-6-

phosphate dehydrogenase). Recently in fission yeast, there was a report of isolation of the glucose dehydrogenase, named Gcd1 (Corkins et al., 2017). Gcd1 was regarded as G6PD at first, but it was revealed that Gcd1 has the activity of glucose dehydrogenase, rather than G6PD activity. The identification of glucose dehydrogenase provides the possibility of the influential role of gluconate shunt in *S. pombe*. Therefore, it would be interesting to find out the undefined role of gluconate metabolism in fission yeast.

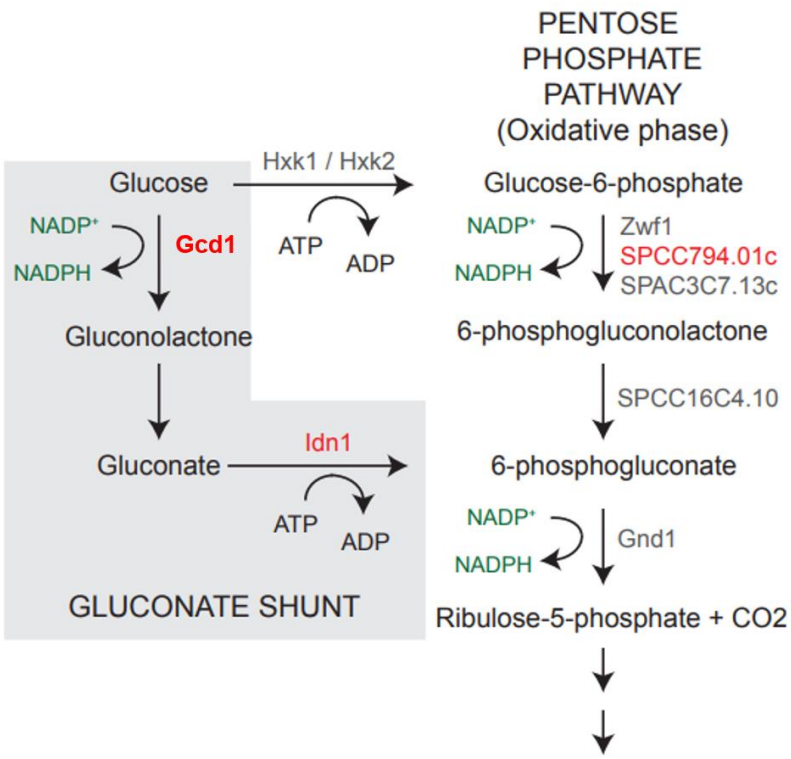


Figure I-6. Pentose phosphate pathway and gluconate shunt in *S. pombe*. Gluconate shunt is another pathway to generate NADPH and 6-phosphogluconate, consuming ATP. This pathway bypasses hexokinases (Hxk1/2) which are critical to form glucose-6-phosphate in PPP. In gluconate shunt pathway, glucose dehydrogenase (Gcd1) and glucokinase (Idn1) are necessary for producing 6-phosphogluconate, the common product of PPP. (Corkins et al., 2017)

I. 8. Zinc Homeostasis

Zinc is an essential metal for structural and functional activities of many proteins. Zinc can stabilize the structures of zinc finger-containing transcription factors. Also, zinc is a cofactor for many enzymes like alcohol dehydrogenase, RNA polymerase, and cytochrome c oxidase (Coleman, 1992). Although zinc is essential for many proteins, excess zinc can be deleterious to cells. As for the essential and toxic nature of zinc, the zinc concentration must be tightly regulated through modulating the uptake, efflux, and storage of zinc.

There are several zinc transporters required for delivering zinc out of or into the cytosol. In eukaryotes, zinc transporters can be categorized to two different families, Zrt/Irt-like protein (ZIP) family or cation diffusion facilitator (CDF) family. Usually, ZIP family facilitates the zinc uptake or the release of zinc from cellular reservoirs, leading to the increase of the cytosol zinc level. Meanwhile, CDF family proteins release zinc out of the cytosol or transport zinc into the lumens of intracellular compartments (Gaither and Eide, 2001; Kambe et al., 2015). In *S. pombe*, Zrt1 is a zinc uptake transporter, whose expression is elevated in response to zinc deprivation. And Fet4 protein is also identified to be involved in zinc uptake together with Zrt1 (Dainty et al., 2008; Schothorst et al., 2017). For CDF family, Zhf1 can transport zinc into the endoplasmic reticulum, and Cis4 and Zrg17 form a heterodimer to transport zinc into the cis-Golgi (Choi et al., 2018). It is revealed that Zhf1 preferentially transports zinc out of the cytosol when labile zinc is present, and that Cis4 and Zrg17 can transport zinc out of the cytosol under zinc-limiting condition. In *S. pombe*, Loz1 plays an important role in maintaining zinc homeostasis (Corkins et al., 2013). Loz1 is a double C_2H_2-type zinc finger protein, and plays as a transcriptional repressor of the target genes in zinc-replete condition. When *loz1* is mutated, the expressions of *zrt1*, *adh4*, SPBC1348.06c, and *adh1AS* increased even under zinc-replete condition, and cells hyper-accumulated zinc.

CHAPTER II.
MATERIALS and METHODS

II. 1. Strains and Culture Conditions

II.1.1. Fission yeast strains, media, and culture conditions

Fission yeast strains used in this study are listed in Table II-1. Prototrophic strains were usually used for experiments, and auxotrophic strains were also maintained depending on the purpose. General growth of fission yeast cells was carried out as previously described (Alfa and Laboratory, 1993; Moreno et al., 1991). The standard YE rich medium (0.5% yeast extract (Bacto™) and 3% glucose (Daejung)) was used to culture fission yeast cells at 30°C. For glucose-starved culture, cells were collected to be resuspended in YED medium (0.5% yeast extract and 0.1% glucose). EMM medium (0.3% potassium hydrogen phthalate (Sigma), 0.56% $Na_2HPO_4 \cdot 12H_2O$ (Junsei), 0.5% NH_4Cl (Daejung), 2% glucose, 20 ml/L salts stock (50 x), 1 ml/L vitamins stock (1,000 x), and 0.1 ml/L minerals stock (10,000 x)) and YES medium were used for auxotrophic strains with appropriate supplements of leucine, adenine, and uracil (concentration of 250 mg/L).

For conjugation, haploid cells of opposite mating types were mixed in vitamins stock solution, and plated on SPAS solid medium (1% glucose, KH_2PO_4 0.4 g/L, 2% agar and three of supplements). And for selection of mutant cells, cells were grown to make colonies in complex solid medium with appropriate antibiotics like G418, nourseothricin, and hygromycin, according to the marker cassette.

II.1.2. Bacterial strain and culture conditions

Escherichia coli DH5α strain was used as the host cell for manipulation and expression of recombinant DNA. *E. coli* was cultured in LB liquid medium (0.5% yeast extract (Acumedia), 1% NaCl (Daejung), and 1% tryptone (Acumedia)) at 37°C. For selection of *E. coli* having recombinant DNA, cells were plated on LB solid medium (LB plus 2% agar (Daejung)) supplemented with ampicillin.

Table II-1. Fission yeast strains used in this study.

Strain	Genotype	Source
ED665	*h-, ade6-M210, leu1-32, ura4-D18*	Lab stock
972	*h-*, wild type	Lab stock
975	*h+*, wild type	Lab stock
JH42	*h-, ade6-M210, ura4-D18*	This study
	h-, ade6-M210, leu1-32, ura4-D18, Δrsv1::kanMX	This study
	h-, Δatf1::hphMX	This study
	h-, Δcgs1::natMX	This study
	h-, Δgcd1::kanMX	This study
	h-, Δloz1:: kanMX	This study
	h-, Δrst2::kanMX	This study
	h-, Δrsv1::hphMX	This study
	h-, Δrsv1::kanMX	This study
	h-, Δrsv1::natMX	This study
	h-, Δsty1::kanMX	This study
	h-, Δtup11:: kanMX	This study
	h-, Δtup12:: kanMX	This study
	h-, Δatf1::hphMX, Δcgs1::natMX	This study
	h-, Δrsv1::hphMX, Δgcd1::kanMX	This study
	h-, Δrsv1::hphMX, Δloz1:: kanMX	This study
	h-, ade6-M210, leu1-32, ura4-D18, EGFP-rsv1::leu1	This study
	h-, atf1-5flag::hphMX	This study
	h-, cpy1-GFP::kanMX	This study
	h-, pcr1-5flag::hphMX	This study
	h-, php2-5flag::hphMX	This study
	h-, rst2-5flag::hphMX	This study
	h-, rsv1-13myc::natMX	This study
	h-, rsv1-5flag::hphMX	This study
	h-, scr1-5flag::hphMX	This study
	h+, ssp2-3HA::kanMX	This study
	h-, sty1-13myc::natMX	This study
	h-, tup11-5flag::hphMX	This study

h-, tup12-5flag::hphMX	This study
h-, atf1-5flag::hphMX, Δrst2::kanMX	This study
h-, cpy1-GFP::kanMX, Δrsv1::natMX	This study
h-, rst2-5flag::hphMX, Δatf1::natMX	This study
h-, rsv1-5flag::hphMX, Δatf1::natMX	This study
h-, rsv1-5flag::hphMX, Δcgs1::natMX	This study
h-, rsv1-5flag::hphMX, Δphp3::kanMX	This study
h-, rsv1-5flag::hphMX, Δphp5::kanMX	This study
h-, rsv1-5flag::hphMX, Δrst2::kanMX	This study
h-, rsv1-5flag::hphMX, Δsty1::kanMX	This study
h-, rsv1-5flag::hphMX, gad8-13myc::natMX	This study
h-, rsv1-5flag::hphMX, sty1-13myc::natMX	This study
h-, rsv1-13myc::natMX, tup11-5flag::hphMX	This study
h-, rsv1-13myc::natMX, tup12-5flag::hphMX	This study

II. 2. Bacterial Transformation and Yeast Transformation

For transformation of competent *E. coli* DH5α cells with recombinant plasmid DNA, heat shock method was used. Cells were mixed with plasmid DNA on ice, and placed in 42°C water bath for 1 min 30 sec. Heat-shocked cells were put on ice, and plated onto LB solid medium with ampicillin.

Fission yeast competent cells were prepared at OD_{600} 0.7 in YE (with supplements if necessary) complex media. Collected cells were kept on ice, and washed with distilled water and 1.2 M sorbitol. After resuspended in 150 μl 1.2 M sorbitol, cells were mixed with recombinant DNA (circular or linear form) and moved to cuvette (MicroPulser™, Bio-Rad) for electroporation. Electroporation was performed with the operation option for fission yeast (MicroPulser electroporator, Bio-Rad), and cells were regenerated overnight in 6 ml of 1/2 water-diluted YE complex media at 30°C. Regenerated cells were plated onto solid YE complex media with appropriate antibiotics.

II. 3. Construction of Recombinant DNA

II. 3. 1. Recombinant DNA construction with site-directed mutagenesis

For construction of fission yeast mutant strains, linear DNA which has the overlap sequences with the targeted regions was prepared. Recombinant DNA was constructed using plasmids containing the resistance gene cassette for selection. Plasmids and procedures used for making recombinant DNA are presented in Table II-2 and Figure II-1, respectively.

To delete the specific gene locus, vector carrying the adequate marker cassette like *kanMX*, *natMX*, and *hphMX* was selected. As the specific gene locus should be substituted to the maker cassette, the promoter site of the gene was inserted just before the cassette and the terminator site was inserted just after the cassette. The promoter site and the terminator site were inserted to the plasmid

one by one. To construct the mutant expressing epitope-tagged protein, the plasmid with the adequate epitope and resistance maker cassette was prepared for cloning. For epitope insertion to C-terminus of the targeted gene, the C-terminal region of the gene was introduced right before epitope sequence, and the terminator region was inserted after the cassette sequence. The ORF C-terminal region and the terminator region were introduced to the vector one by one. Usually, the plasmid was cut with Xma1 restriction enzyme for insertion of the C-terminus of the gene ORF, and Cla1 was used for insertion of the terminator region of the gene.

II. 3. 2. Construction of cells expressing EGFP-Rsv1 protein

To construct the cells that express EGFP-Rsv1 under *rsv1* gene promoter, pJK148 vector with *leu1* site was used. Promoter region of *rsv1* gene (1.5 Kb), EGFP DNA sequences, *rsv1* ORF, and *rsv1* terminator region (500 bp) were inserted into pJK148 vector. The recombinant plasmid was cut with Tth111I restriction enzyme (New England Biolabs) at 65°C, and transformed into ED665 wild-type cells and *Δrsv1* deletion (ED665 type) cells.

II. 3. 3. DNA manipulation

The chromosomal DNA of fission yeast cells was prepared with the extraction buffer (0.5 M NaCl, 0.2 M Tris-Cl (pH 7.6), 0.01 M EDTA, 1% SDS). After the cells were resuspended with 200 µl of extraction buffer and 200 µl of phenol, cells were vortexed vigorously. The supernatant was obtained after the centrifugation, and genomic DNA was precipitated with 2 volume of 100% ethanol. DNA pellet was washed with 70% ethanol, and dissolved in TDW. The extraction of *E. coli* plasmid DNA was done with Labopass™ Plasmid Mini kit according to the manufacturer's instructions.

The restriction enzymes were used for digestion of DNA according to the

manufacturer's recommendation (New England Biolabs). For ligation of DNA fragments, T4 ligase with rapid ligation buffer was used (Thermo Fisher Scientific). For another way of ligation of DNA fragments, Gibson Assembly kit was also used according to the manufacturer's instructions (New England Biolabs). For using Gibson Assembly, adequate primers were designed for obtaining DNA fragments.

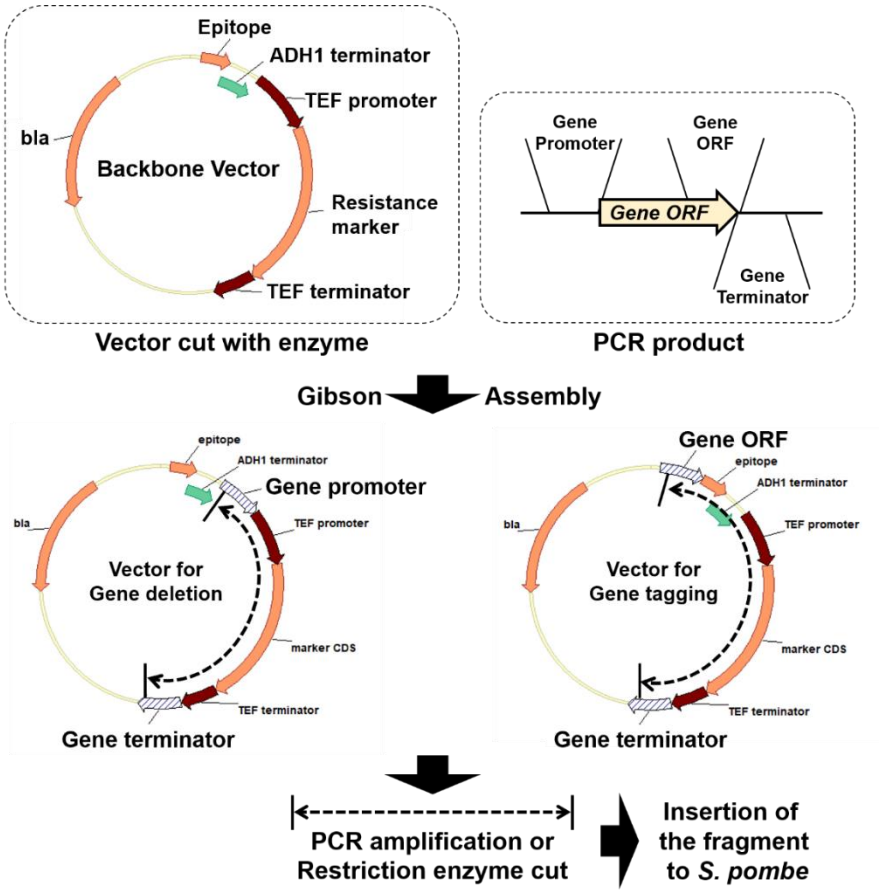


Figure II-1. Construction of recombinant plasmids for mutant strains.
Description of the procedures for construction of recombinant plasmids. For deleting the specific gene ORF, the promoter and terminator sites of the gene were inserted to the both ends of the marker cassette. And for epitope tagging, the gene ORF sequence was introduced to the front of the marker cassette gene, and the terminator sequence was inserted to the end of the cassette sequence. The constructed DNA fragments were obtained with the adequate restriction enzyme cut or PCR method, and inserted to fission yeast competent cell.

Table II-2. Plasmids used in this study.

Name	Description	Source
pFA6a-5flag-hph	Backbone vector	Lab stock
pFA6a-13myc-nat	Backbone vector	Lab stock
pFA6a-GFP-kan	Backbone vector	Lab stock
pFA6a-3HA-kan	Backbone vector	Lab stock
pJK148	Backbone vector	Lab stock
pREP42	Backbone vector	Lab stock
pFA6a-kan-P81nmt-GST	Backbone vector	Lab stock
** P; promoter sequence (partial), O; end of ORF (partial), T; terminator sequence (partial)*		
pJK148-P*rsv1*-EGFP-*rsv1*-T*rsv1*	Epitope tagging	This study
pFA6a-O*scr1*-5flag-hph-T*scr1*	Epitope tagging	This study
pFA6a-O*pcr1*-5flag-hph-T*pcr1*	Epitope tagging	This study
pFA6a-O*atf1*-5flag-hph-T*atf1*	Epitope tagging	This study
pFA6a-O*sty1*-13myc-nat-T*sty1*	Epitope tagging	This study
pFA6a-O*rsv1*-13myc-nat-T*rsv1*	Epitope tagging	This study
pFA6a-O*rsv1*-5flag-php-T*rsv1*	Epitope tagging	This study
pFA6a-O*ssp2*-3HA-kan-T*ssp2*	Epitope tagging	This study
pFA6a-O*php2*-5flag-hph-T*php2*	Epitope tagging	This study
pFA6a-O*rst2*-5flag-hph-T*rst2*	Epitope tagging	This study
pFA6a-O*gad8*-13myc-nat-T*gad8*	Epitope tagging	This study
pFA6a-O*tup11*-5flag-hph-T*tup11*	Epitope tagging	This study
pFA6a-O*tup12*-5flag-hph-T*tup12*	Epitope tagging	This study
pFA6a-13myc-P*atf1*-nat-T*atf1*	Gene deletion	This study
pFA6a-13myc-P*cgs1*-nat-T*cgs1*	Gene deletion	This study
pFA6a-13myc-P*sty1*-nat-T*sty1*	Gene deletion	This study
pFA6a-5flag-P*rsv1*-hph-T*rsv1*	Gene deletion	This study
pFA6a-P*rst2*-kan-T*rst2*-P81nmt-GST	Gene deletion	This study

II. 4. RNA Preparation and Analysis

II. 4. 1. RNA extraction

General hot phenol method was used for RNA extraction. Cells were centrifuged at 5000 rpm, and washed with TDW to collect cell pellet. Cell pellet was resuspended in 360 µl of AE buffer (50 mM NaOAC pH 5.2, 10 mM EDTA) with 40 µl of 10% SDS at 4°C. Equal amount of phenol:chloroform 5:1 (P1944, Sigma) was added and vortexed thoroughly. Samples were incubated at 65°C for 5 min, and kept on ice. After samples were centrifuged for 10 min at 4°C at top speed, the upper aqueous layer was transferred to a new tube. Phenol:chloroform 5:1 solution was added and vortexed again, and the upper layer was obtained as above. Aqueous phase was mixed with chloroform (28560-0350, Junsei), and moved to a new tube after centrifugation. RNA in the solution was precipitated with double volume of 100% ethanol (412701, Carlo Erba), and collected RNA pellet was washed with 70% ethanol. After air dry, RNA pellet was dissolved in RNase-free water.

II. 4. 2. cDNA synthesis and qPCR

To eliminate genomic DNA, we used DNA-free™ kit (AM1906, Thermo Fisher Scientific) according to the manufacturer's instructions.

For cDNA synthesis, random hexamer primer was used for the extension and RevertAid™ reverse transcriptase (EP0441, Thermo Fisher Scientific) was used as manufacturer's instructions. TOPreal™ qPCR premix (RT500S, Enzynomics) was used for the qPCR reaction. RT-qPCR primer sets used in this study were presented in Table II-3.

Table II-3. Primers for RT-qPCR

Gene	Forward (5′-3′)	Reverse (5′-3′)
act1	GGATTGGTGGATCCATTCTT	ATACCAGGTCCGCTCTCATC
atf1	CCGCTCCTTCACCTGGTACT	TTGCTGCTGATCGGGAACCT
cgs1	TATCAAGCCGGTAGCATCGT	CGCCTTTGCCATTCTTCACT
gcd1	CAAAGTCTCAAGATGCCGAA	GGACCTTTCCATTTGCACTT
gnd1	CTGTGACTGGGTTGGTGAAC	TACCAAGGCCACGTTTCATA
hxk2	GGTTGAACGTTCTGCTGAGA	AGACATACGAGCAGCACGAC
idn1	GGAGCATCGTCCTGCAATTT	TCCATTCTCAACGCTAATGGT
loz1	GAACTGGCGCTTGCTAATCA	TATGGGAGCGGCAGGAATAG
pcr1	TCGCATTGCCGCTTCTAAAT	ACTGAAGACGCTTGGATTGC
rst2	CTGGTTTCTGCCGGTAATGG	ACACCGAGACGTTGATCTGA
rsv1	TTCTCCAGGCAGTTCATTTG	ATCGGCCCTTAGTAATGTGG
rsv1-5′end	ATCGGCCTTTTCCACTCTAC	TGCGGGAGAACCAAAAAGCA
scr1	GACCCAGCGTATCCAATGGC	GGACTACTCGGTGCAGTGCT
sty1	GCTGATGCTTTGGCTCATAA	ACCTTCCAAGTCTCCACAGG
zrt1	TTGGTGCCAAACAACACAGT	TCAACACCATCGGCACATTC
zwf1	TAAAGCCATGGATATTGGCA	TCTGGCTGAACACGGATTAC
zym1	CGGTTGCCAAGATTGCAAAG	TGCAAGAAGAGCACTTGCAG
SPCC16C4.10	CAGTCACTGATTCGCCCAAA	ACCAGCACCTGTGGTAACAA

II. 5. Cell Extract Analysis

II. 5. 1. Protein preparation

For protein extraction, fission yeast cells were collected at indicated time points, and washed with water to be preserved at -70°C. Cells were resuspended with ice-cold protein lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM PMSF, proteinase inhibitor cocktail (s8830, sigma)), and filled with glass beads. Cells were subjected to bead beating for 5 times for 15 sec, with 1 min breaks between. Lysate was centrifuged for 10 minutes at 4°C, and supernatant was moved to a new tube. The amount of protein was quantified using Nanodrop (Thermo Fisher Scientific).

II. 5. 2. Gel analysis, protein staining, and western blotting

Protein samples boiled with SDS buffer solution were loaded and separated on 10% SDS-PAGE gel, and transferred onto nitrocellulose membrane (Protran 0.2 μm, Amersham). Following 1 hour incubation with 5% skim milk (MP Biomedicals) in TBST (TBS with 1% tween-20) buffer, membrane was incubated with mouse anti-DDDDK (MBL) (1:5000) or mouse anti-myc (MBL) (1:5000) in TBST-BSA buffer (TBST with 0.5% BSA) for 1 hour. After washed for 3 times with TBST buffer, membrane was treated with rabbit anti-mouse antibody (Thermo Fisher Scientific) (1:5000) in TBST-BSA buffer for 1 hour. After washed three times with TBST buffer, membrane was incubated with ECL solution (EZ-Western Lumi Femto, DoGen) according to the instructions, and immuno-reactive bands were detected with chemiluminescent imaging system (Atto, LuminoGraph II).

II. 5. 3. Flag immunoprecipitation and protein identification

Wild-type cells and Rsv1-5flag cells were grown in YE media and transferred

to YED media for starvation. Cells were harvested and washed with TDW. Cells were resuspended in 20 ml of ice-cold lysis buffer (30 mM HEPES/KOH pH 7.6, 100 mM KCl, 2 mM EDTA, 0.2% NP40, 2 mM PMSF, 0.2 mM DTT, protease inhibitor). Glass bead 70 ml was added to cell suspension, and cells were lysed using bead beater (Biospec). Cell lysates were collected in 50 ml tube and centrifuged at 12000 rpm for 20 min at 4°C. The supernatant was transferred to a centrifuge tube (26.3 mM capacity, Beckman Coulter). The supernatant was centrifuged at 55000 rpm for 1 hour (Beckman Coulter). The newly obtained supernatant was moved to a new 50 ml tube, and incubated with anti-FLAG M2 affinity gel (Sigma) for 2 hours. Beads were collected at 3000 rpm, and washed intensively twice with lysis buffer, and once with lysis buffer of 100 mM KCl. Beads were transferred to a new e-tube, and incubated with 200 μl of 1 mg/ml FLAG peptide solution (Sigma) for overnight at 4°C. The eluted supernatant was obtained, and the protein extracts were precipitated with TCA solution (final concentration of 10% TCA) for overnight. Protein pellet was washed with 70% acetone, and dried. LC-MS/MS analysis was conducted in Proteomic Core Facility in Seoul National University.

II. 6. Co-Immunoprecipitation (Co-IP)

Epitope-tagged cells were harvested after the cells were glucose-starved for three hours in YE complex media. After cell pellets were washed twice with TDW, cells were resuspended in protein lysis buffer for protein extraction. Protein extract was prepared as above, and Co-IP was conducted with Dynabeads® (Thermo Fisher Scientific), according to the manufacturer's instructions.

II. 7. Chromatin Immunoprecipitation (ChIP)

Chromatin IP experiment was performed as described previously with brief modifications (Hirota et al., 2006). After glucose starvation, cells were fixed with 1% formaldehyde for 15 minutes at room temperature, and quenched with 125 mM glycine for 5 minutes. Cells were collected, and washed with TBS buffer (20 mM Tris-Cl pH 7.6, 150 mM NaCl, autoclaved). Cells were lysed with glass bead beater (Minibeadbeater, Biospec), and the cell extract was sonicated 5 times for 20 seconds with 10 seconds breaks between (Qsonica). After centrifugation for 20 minutes at 4°C, the supernatant was collected and precleared with 15 µl A/G agarose (sc-2003, Santa Cruz) for 1 hour. Extracts were immunoprecipitated with 2 µg BSA, 20 µl A/G agarose, and 2 µl anti-DDDDK antibody (MBL) overnight at 4°C. The agarose precipitates were washed twice with TBS-tween20 buffer, and twice with TBS buffer. Bead-bound DNA was extracted in EB buffer (1% SDS and 250 mM NaCl in TE (100 mM Tris-Cl, 10 mM EDTA)) twice for 30 minutes at 65°C. Supernatants were moved to a new tube and treated with RNase A (sigma) for 1 hour at 37°C. Proteinase K (20 mg/ml, MP biomedicals) 5 µl was treated for 2 hours at 55°C, and overnight at 65°C. Protein was eliminated through PCIAA and chloroform, and DNA was obtained with 2-volume 100% ethanol at -20°C. Quantitative PCR was performed using primers presented in Table II-4. TOPreal™ qPCR premix (RT500S, Enzynomics) was used for the reaction.

II. 8. Cell Viability Assay

Cells were prepared both in 3% YE complex media or 2% EMM media. To measure the cell viability, cells were glucose-starved and incubated until cells reached the plateau phase. From this time, same numbers of cells were taken daily and plated onto the solid YE complex media to grow cell colonies. The

number of colonies was counted, and colony number of day 0 was regarded as 100% viability rate.

II. 9. Microscopy

EGFP DNA sequence was tagged to N-terminus of *rsv1* start codon with the promoter and terminator sequences of *rsv1* gene via JK148 vector, and the vector digested with Tth111 Ⅰ (NEB) was transformed to 665 wild-type cells. Transformed cells were selected with *leu* marker in EMM-leu media. Selected cells were cultured for indicated times, and aliquots were taken. Cells were placed onto the slide glass and sealed with cover glass and manicure. Cellular EGFP signal was detected by LSM 700 Confocal Laser Scanning Microscope from Carl Zeiss.

Table II-4. Primers for ChIP-qPCR

Gene	Forward (5′-3′)	Reverse (5′-3′)
rsv1 (1)	CGCTACCCTCAGAGCCATTT	CCCCTCTCATCTGAGAAATTC
rsv1 (2)	GGAATTAGGGGTTGAACCTT	GGCTAATTTGGAGGTAAGGC
rsv1 (3)	GGGGTAAATCACCTAGTTTC	GTAAGCTGCAACCACCTTCG
rsv1 (4)	TCCACTCTTCAAGCCTCGTA	TAGAAGAAAAGGCAGAGGCT
rsv1 (5)	CTAGCGCTGGTCTCTTCATT	TGGAAACAAGTAAACGCCCG
rsv1 (6)	CTTCCCCCACAATCTTAATTC	AGTGGAAAAGGCCGATTCCA
rsv1 (7)	CTTGCAAGCCCAATCTAAGC	CGCCTGCTGATTCTGAAATAG
rsv1 (8)	TGAGGTAACATTTCCGCAGC	GACGAAGAGGGTAAACGAAAC
rsv1 (9)	ACGTCCTCGGAAATCCAACT	CGCCGTTAGTGTGAAATGAAG
dak2 (1)	CTTGTTCCGCTATGGAAACGGA	GGATGGATCGCCCAATTGGAAA
dak2 (2)	TGCAAGTGGTCCAGATAACCGA	GATTTGCGCTTAAGATTCGTC
gcd1 (2)	GTTTCCCATAAACCTTATGC	GAAACGCAGGTGCCAATGAA
gcd1 (3)	GGTAGACGCAAAGTGAATCA	GAAGAAAAGGAGAGAACAGG
ght1 (1)	CAGGCATAGAGGAAGTGAATGG	GTCGAGACACTTAGTGTGCAA
ght1 (2)	CATCATCTTCCCATAGCGAAAC	GGCTAAGCGATCGTTTAGGT
ght4 (1)	CGCTTGTGCAACAATTAATGA	GCTGCAGTACAAAGGTGTGG
ght4 (2)	TCTTCCAGCTTCTTGTTCCC	GCATCCAATCGCATGAAG
ght5 (1)	ATGGCATGACAGCCTAGACA	TTGGAAAGGCATGAGAGAGA
ght5 (2)	GGGAGAGTGTTCCCATTTGT	TTGGAGACACGCATTCTGAT
ght7 (1)	CAAACCCTTTACCCAATTACG	ACGAGGTGCTCTATCTTTATGGT
ght7 (2)	CGCAGCGGAGTACATCTAAA	TGCATAGCTGGTAAATGCAGA
gnd1	CGTAGCAAAACGATGCACATG	TTCTTCAGCTCACCCAAGTTC
hxk2 (1)	CTTTGCTTTACCCCTGCTTGT	ATACCGAAACACTGTACCAGAG
hxk2 (2)	AAGCCTGGTCAAAGAAGTAAGC	ATTCCGTCTCACTTAACCGTC
idn1	GTATTAAAGGGACGAGAAGCC	TGCAGACACTTACGACTTGGT
zrt1	GCACATCATTAGAATGACAGTC	CGTACTCTAACAAAACCTAAC
zwf1	ATTATAGTGGCATCGTTGGCG	ATGGAATGAAAGGGGTGAGGC
zym1	GTATATGCATTTGCCTCTCC	GGTGAGAGTAGGGTGCCTTTA
SPCC16C4.10	AGCGATGCAGACGTATTGCT	GTACGATTACGGCATTATCACG

II. 10. Sequencing Data Analysis

II. 10. 1. Genome-wide sequencing

For RNA-seq samples, 972 wild-type cells and Δ*rsv1* deletion cells were grown in YE complex media until OD_{600} 0.7 (exponential phase samples). Cells were collected and moved to YED media (glucose starvation samples). Cells were incubated for 2 hours and harvested for RNA extraction. RNA extraction was conducted as described in section II. 4, and RNA was dissolved in TDW.

972 wild-type cells and cells expressing Rsv1-5flag were used for ChIP-seq. 2L of each sample was cultured in YE complex media. After moved to YED media and cultured for 2 hours, cells were cross-linked with formaldehyde. Subsequent procedures of ChIP assay were conducted as described in section II. 6. Extracted DNA samples were prepared in TDW.

Genome-wide RNA-seq and ChIP-seq were performed at Macrogen Sequencing Facility (Macrogen Inc., South Korea).

II. 10. 2. RNA-sequencing data analysis

Fastq.gz raw files were obtained from Macrogen. As pair-end sequencing was conducted, forward/reverse files were generated. For the raw data analysis, Galaxy program was used in part of the analysis processes (FastQC, Trimmomatic, HISAT2) (https://usegalaxy.org). Gz raw files and genome fna/gene annotation gff files of *S. pombe* were uploaded to Galaxy. Fna file and gff file were downloaded from EnsemblFungi (fungi.ensembl.org). FastQC tool was used for checking quality control of the samples with default options. FastQC was performed both before and after the trimming process. Trimming process of the adaptor sequences was carried out with Trimmomatic tool (paired-end, sliding window for window size=4/mean quality=15, and min length=36). After trimming procedure, the results were aligned to the genome of *S. pombe* via HISAT2. Aligned data were obtained and counted via

FeatureCounts with pair-end and reverse-strand options. Subsequently obtained counting data were loaded to R program, and differential gene expression analysis was performed with DESeq2. Heatmap of gene expression result was generated by Heatmap.2.

II. 10. 3. ChIP-sequencing data analysis

For chromatin immunoprecipitation, we used the tagging strain in which the coding sequencing of 5-flag protein is chromosomally integrated to 3'-end of *rsv1* ORF. 972 wild-type cell was used as a no-tag control. After the ChIP procedures as described above, DNA samples after ethanol precipitation were obtained from both wild-type sample and Rsv1-5flag sample. DNA sequencing was performed at the Macrogen sequencing facility (Macrogen Inc., South Korea). Sequencing libraries were prepared using KAPA library preparation kit (Roche), and DNA sequences were read as paired-end. Sequence quality check was performed by FastQC, and Trimmomatic was applied to remove adaptor sequences. Reads mapping process was done through BOWTIE, and aligned reads were subjected to Picard (MarkDuplicates) to identify duplicate reads. MACS2 was applied for peak calling process, and ChIPpeakAnno was used for annotation of the peaks from ChIP-seq experiments. For specific analysis for transcription factor, HOMER with 'factor' option was used for peak calling.

CHAPTER III.
RESULTS and DISCUSSION

III. 1. Characteristics of Rsv1 under Glucose Starvation

III. 1. 1. Function of Rsv1 under glucose starvation

The main function of Rsv1 has been studied at stationary phase to be necessary for maintaining cell viability (Hao et al., 1997). Actually at stationary phase, many nutrients like glucose, nitrogen, phosphate, and sulfur become deprived. Among them, only glucose deprivation showed the deleterious effect to cells who are deleted with *rsv1* gene. So the effect of glucose starvation to *Δrsv1* deletion cells was investigated specifically.

To clarify the effect of Rsv1 under glucose starvation, we tested the survival rate of *Δrsv1* cells after glucose depletion both in complex media and minimal media. First, we checked the growth rates of wild-type cells and *Δrsv1* cells after glucose starvation. Normally, 972 wild-type cells can divide twice more after they are moved to 0.1% glucose YED media from 3% glucose YE media at OD_{600} 0.7. When we checked the growth rates of wild-type cells and *Δrsv1* cells, there was no difference in their growth rates (Fig. III-1A). So it seems that Rsv1 is not critical for cell growth until the cell reaches the plateau phase after glucose depletion. We checked cell viability under starvation condition. As shown in Fig. III-1B and C, *Δrsv1* cells lost their viability faster than wild-type cells in both complex media and minimal media. The degree of losing viability was bigger in complex media. So we confirmed that Rsv1 is important for maintaining cell viability under glucose-limited condition.

III. 1. 2. Expression of Rsv1

In fission yeast, there are three C_2H_2-type zinc finger proteins related to glucose signaling; Rst2, Scr1, and Rsv1. They all have the conserved zinc finger sequences in their N-terminus, and they also have the conserved sequences for recognition of target DNA (Fig. I-2). Different from Rst2 and Scr1, which are controlled by posttranslational modification (Higuchi et al., 2002; Hirota et al.,

2006), Rsv1 was barely detected when glucose was abundant, and regulated mainly at transcriptional level (Fig. III-2A). After being moved to glucose-depleted condition, the level of *rsv1* transcript began to increase immediately, and the level of transcript was maintained for a long time (Fig. III-2A). Interestingly, the transcript level of *rsv1* showed temporal decrease at 1 hr of glucose starvation. As Rsv1 regulates its own transcription negatively (Figure III-14), it is supposed that this decrease of expression may be caused by Rsv1 itself.

Similar to the transcript level, the amount of Rsv1 protein increased after glucose limitation, and the protein level was maintained for up to 10 hours (Fig. III-2B). Also, expression of Rsv1 under various concentrations of glucose was investigated (Fig. III-3). When cells were transferred to water at exponential phase, Rsv1 protein was not induced at all. Also, Rsv1 was not expressed under 0% glucose condition as in water. But when cells were starved for glucose of 0.02%, 0.08%, 0.2%, and 1%, Rsv1 protein was expressed. Especially, Rsv1 protein was detected sooner under lower concentrations of glucose. Therefore, it seems that the presence of some glucose is necessary for expression of Rsv1, and Rsv1 can be induced more rapidly at lower concentrations of glucose.

III. 1. 3. Localization of Rsv1

To find out the cellular localization of Rsv1 protein according to glucose concentration, we checked the EGFP-Rsv1 localization. As shown in Fig. III-4, EGFP-Rsv1 was barely detected in exponential phase cells. But when cells were moved to glucose-starved media, EGFP-Rsv1 started to be detected particularly in the nucleus. And the nuclear EGFP-Rsv1 signal disappeared rapidly when cells were shifted to glucose-rich media again. So, Rsv1 is eliminated in cells under the condition of high concentration of glucose. Other zinc finger proteins, Rst2 and Scr1, can reside both in the nucleus and the

cytosol, according to the glucose concentration. But different from these proteins, Rsv1 seems to be degraded under glucose-rich condition by undefined mechanisms. Taken together, Rsv1 is expressed exclusively under glucose-limited condition, and localizes in the nucleus. And Rsv1 is rapidly eliminated under glucose-rich condition.

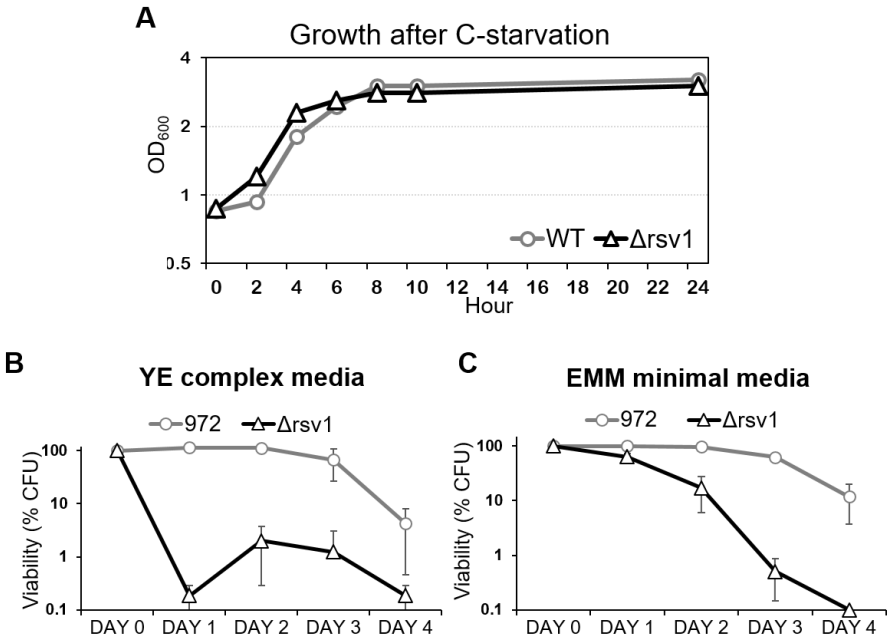


Figure III-1. Phenotypic analysis of wild-type cells and *Δrsv1* deletion cells.
(A) Growth of wild-type cells and *Δrsv1* deletion cells after glucose starvation. Cells were grown to exponential phase of OD_{600} 0.7 in YE complex media, and transferred to 0.1% glucose YED media. After media change, the density of the cells were measured with spectrophotometer.

(B, C) Long-term viability of wild-type cells and Δ*rsv1* deletion cells under glucose-depleted condition. Cells were grown in YE complex media or EMM minimal media until OD_{600} of 0.7. Cells were collected and transferred to YED (YE of 0.1% glucose) media or EMM media of 0.2% glucose, respectively. From this time point (DAY 0), aliquots were take daily and plated onto YE solid media. The number of colonies each day was expressed as a percentage of the colony number of DAY 0. Results were presented as means ± SEM from three independent assays.

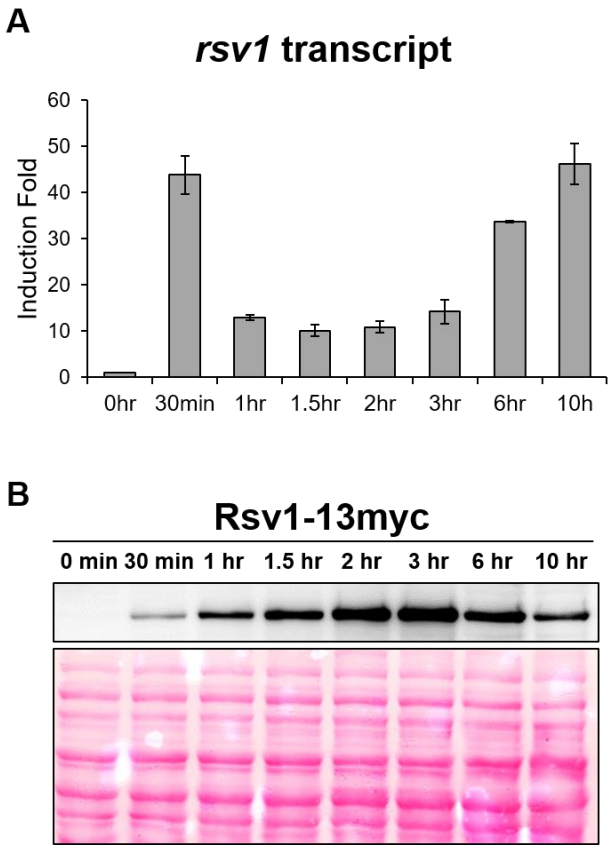


Figure III-2. Expression of *rsv1* under glucose starvation.

(A) Expression of *rsv1* gene under glucose-depleted condition. 972 wild-type cells were grown in YE complex media and moved to YED media at OD_{600} 0.7 (0 hr). Cells were collected at indicated times after media transfer. Amount of *rsv1* transcript was normalized to the level of *act1* gene transcript. Error bars indicate the SEM from three independent experiments.

(B) Rsv1 protein induction under glucose-starved condition. Cells expressing Rsv1-13myc were obtained as in (A). Cell extracts were collected and Rsv1-13myc was detected with anti-myc antibody. Ponceau S blot is presented as a loading control.

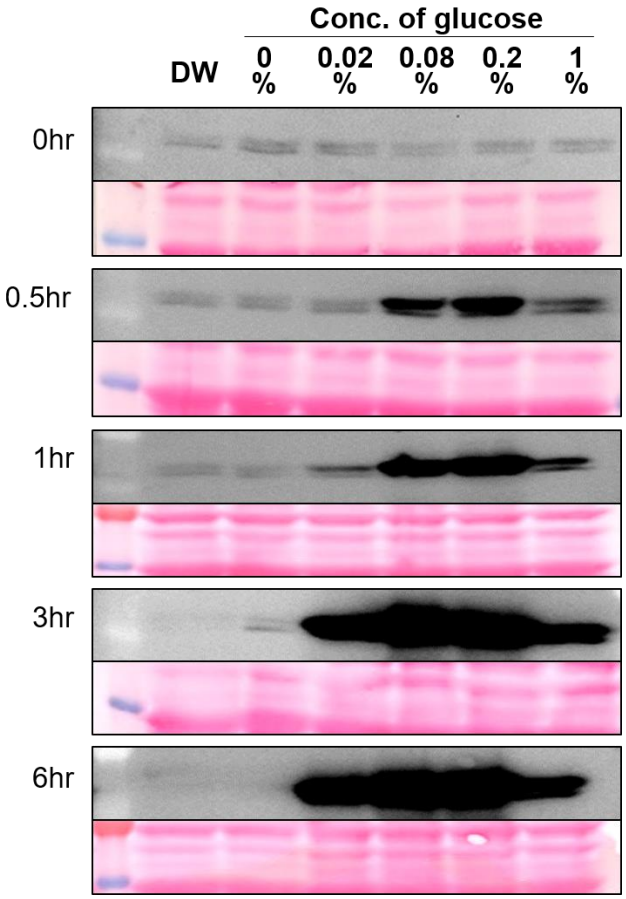


Figure III-3. Expression of Rsv1 under various concentrations of glucose.
Cells expressing Rsv1-5flag were grown in EMM media, and were glucose-starved as indicated times under various concentrations of glucose (EMM media and water). Rsv1 protein was detected with anti-flag antibody. Ponceau S staining blot is shown as a loading control.

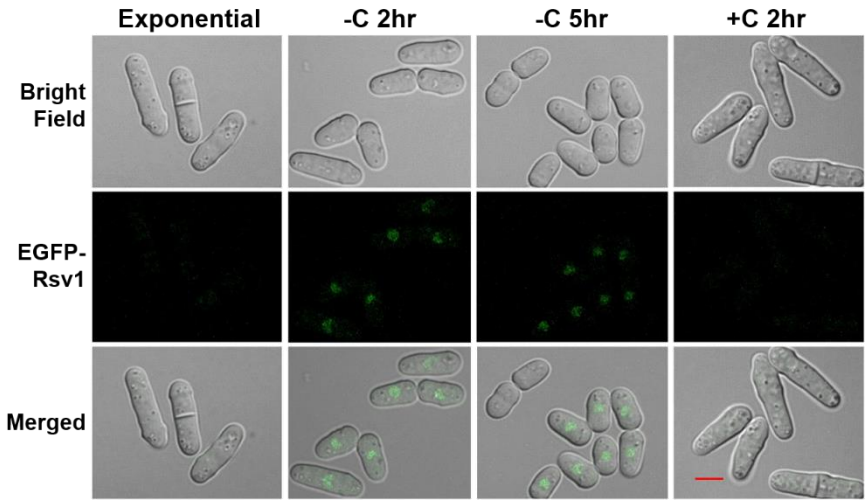


Figure III-4. Expression and localization of EGFP-Rsv1.

Cells expressing EGFP-Rsv1 were maintained in YE complex media. Cell were collected at exponential phase (Exponential), and moved to glucose-starved YED media to be incubated for 2 hours and 5 hours (-C 2 hr and -C 5 hr). After 5 hours of incubation in YED media, cells were transferred again to YE complex media and incubated for 2 hours (+C 2 hr). Fluorescence images were taken at indicated times with confocal microscopy. Scale bar; 5 μm.

III. 1. 4. Stress accumulation in *Δrsv1* cells

Glucose deprivation is known to trigger cellular ROS (reactive oxygen species) in many organisms (Graham et al., 2012; Liu et al., 2003; Marambio et al., 2010; Zhao et al., 2017). ROS is the byproduct of energy production process, and is mainly generated in mitochondria by oxidative metabolism (Zorov et al., 2014). Under glucose starvation, reduced glucose influx results in the reduced glycolysis and facilitates the respiration for energy supply. And the increase of respiration rate causes the production of ROS. It is well known that cells suffer the ROS under glucose starvation, and it is important for cells to balance the metabolic defect and antioxidant responses.

To detect ROS generation in *Δrsv1* cells, wild-type cells and *Δrsv1* cells were grown in minimal media, and ROS (especially superoxide) was detected with DHE (dihydroethidium) assay (Fig. III-5). When cells were moved to glucose-starved media, cells showed the decreased level of ROS in both wild-type and *Δrsv1* cells (Fig. III-5A, B). This may be caused by the increased production of antioxidant genes, as the elevated expression of the genes was detected both in wild-type and *Δrsv1* cells (data not shown). But when the starvation condition continues, *Δrsv1* cells showed the higher level of ROS at 9.5 hour, day 1, and day 2. So it is assumed that *Δrsv1* cells accumulate more ROS under glucose starvation. Also, we detected the protein carbonylation after glucose deprivation (Fig. III-5C). Protein carbonylation is a type of protein oxidation that can be promoted by reactive oxygen species. When protein carbonylation was detected under long-term glucose starvation condition, wild-type cells and *Δrsv1* cells showed the similar carbonylation patterns until 7 hours. But when starved for longer, *Δrsv1* cells showed the stronger carbonylation patterns than wild-type cells (24 hr and 72 hr). Taken together, *Δrsv1* cells showed the more severe defects like ROS accumulation and protein carbonylation than wild-type cells.

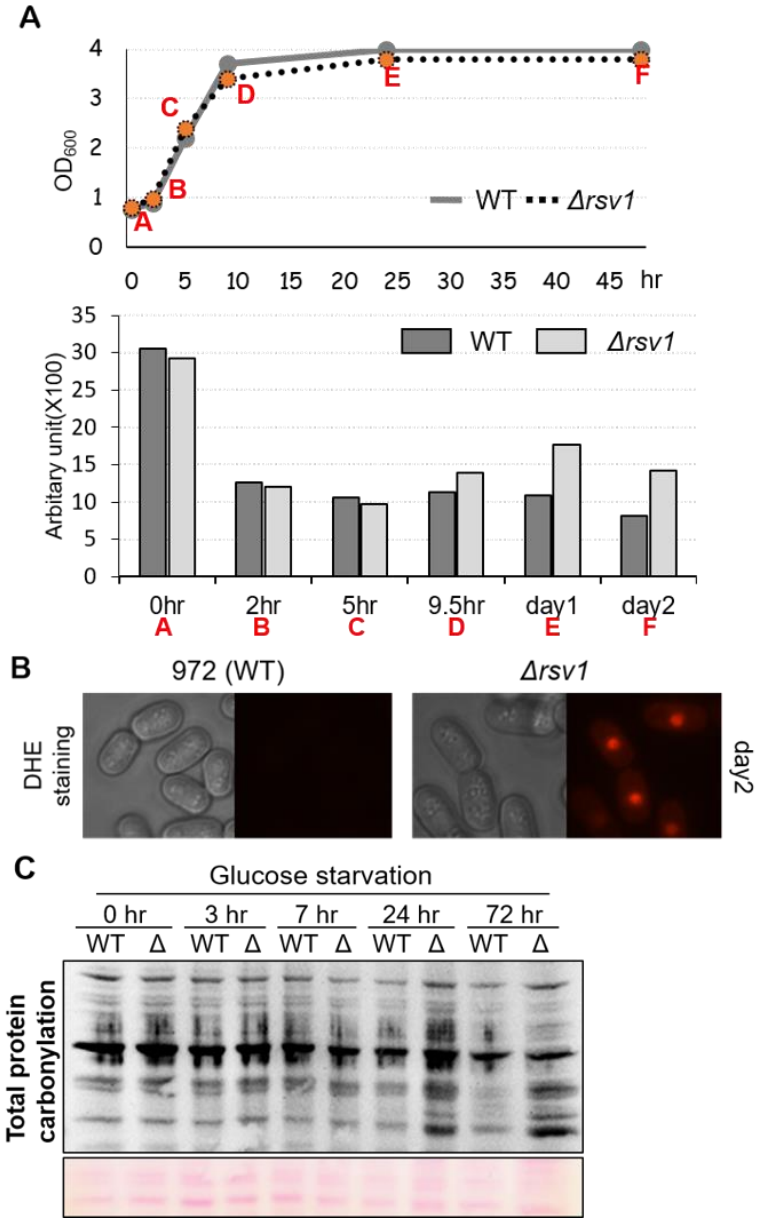

A
OD600
WT
Δrsv1
hr
Arbitary unit(X100)
0hr
2hr
5hr
9.5hr
day1
day2
B
972 (WT)
Δrsv1
DHE staining
day2
C
Glucose starvation
0 hr
3 hr
7 hr
24 hr
72 hr
WT Δ
Total protein carbonylation

Figure III-5. Stress accumulation in wild-type cells and *Δrsv1* deletion cells.
(A) ROS generation in wild-type cells and *Δrsv1* deletion cells in minimal media. Cells were collected at exponential phase, and moved to glucose-starved media and incubated for indicated times. To investigate the production of superoxide, 50 μM of DHE (dihydroethidium) was treated for 20 minutes. FACS analysis was used for detection of the DHE signal at indicated times (A to F).
(B) Microscopic observation of DHE signal. Wild-type cells and *Δrsv1* deletion cells were incubated with 50 μM of DHE at day 2, and DHE signal was detected under confocal microscopy.
(C) Detection of protein carbonylation states. Total cell extracts from wild-type cells and *Δrsv1* deletion cells were prepared for measuring the overall protein carbonylation. Cell extracts were obtained at indicated times after glucose starvation. Protein carbonylation pattern was detected with anti-DNP antibody, which can detect stable dinitrophenyl hydrazone product. Ponceau S blot was presented as a loading control.

III. 2. Regulatory Mechanisms for Rsv1

III. 2. 1. Protein modifications of Rsv1

As the expression of *rsv1* is controlled by glucose, there may be some regulators for *rsv1* gene induction or Rsv1 protein modification which are activated when glucose becomes limited. And there are some notable kinases, like Pka1 or Sty1, which can modulate target proteins depending on the glucose signal. Moreover, Scr1 and Rst2, the homologues of Rsv1, were reported to be regulated via phosphorylation, by Ssp2 and Pka1, respectively (Higuchi et al., 2002; Matsuzawa et al., 2012). To find out whether Rsv1 is also regulated directly by protein modification, we first isolated Rsv1 protein through immunoprecipitation in fission yeast to discover the modification status of Rsv1 (Fig. III-6). As the amount of cellular Rsv1 protein is low, 24 L cells were prepared for immunoprecipitation of Rsv1 protein. Wild-type cells and cells expressing Rsv1-5flag were grown for immunoprecipitation, and the obtained extracts were analyzed in SDS protein gel. Rsv1 protein band was cut and analyzed for post-translational modifications (Fig. III-6C, arrow).

We could detect some phosphorylated amino acid residues in Rsv1 protein. Especially, Rsv1 had the phosphorylated S382 and S405 residues, which are the consensus phosphorylation sites of MAPK (Fig. III-6). The phosphorylation site of MAPK is well conserved in eukaryotes; a serine or a threonine immediately followed by a proline; (S/T)P (Roux and Blenis, 2004). And Sty1, the MAPK protein in fission yeast, was supposed to be the modulator for Rsv1 because Sty1 is activated by environmental stresses like oxidative stress and glucose starvation (Madrid et al., 2004). Although Rsv1 has the phosphorylation site of Sty1, we could not confirm the direct phosphorylation of Rsv1 by Sty1 with kinase assay and anti-phospho antibody detection (data not shown).

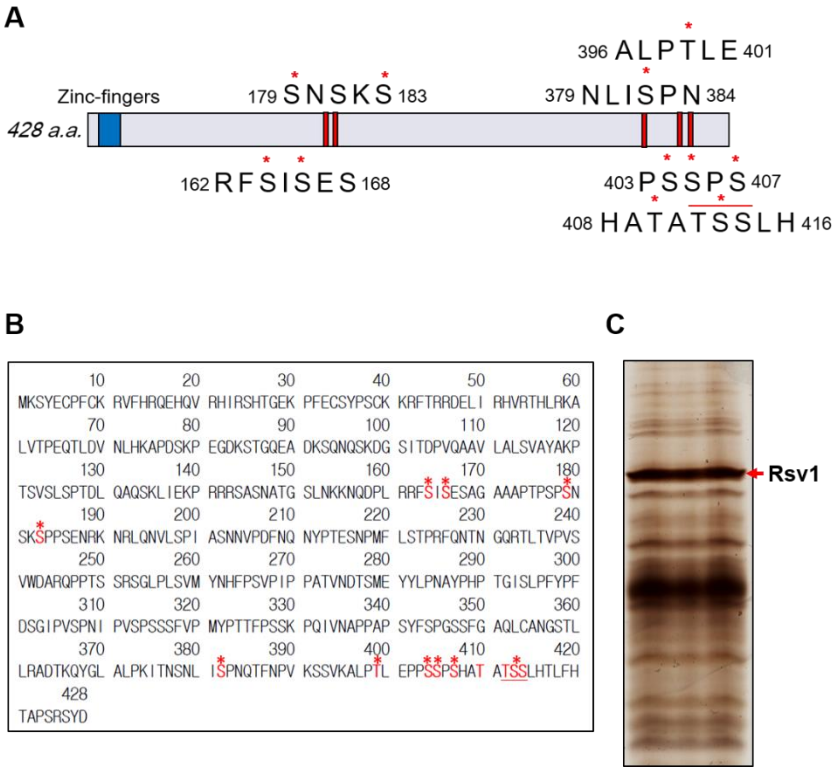


Figure III-6. Phosphorylation of Rsv1 protein.

(A, B) Location of phosphorylated residues in Rsv1 protein. Rsv1 protein was obtained with immunoprecipitation under glucose-starved condition, and subjected to identify post-translational modifications. Red asterisks mean the phosphorylated serine or threonine sites identified in LC-MS/MS analysis.

(C) SDS-PAGE gel of immunoprecipitated Rsv1 protein. 28 L cells expressing Rsv1-5flag were lysed and immunoprecipitated in the condition of glucose starvation of 2 hours. Immunoprecipitated extracts were separated with SDS-PAGE. Proteins were detected with silver staining method, and Rsv1 protein band (arrow) was cut and analyzed with LC-MS/MS.

III. 2. 2. MAPK pathway is involved in regulation of *rsv1* expression

Although the direct phosphorylation of Rsv1 by Sty1 was not confirmed, we tested the possibility that Sty1 (MAPK pathway) may be involved in the regulation of *rsv1* expression, and we could find that Sty1 is important for full induction of *rsv1* transcript. As shown in Fig. III-7, the full inductions of *rsv1* transcript as well as Rsv1 protein were hindered in *Δsty1* mutant cells. So, although we could not prove the direct phosphorylation of Rsv1 by Sty1, it seemed that MAPK signaling pathway involving Sty1 is necessary for positively regulating the induction of *rsv1*. It is known that Sty1 can be recruited to stress-induced target genes directly (Reiter et al., 2008). As Sty1 was revealed to be important for *rsv1* induction, we conducted ChIP-qPCR assay with cells expressing Sty1-13myc protein (data not shown). We found that Sty1 does not bind to *rsv1* promoter directly, suggesting that another factor activated by Sty1 is involved in the regulation of *rsv1* expression.

The transcription factor Atf1 is the main effector protein of Sty1, and Atf1 is phosphorylated by Sty1 after glucose depletion (Madrid et al., 2004). It has recently been revealed that phosphorylation of Atf1 by Sty1 enhances interaction with the transcriptional machinery to promote the transcription of target genes (Sánchez-Mir et al., 2017). As we confirmed that presence of Sty1 is important for *rsv1* gene expression, we checked the possibility that Atf1, the effector protein of Sty1, is involved in transcriptional activation of *rsv1* gene. As shown in Fig. III-7C, the full expression of *rsv1* gene was curtailed in *Δatf1* deletion cells. Moreover, Rsv1 protein level was lower in *Δatf1* deletion cells (Fig. III-7D). These results demonstrated that Rsv1 is controlled transcriptionally by MAPK pathway, and that Atf1 may be the direct transcription factor for *rsv1* expression.

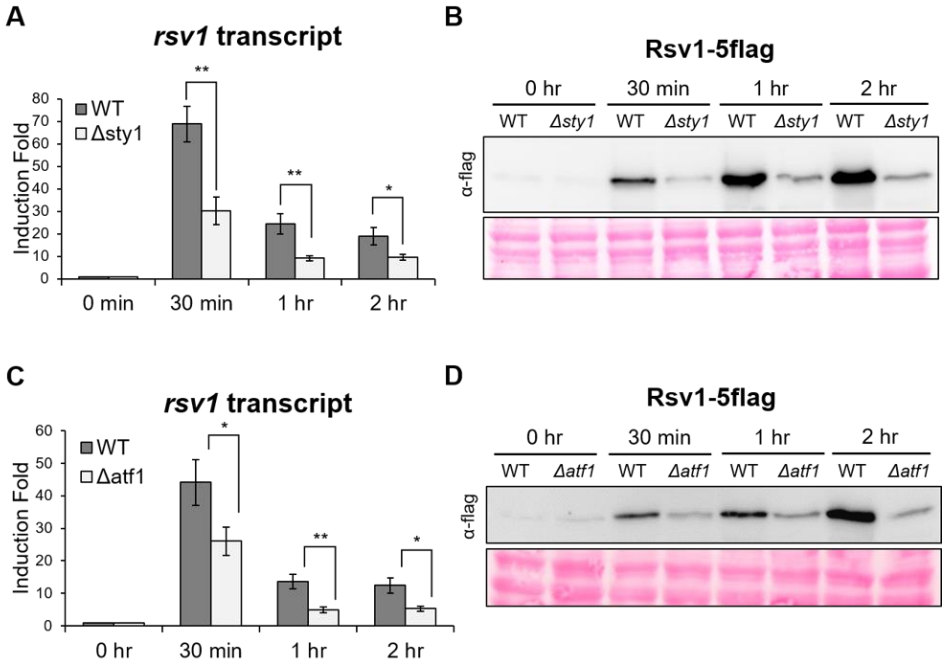


Figure III-7. MAPK pathway is involved in the induction of *rsv1*.

(A) The RT-qPCR assay of *rsv1* transcripts in wild-type cells and *Δsty1* deletion cells. Cells were grown in YE complex media and transferred to YED media at exponential phase. Cell aliquots were collected after glucose starvation at indicated times for total RNA preparation. *rsv1* gene-specific primer set was used for RT-qPCR. Results were presented as means ± SEM from three independent assays. **P*<0.1, ***P*<0.05.

(B) Expression of Rsv1-5flag protein in wild-type cells and *Δsty1* cells. Wild-type cells and *Δsty1* cells expressing 5flag-tagged Rsv1 were prepared as in (A). Rsv1-5flag protein was detected with an anti-flag antibody. Ponceau S stained-blot was used as a loading control.

(C) The RT-qPCR assay of *rsv1* transcripts in wild-type cells and *Δatf1* deletion cells. Experiments were conducted as in (A). **P*<0.1, ***P*<0.05.

(D) Expression of Rsv1-5flag protein in wild-type cells and *Δatf1* cells.

III. 2. 3. PKA pathway is involved in regulation of *rsv1* expression

cAMP/PKA pathway is the highly conserved glucose sensing pathway important for adapting to the environmental nutrient condition, and is also known to control the chronological lifespan (Roux et al., 2006). And it has been reported that the expression of *rsv1* gene is dependent on PKA pathway at stationary phase (Hao et al., 1997). To address the regulation of *rsv1* gene induction by PKA pathway under glucose-starved media, we examined the expression of *rsv1* when PKA pathway is constitutively active. We deleted the *cgs1* gene, encoding the regulatory subunit of PKA, to trigger the constitutively active PKA signaling system (active Pka1 catalytic subunit) even under glucose starvation condition (Hoffman, 2005). As shown in Fig. III-8A, the expression level of *rsv1* gene was lower in *Δcgs1* deletion cells than in wild-type cells. Rsv1 protein level also decreased in *Δcgs1* deletion cells (Fig. III-8B). These data demonstrate that PKA pathway is implicated in the suppression of *rsv1* gene expression. And when PKA pathway becomes inactive under glucose starvation, the transcription of *rsv1* would be enhanced.

We also inspected whether Rst2 is related to transcriptional activation of *rsv1*. Rst2 leads to the transcriptional activation of downstream target genes when PKA activity is reduced (Higuchi et al., 2002). When the phosphorylation of Rst2 by Pka1 is inhibited under glucose-starved condition, Rst2 can be imported to the nucleus to activate target genes. As shown in Fig. III-8, *Δrst2* deletion resulted in the decreased expressions of *rsv1* gene and Rsv1 protein. The degree of reduced expression of *rsv1* was bigger in *Δrst2* cells than in *Δatf1* cells. So it seems that PKA pathway plays more important role in regulating *rsv1* expression. To summarize, Rst2 is necessary for up-regulation of *rsv1* transcription in glucose-deprived condition, and Rst2 may act as the direct transcriptional regulator of *rsv1* in this condition.

To investigate the relevance of *atf1* and *cgs1* which are responsible for

activation of *rsv1* transcription, the expression of *rsv1* in *Δatf1Δcgs1* double deletion cells was monitored in long-term starvation condition (Fig. III-9). The degree of *rsv1* transcription in *Δatf1Δcgs1* cells was similar to that in *Δcgs1* cells under the short-term starvation (30 min). And when cells were starved for 6 hours, the amount of *rsv1* transcript became lower in *Δatf1Δcgs1* double deletion cells, suggesting that the two pathways can regulate *rsv1* transcription independently.

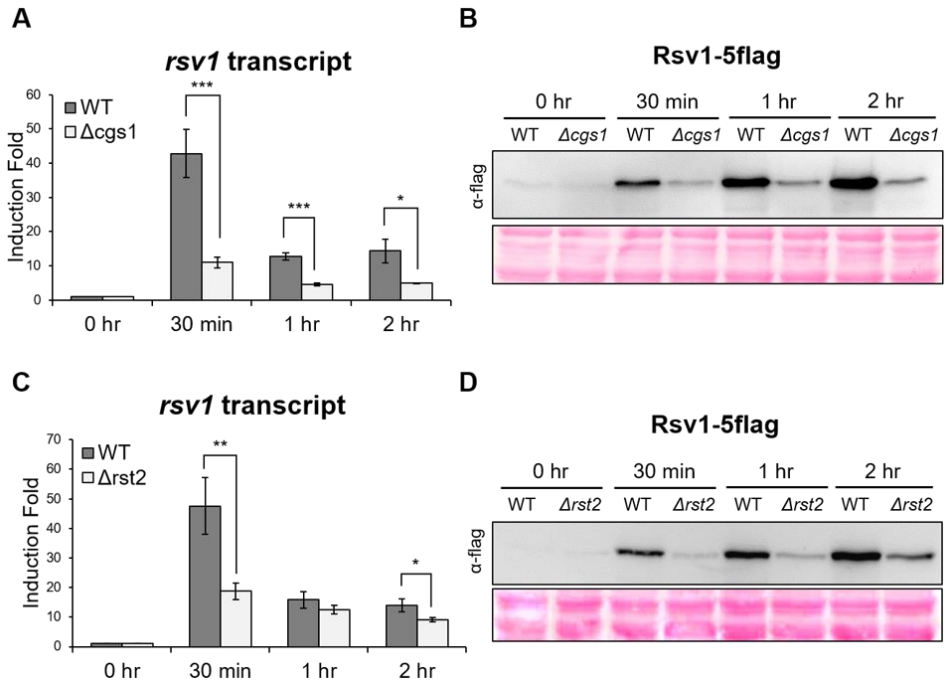


Figure III-8. PKA pathway is involved in the expression of *rsv1*.

(A) Induction of *rsv1* transcript in wild-type cells and *Δcgs1* deletion cells. Total RNAs were extracted to synthesize cDNAs. The results were depicted as means ± SEM from four independent experiments. **P*<0.1, ****P*<0.01.

(B) Rsv1-5flag protein expression in wild-type cells and *Δcgs1* cells. Ponceau S stained-blot was used as a loading control.

(C) Expression of *rsv1* transcript in wild-type cells and *Δrst2* deletion cells. The expression was depicted as means ± SEM from four independent experiments. **P*<0.1, ***P*<0.05.

(D) Induction of Rsv1-5flag protein in wild-type cells and *Δrst2* cells. Ponceau S stained-blot was presented as a loading control.

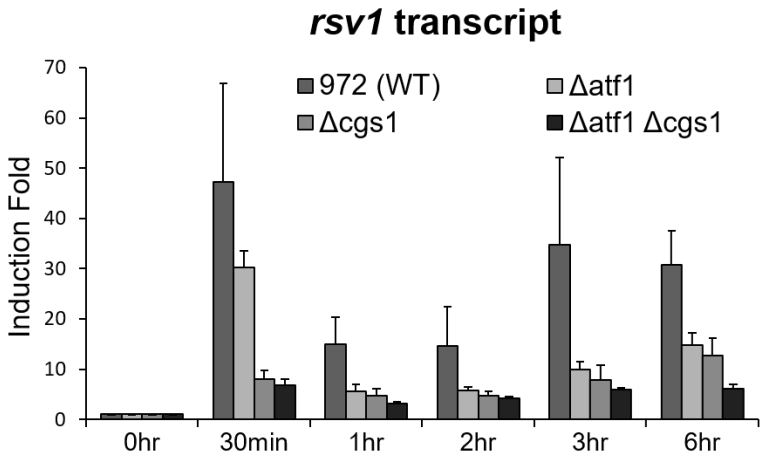


Figure III-9. Expression of *rsv1* in *Δatf1Δcgs1* cells.

The RT-qPCR assay of *rsv1* transcript in wild-type cells, *Δatf1* cells, *Δcgs1* cells, and *Δatf1 Δcgs1* double deletion cells. Cells were grown in YE complex media and moved to YED media at exponential phase. Cell were obtained after glucose starvation at indicated times for total RNA preparation. *rsv1* gene-specific primer set was used for measuring the transcription level. Results were presented as means ± SEM from three independent assays.

III. 2. 4. Enrichment of Atf1 and Pcr1 proteins to *rsv1*

We verified the signaling pathways important for regulating the expression of *rsv1* gene. And transcription factors Atf1 and Rst2 were revealed to be involved in this process (Fig. III-7, 8). We wanted to assess whether these factors are also responsible for the direct regulation of *rsv1* by binding the promoter region. To characterize the direct regulatory mechanisms of *rsv1* expression, we performed the ChIP-qPCR assay *in vivo*, using qPCR primer sets of 1~9 as described in Fig. III-10A. The primers each span 300 bp upstream of *rsv1* (1-6), downstream of *rsv1* (8-9), and the coding region of *rsv1* (7). And as depicted in Fig. III-10A, there are several consensus motifs of Atf1 (TGACGT) and Rst2 (CCCCTC) in the promoter region of *rsv1* gene (Eshaghi et al., 2010; Kunitomo et al., 2000). We could find the consensus motif of Atf1 in the regions of 1, 3, 4, 8, and 9, and the consensus motif of Rst2 in the regions of 2 and 3. So we confirmed that *rsv1* has the binding motifs of Atf1 and Rst2.

We first checked the binding of Atf1 to *rsv1* gene (Fig. III-10B). ChIP assay of Atf1 was conducted at three conditions; exponential phase, glucose starvation condition, and glucose-replete condition. As shown in Fig. III-10B, Atf1 was found to be recruited to *rsv1* gene upstream regions, especially to regions of 1 to 3. In these regions, Atf1 was enriched at the basal level even under glucose-rich condition (LogPhase). This phenomenon is not unusual, because Atf1 has been reported to bind to the stress-regulated target promoters even before stress exposure (Sansó et al., 2008). And when cells were glucose-starved, Atf1 became highly enriched at the regions of 1 to 3, and the enrichment was reduced to the basal level when cells were moved to glucose-rich condition. Unexpectedly, Atf1 was found to be bound to the terminator region (8 and 9) of *rsv1*. And the binding was also specific to the condition of glucose starvation, although the function of this binding is not understood. Therefore, we could conclude that Atf1 is involved in the direct regulation of *rsv1* transcription

upon glucose starvation.

Meanwhile, Atf1 has been reported to form a heterodimer with Pcr1, and they cooperatively regulate the stress-responsive target genes. In many cases, Atf1 and Pcr1 are recruited to target promoters simultaneously, but they can also perform distinct roles without each other (Kato et al., 2013; Sansó et al., 2008; Wahls and Smith, 1994; Watanabe and Yamamoto, 1996). To find out whether Pcr1 is involved in regulation of *rsv1* as a heterodimer with Atf1, we conducted ChIP-qPCR of Pcr1 protein (Fig. III-10B). But unlike Atf1, Pcr1 was not enriched to *rsv1* upstream. So, we concluded that Pcr1 is not involved in the regulation of *rsv1* expression. Therefore, Atf1 regulates the transcription of *rsv1* without the partner protein Pcr1.

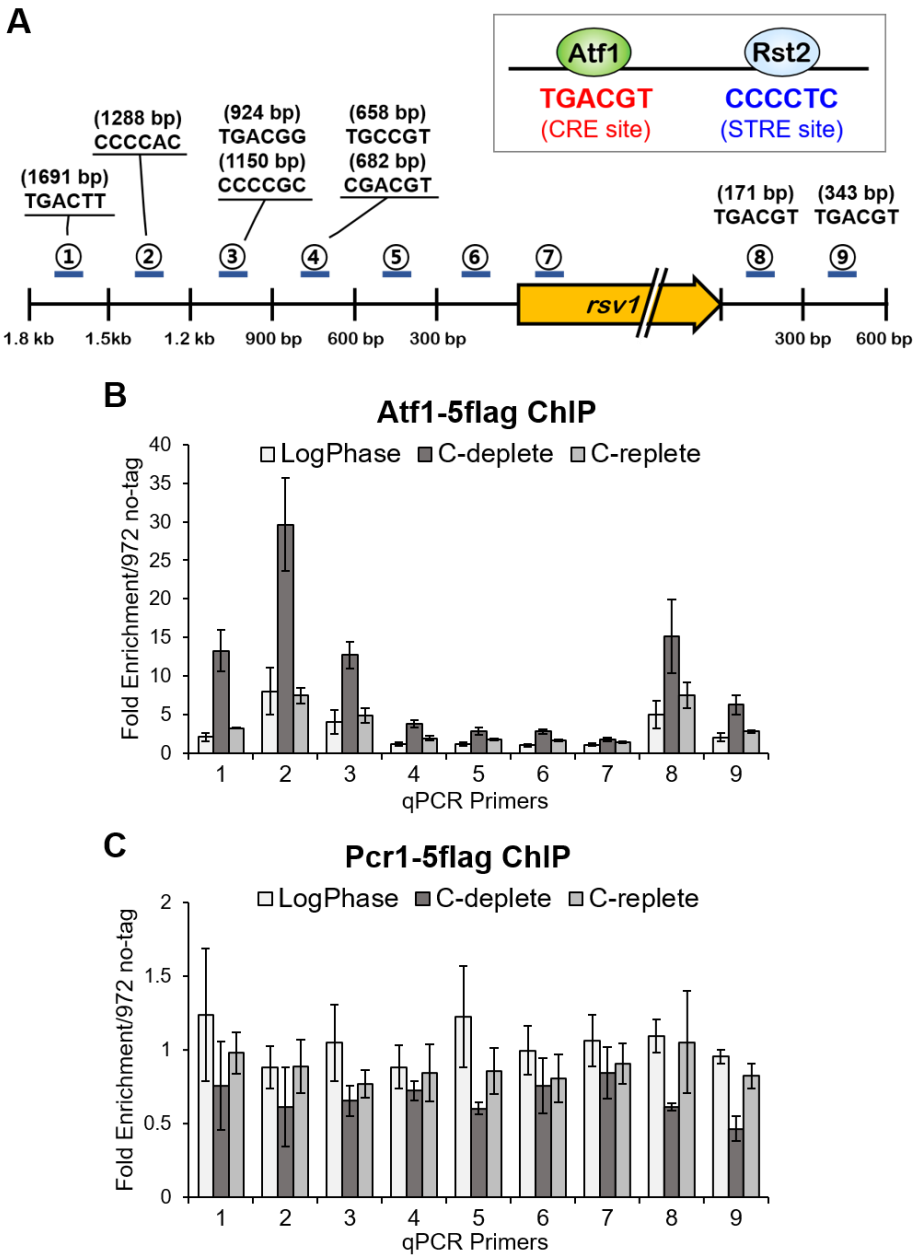

A
Atf1
Rst2
TGACGT
(CRE site)
CCCCTC
(STRE site)
(1691 bp)
TGACTT
(1288 bp)
CCCCAC
(924 bp)
TGACGG
(1150 bp)
CCCCGC
(658 bp)
TGCCGT
(682 bp)
CGACGT
(171 bp)
TGACGT
(343 bp)
TGACGT
1
2
3
4
5
6
7
8
9
rsv1
1.8 kb
1.5kb
1.2 kb
900 bp
600 bp
300 bp
300 bp
600 bp
B
Atf1-5flag ChIP
LogPhase
C-deplete
C-replete
Fold Enrichment/972 no-tag
qPCR Primers
C
Pcr1-5flag ChIP
LogPhase
C-deplete
C-replete
Fold Enrichment/972 no-tag
qPCR Primers

Figure III-10. Enrichment of Atf1 and Pcr1 heterodimer to *rsv1*.

(A) Description of primer sets used in ChIP-qPCR assay. Primer sets of 1~6 were designed for every 300 bp interval in upstream of *rsv1* gene start-codon. Primer sets of 8 and 9 were also designed for 300 bp intervals from the end of *rsv1* gene, and primer set 7 is in the *rsv1* internal region. Consensus sequences of Atf1 (TGACGT) and Rst2 (CCCCTC) are also described with their locations around *rsv1*.

(B) ChIP-qPCR assay of Atf1-5flag protein around *rsv1* region. Cells expressing Atf1-5flag were cross-linked with formaldehyde at exponential phase (LogPhase), 1 hour after glucose reduction (YE to YED; C-deplete), and 1 hour after glucose repletion (YED to YE; C-replete). Cell lysates were immunoprecipitated with an anti-flag antibody, and protein-bound DNA fragments were analyzed with primer sets of 1~9 as depicted in (A). Results were presented as means of fold enrichment over 972 no-tag control. Error bars indicate the SEM from three independent assays.

(C) ChIP-qPCR assay of Pcr1-5flag protein over *rsv1* gene region. Assay was performed as in (B). Results were presented as means ± SEM from three independent experiments.

III. 2. 5. Direct binding of Rst2 and Scr1 to *rsv1*

As we have found the relevance of Rst2 transcription factor in regulating *rsv1* expression (Fig. III-8), we performed the ChIP assay of Rst2 protein. There are motif sequences of Rst2 in region 2 and 3 of *rsv1* (Fig. III-10A). As shown in Fig. III-11A, Rst2 was shown to be enriched to the regions of 1-3 under glucose starvation, but Rst2 seemed not to reside to those regions at exponential phase unlike Atf1. Also, Rst2 showed the enrichment in regions of 8 and 9 wherein no consensus motif is present, and the recruitment pattern of Rst2 was very similar to the pattern of Atf1. As no consensus sequences of Rst2 exist in the terminator region of *rsv1*, Rst2 may be recruited here by other binding factors like Atf1. Therefore, we concluded that Rst2 is the direct regulator of *rsv1* gene expression under glucose starvation.

In fission yeast, there is another zinc finger protein, Scr1, which recognizes the same DNA sequence with Rst2. Scr1 was reported to share the same regulatory site of *fbp1* gene with Rst2 (Hirota et al., 2006). As Scr1 protein acts as a repressor under glucose-rich condition, we decided to find out whether Scr1 shares the same sites with Rst2 to reciprocally bind to *rsv1* promoter (Fig. III-11B). When we tested the recruitment of Scr1 to the regions of 1-9 at exponential phase and glucose-starved condition, we could find that Scr1 was enriched to the *rsv1* promoter regions of 1-3 under glucose-rich condition. So, the binding sites of Scr1 were shared with Rst2. When cells were glucose-starved for 2 hours, Scr1 showed the decreased enrichment to *rsv1*. Therefore, Scr1 acts as a repressor to regulate *rsv1* expression, sharing the *rsv1* binding sites with Rst2. Overall, Rst2 and Scr1 are enriched to the same sites of *rsv1* to act as an activator and a repressor respectively, suggesting the reciprocal role of Rst2 and Scr1 for *rsv1* gene induction.

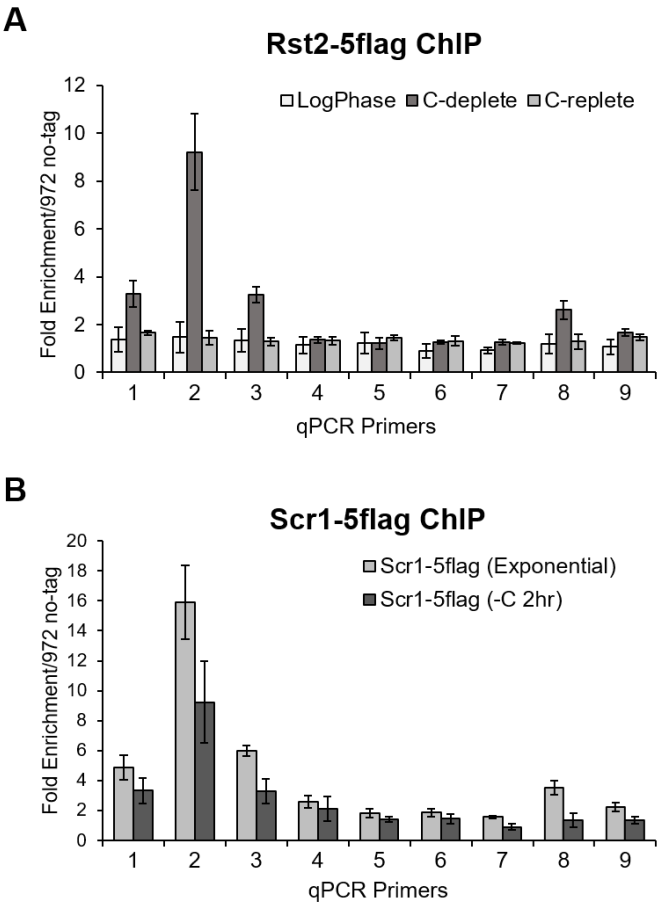


Figure III-11. Recruitment of Rst2 and Scr1 to *rsv1*.

(A) ChIP-qPCR assay of Rst2-5flag protein around *rsv1* regions (1~9). Assays were performed as in Fig. III-10B. Results were presented as means ± SEM from three independent experiments.

(B) Enrichment of Scr1-5flag protein to *rsv1*. Cells were fixed at exponential phase in complex media (Exponential). For starvation condition, cells were transferred to YED media and incubated for 2 hours for fixation (-C 2 hr). Error bars were obtained as means ± SEM from three independent experiments.

III. 2. 6. Recruitment of Tup11 and Tup12 proteins to *rsv1*

Tup1-like proteins, Tup11 and Tup12, are well studied to be involved in the regulation of *fbp1* gene, and are bound to the same *fbp1* promoter binding site with Scr1 and Rst2 (Hirota et al., 2006; Mukai et al., 1999). Also, Tup11/12 proteins are reported to have the role of corepressors, obstructing the recruitment of Atf1 to the target genes, which is antagonized by upstream lncRNA of target genes (Takemata et al., 2016). So, to verify whether Tup11/12 proteins are also recruited to *rsv1* promoter site like Atf1, Rst2, and Scr1, we performed the ChIP-qPCR assay of Tup11/12 proteins. As shown in Fig. III-12, Tup11/12 were bound to *rsv1* gene regions of 1-3 at exponential phase (repression condition), showing the role of corepressors under glucose-rich condition. And when cells were glucose-starved (derepression condition), the bindings of Tup11/12 became slightly stronger, indicating Tup11/12 proteins did not dissociate from DNA under glucose starvation. There are several reports that Tup1 in *S. cerevisiae* and Tup11/12 in *S. pombe* are constitutively bound to target regions under derepression condition as well as repression condition. Under derepression condition, they are important for the chromatin remodeling or mediator recruitment (Asada et al., 2014; Buck and Lieb, 2006; Hirota et al., 2006; Papamichos-Chronakis et al., 2004; Wong and Struhl, 2011). So it is supposed that Tup11/12 mediate the repression of *rsv1* under glucose-rich condition, and help the transcriptional activation of *rsv1* under glucose-starved condition by recruiting the transcriptional machinery. Taken together, Tup11/12 proteins are bound to *rsv1* gene upstream region under glucose-rich condition, and their binding sites were shared with Atf1 and Rst2. This means that Tup11/12 proteins work antagonistically to Atf1 and Rst2 for transcriptional regulation of *rsv1*. Also, Tup11/12 proteins still reside in the same sites under glucose-starved media, proposing the function of Tup11/12 for transcriptional activation of *rsv1*.

Meanwhile, the interaction between Tup11/12 proteins and Rsv1 protein was examined via CoIP assay. Tup11/12 proteins are recruited to target regions via transcription factors. They are occupied to *fbp1* gene promoter with Scr1 under glucose-rich condition to repress *fbp1* gene. And when glucose becomes scarce, the Scr1 is replaced with Rst2 (Hirota et al., 2006). So like other zinc finger proteins, Rsv1 may interact with Tup11/12 proteins for the regulation of target genes. To find out whether Rsv1 can interact with Tup11/12 proteins, CoIP assay was conducted (Fig. III-13). As shown here, Rsv1 did not seem to interact with Tup11/12 proteins under glucose starvation, unlike other homologous proteins.

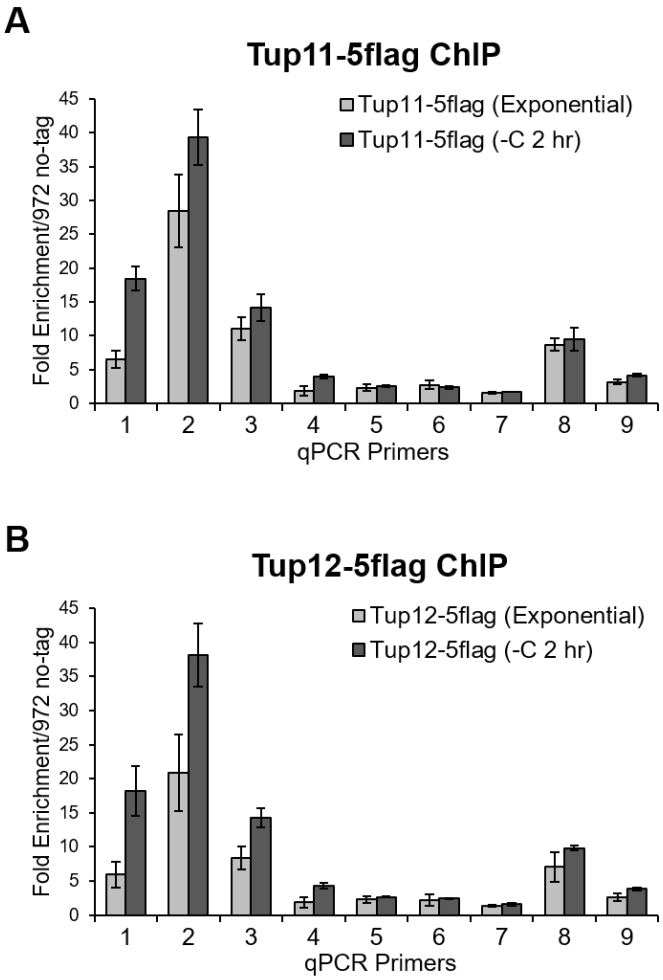


Figure III-12. Enrichment of Tup11/12 proteins to *rsv1*.

(A, B) ChIP-qPCR assays of Tup11-5flag and Tup12-5flag around *rsv1* gene. Cells were prepared for ChIP assay at exponential phase (Exponential) and after 2 hours of glucose starvation (-C 2 hr). Error bars were obtained as means ± SEM from three independent experiments.

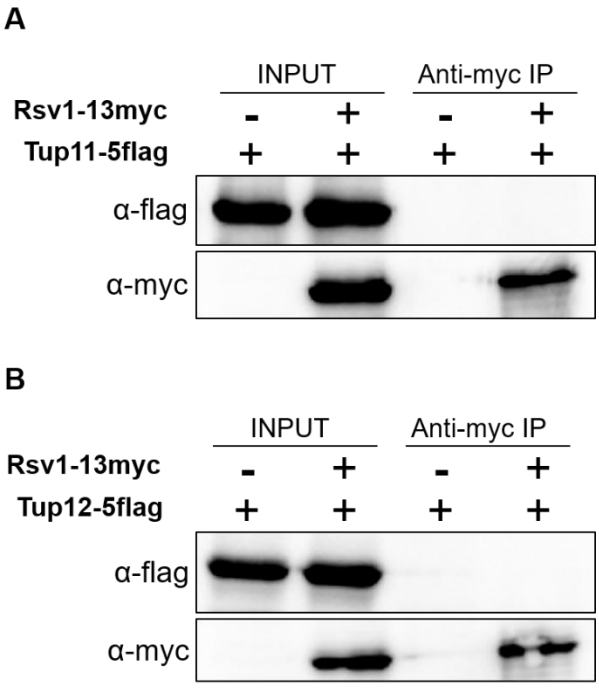


Figure III-13. Interaction between Tup11/12 and Rsv1.

(A, B) Co-immunoprecipitation assays for detection of interaction between Tup11/12 and Rsv1. Cells expressing Rsv1-13myc and Tup11/12 protein tagged with 5flag were prepared, and cell extracts were immunoprecipitated with an anti-myc antibody. For detecting co-precipitated Tup proteins, the immunoprecipitated extracts were analyzed with an anti-flag antibody.

III. 2. 7. Autoregulation of Rsv1

Mig1, the orthologue of Rsv1 in *S. cerevisiae*, is known to regulate itself by repressing the transcription of *MIG1* (Kaniak et al., 2004). But in *S. pombe*, to our knowledge, the homologous proteins Scr1 and Rst2 are not reported to autoregulate their own transcription. In upstream of *rsv1*, there are two motif sites of 'CCCCNC' which are known to be recognized by Rst2 (Kunitomo et al., 2000), and we confirmed that Rst2 is actually recruited to *rsv1* upstream (Fig. III-11). And interestingly, through ChIP-seq analysis, we have found that Rsv1 can recognize the same motif sequence as Rst2 (Fig. III-16). This was predictable because Rst2 and Rsv1 have the same amino acid sequences for DNA recognition in N-terminus. So we checked the possibility that Rsv1 can regulate its own gene transcription by binding *rsv1* upstream.

We first checked the RNA-seq results of wild-type cells and *Δrsv1* deletion cells under glucose-starved condition (Fig. III-14A, upper 4 lanes). As *Δrsv1* deletion strain was constructed by replacing *rsv1* CDS region with the hygromycin resistance gene cassette, *rsv1* CDS transcript was absent in *Δrsv1* cells under glucose starvation. Moreover, we could find that the transcription of 5′ region right before *rsv1* CDS was accumulated more in *Δrsv1* deletion cells than in wild-type cells. We also verified the increased expression of the 5′ upstream region of *rsv1* with RT-qPCR (Fig. III-14B). The transcription of the 5′ region of *rsv1* increased even more after 6 hours of glucose starvation in *Δrsv1* cells. As this upstream region may contain the transcription start region of *rsv1*, the increased transcription of this region reflects the activated *rsv1* induction in absence of Rsv1.

Meanwhile, Rsv1 was identified to be enriched to the upstream of *rsv1* in the ChIP-seq result (Fig. III-14A, bottom 2 lanes). The Rsv1 ChIP-peak summit resided in the region 2, wherein the 'CCCCTC' motif exists (Fig. III-10A). We verified the enrichment of Rsv1 over the regions of 1-9 with ChIP-qPCR (Fig.

III-14C). Rsv1 was strongly recruited to the regions of 1-3 and 8-9, which showed the very similar pattern to Rst2. So, like Rst2, Rsv1 can bind to *rsv1* regulatory region upon glucose starvation. For transcriptional regulation of *rsv1*, Rst2 acts as an activator, but Rsv1 acts as a repressor upon glucose starvation. As they share the same binding sites, they are thought to compete to bind to *rsv1* upstream. So far, Rsv1 is the only known repressor of *rsv1* transcription upon glucose starvation. To summarize, Rsv1 can bind to its own gene regulatory region, resulting in the repression of its transcription.

III. 2. 8. Model for the regulation of *rsv1* expression

Unlike Rst2 and Scr1, Rsv1 is mainly regulated at the transcriptional level. For the regulation of *rsv1* expression, three major pathways are important; MAPK pathway, AMPK pathway, and PKA pathway (Fig. III-15). When glucose is plentiful, Scr1 acts as a repressor by binding to the same site as Rst2. Rst2 is phosphorylated by Pka1 under glucose-rich condition, which blocks the import of Rst2 to the nucleus. When glucose becomes limited, MAPK protein Sty1 is activated to phosphorylate the target effector Atf1. Although Atf1 exists in *rsv1* upstream at the basal level under repression condition, Atf1 becomes more enriched upon glucose starvation, activating the transcription of *rsv1*. When glucose signal is absent, Ssp2 is phosphorylated to activate the phosphorylation of Scr1. Phosphorylated Scr1 is exported to the cytosol, exposing the binding motif. Upon glucose limitation, Pka1 is inactivated by Cgs1 regulatory subunit, resulting in the import of Rst2 to the nucleus. Rst2 can bind to the motif formally occupied with Scr1, activating the expression of *rsv1*. Induced Rsv1 also regulates its own transcription as a repressor.

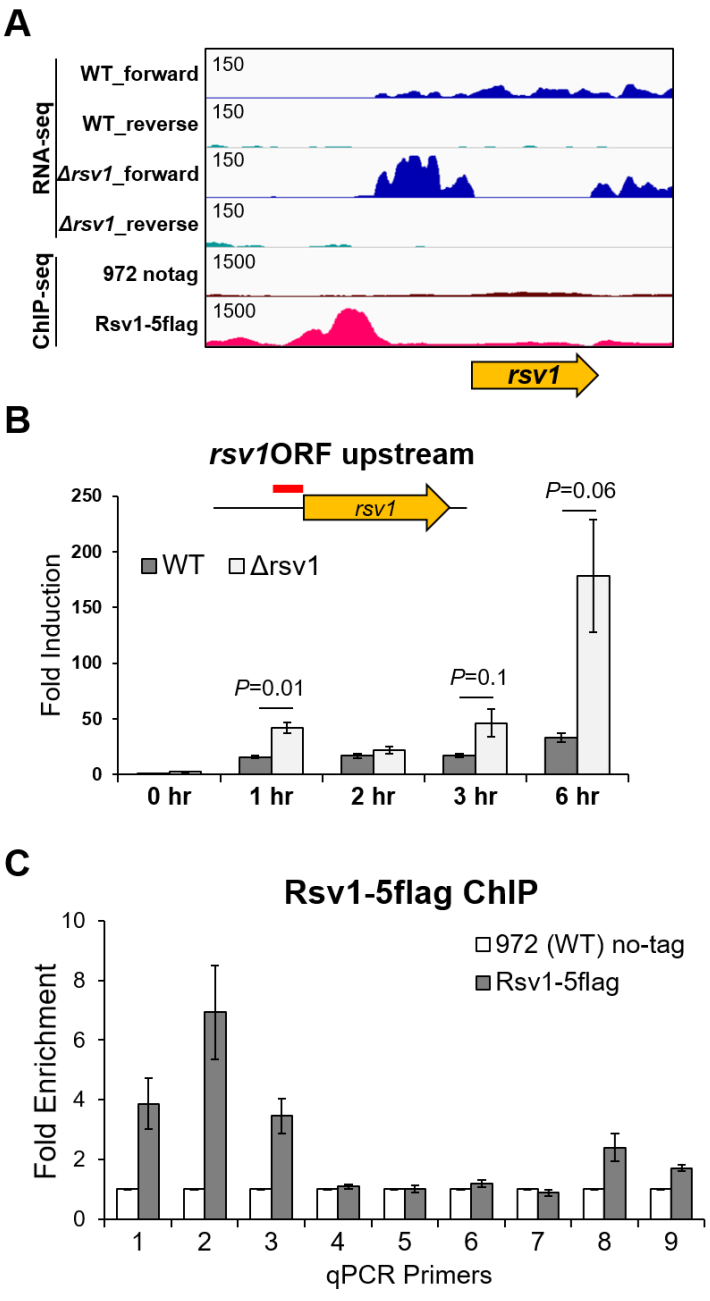

A
RNA-seq
WT_forward
WT_reverse
Δrsv1_forward
Δrsv1_reverse
ChIP-seq
972 notag
Rsv1-5flag
150
150
150
150
1500
1500
rsv1
B
rsv1ORF upstream
rsv1
P=0.06
WT
Δrsv1
Fold Induction
250
200
150
100
50
0
P=0.01
P=0.1
0 hr
1 hr
2 hr
3 hr
6 hr
C
Rsv1-5flag ChIP
972 (WT) no-tag
Rsv1-5flag
Fold Enrichment
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
qPCR Primers

Figure III-14. Rsv1 autoregulates its own gene expression.

(A) Representative image of RNA-seq and ChIP-seq results around *rsv1* gene locus. IGV views of strand-specific RNA-seq results of wild-type cells and *Δrsv1* deletion cells under the condition of 2 hours of glucose starvation are presented. All RNA-seq coverage tracks are normalized with size-factor calculated by DESeq2. ChIP-seq results were obtained under the same condition of RNA-seq from wild-type cells and cells expressing Rsv1-5flag. Schematic genomic region of *rsv1* (bottom) is also presented.

(B) Induction of the 5′ transcript of *rsv1*. Total RNA was extracted from 972 wild-type cells and *Δrsv1* deletion cells after glucose starvation for indicated times. Primer set was designed to detect the upstream of *rsv1* start codon (upper; bar). RT-qPCR results are presented as means ± SEM from three independent experiments (*P*-values from t-test).

(C) Rsv1-5flag enrichment around *rsv1* region. 972 no-tag cells and cells expressing 5flag-tagged Rsv1 were cross-linked after 2 hours of glucose starvation. Cells were collected and cell lysates were immunoprecipitated with an anti-flag antibody. Protein-bound DNA fragments were analyzed with primer sets of 1~9 as indicated in Fig. III-10A. Data are means ± SEM from three independent experiments.

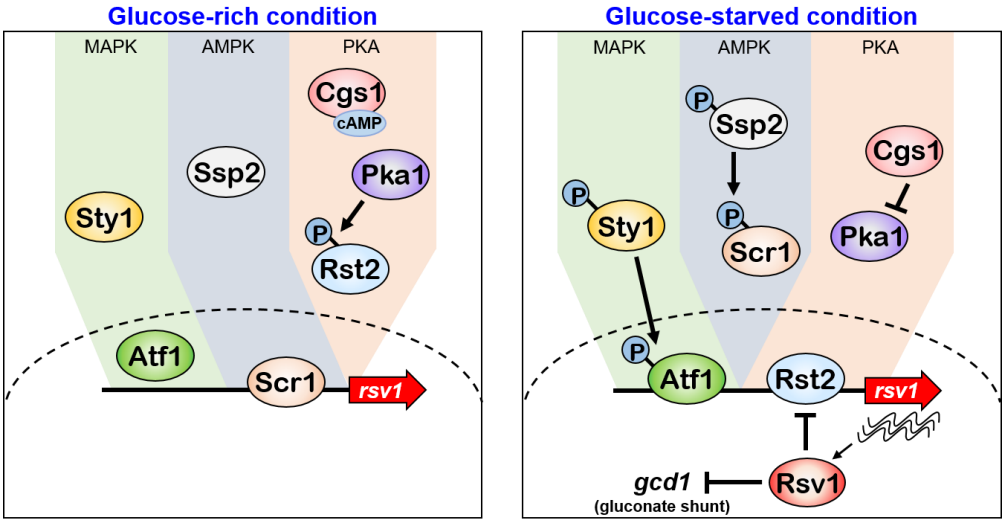


Figure III-15. Model for the transcriptional activation of *rsv1*.

Description of transcriptional regulation of *rsv1* under glucose starvation. Upon glucose starvation, phosphorylated Atf1 by Sty1 can bind to *rsv1* upstream regulatory region to induce its expression. Also, Rst2 can be recruited to *rsv1* upstream under glucose-starved condition to activate *rsv1* induction. Enrichment of Atf1 and Rst2 was restricted to the condition of glucose reduction. Besides, under glucose-rich condition, Scr1 is bound to *rsv1* gene shading the same binding sites as Rst2, which represses the expression of *rsv1*. But when glucose becomes depleted, the motif is occupied with Rst2, showing the function of Scr1 and Rst2 for reciprocal regulation of *rsv1* expression.

III. 3. Target Gene Regulation of Rsv1

III. 3. 1. Differentially expressed genes in *Δrsv1* cells; RNA-sequencing

To identify genes whose transcription are regulated by Rsv1, we performed RNA-seq experiments of gene expression from wild-type cells and *Δrsv1* deletion cells after 2 hours of glucose starvation. RNA expression results from two independently collected samples were analyzed to investigate the differentially expressed genes in *Δrsv1* deletion cells. Through DESeq2 analysis, a total of 232 genes was obtained. Among them, 74 genes were up-regulated more than 0.7 fold (value of $\log_2$FC), and 158 genes were down-regulated more than -0.7 fold (value of $\log_2$FC) in *Δrsv1* deletion cells than in wild-type cells. To examine which cellular processes are changed in *Δrsv1* cells, the DAVID GO analysis about the 232 differentially expressed genes was performed (Fig. III-16A). As shown here, the genes involved in transmembrane transport process, pentose phosphate pathway, and flocculation were up-regulated in *Δrsv1* cells. And the genes of galactose metabolic process, cell detoxification process, pyruvate metabolic process, and process for generating precursor metabolites were down-regulated in *Δrsv1* cells (Fig. III-16A). This indicates that diverse cellular metabolic processes are affected by the absence of Rsv1, suggesting that Rsv1 plays an important role in regulating various cell metabolic processes under glucose starvation condition.

III. 3. 2. Genome-wide enrichment of Rsv1; ChIP-sequencing

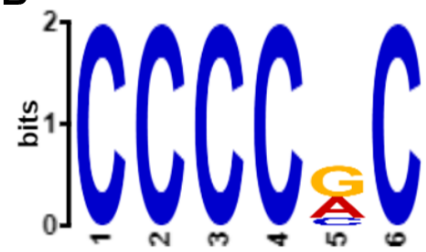
We next asked which genes Rsv1 can control directly to regulate cellular long-term lifespan under glucose-scarce condition. As *Δrsv1* deletion cells showed the accelerated cell death rate, it was supposed that the target genes regulated by Rsv1 may play a crucial role in maintaining cell survival in response to glucose depletion. To understand the regulatory network of Rsv1 under glucose-starved condition, we performed ChIP-seq analysis with wild-type

cells and cells expressing Rsv1-5flag for genome-wide identification of the regulatory regions of Rsv1. Through ChIP-seq analysis of peak-calling with Homer for transcription factors, we could identify that 71 genes have the Rsv1 enrichment peaks (*P*-value<0.01). Also, the FASTA file of the 71 peak regions was analyzed with MEME to search for the consensus motif sequences of Rsv1. As shown in Fig. III-16B, the sequence 'CCCCNC' was highly enriched at Rsv1 peak sequences. In fact, the presence of 'CCCCNC' sequence was predictable because Rst2 and Scr1, the homologous proteins of Rsv1, were reported to recognize the sequence 'CCCCNC'. As Rsv1, Rst2, and Scr1 have the conserved amino acid sequences for DNA recognition, it was supposed that they also recognize the same target DNA sequences. So via ChIP-seq, it was confirmed that the three zinc finger proteins, Rsv1, Rst2, and Scr1, can actually bind to the same DNA sequences.

A

	Biological Process	Count	P-Value
Repressor	transmembrane transport	6	2.30E-04
	meiotic cell cycle	6	2.90E-02
	pentose-phosphate shunt, oxidative branch	2	4.30E-02
	flocculation	2	5.70E-02
Activator	galactose metabolic process	4	2.80E-05
	cellular detoxification	5	2.00E-03
	dephosphorylation	3	5.40E-03
	pyruvate metabolic process	3	9.80E-03
	protein galactosylation	3	1.50E-02
	generation of precursor metabolites and energy	3	1.90E-02
	meiotic cell cycle	10	4.30E-02
	oxidation-reduction process	3	5.20E-02
	arginine catabolic process to proline via ornithine	2	5.80E-02
	galactose catabolic process via UDP-galactose	2	5.80E-02
	negative regulation of G2/M transition of mitotic cell cycle	3	6.20E-02
	extracellular polysaccharide metabolic process	3	6.20E-02
	oligosaccharide catabolic process	2	7.60E-02
	glycerol catabolic process	2	7.60E-02
	asparagine catabolic process	2	7.60E-02
	trehalose metabolism in response to stress	2	9.40E-02

B



E-value: 5.0e-002
Site Counts: 52 (73%)

C

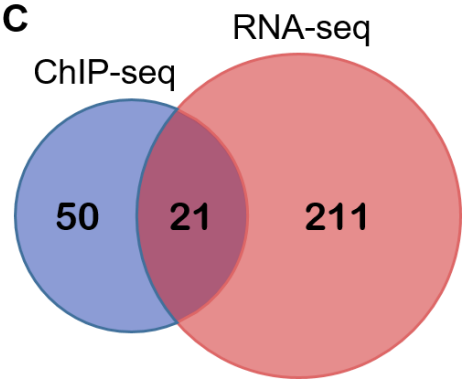


Figure III-16. Results of RNA-sequencing and ChIP-sequencing.

(A) GO analysis of RNA-seq result. The 74 genes which are up-regulated in *Δrsv1* cells (Repressor) and the 158 genes down-regulated in *Δrsv1* cells (Activator) were analyzed with DAVID separately. The number of genes involved in the specific biological process is presented (count) with *P*-value.

(B) MEME analysis for the consensus motif of Rsv1 using ChIP-seq result. The FASTA file of the upstream sequences of 71 genes with the Rsv1-5flag peak was used for MEME (537 base pair-long). The width was fixed to 6. *E*-value and site-counts of the presented motif are shown.

(C) Venn diagram of ChIP-seq and RNA-seq results. The list of genes with Rsv1 peak from ChIP-seq (71 genes) and the list of genes differentially expressed in *Δrsv1* cells (232 genes) were compared for the isolation of direct target genes of Rsv1. Among them, 21 genes were overlapped, showing that the 21 genes are the direct target of Rsv1 under glucose starvation.

III. 3. 3. Direct target genes of Rsv1

For discovering the direct regulon of Rsv1, we compared the 232 genes from RNA-seq result with the 71 genes which have the Rsv1 ChIP-seq peaks. And we could find that 21 genes are the direct target genes upon glucose starvation for 2 hours, whose transcription is dependent on Rsv1 (Fig. III-16C). Among the 21 target genes, Rsv1 repressed the induction of 13 genes and activated the expression of 8 genes when cells were starved for glucose (Fig. III-17). These genes were analyzed with KEGG pathway to figure out the metabolic pathways in which Rsv1 is involved. As a result, it was found that the carbon metabolic pathway is significantly modulated by Rsv1 (Fig. III-18). Here, the central glycolysis pathway, pentose phosphate pathway, gluconate shunt pathway, and glycerol metabolic pathway are shown to be regulated by Rsv1. Some genes like *gcd1*, *gnd1*, *gut2*, and *dak2* were revealed to have the consensus motif of Rsv1, and the expression of these genes was regulated by Rsv1.

Among the direct target genes of Rsv1, the expression of *gcd1* gene was highly dependent on Rsv1, and Rsv1 was prominently enriched to *gcd1* promoter site. Gcd1 was identified as a glucose dehydrogenase, and thus the presence of gluconate shunt pathway became defined in *S. pombe* (Corkins et al., 2017). As shown in Fig. III-18, glucose can be used to generate 6-phosphogluconate via both pentose phosphate pathway and gluconate shunt pathway in *S. pombe*, producing NADPH. And Rsv1 blocks the expression of *gcd1* and *gnd1* under glucose starvation condition. Actually, the inductions of these two genes are activated immediately when cells are starved of glucose. When the RNA-seq results of 972 wild-type cells are compared at exponential phase and at starvation condition (2 hours of glucose starvation), the induction of *gcd1* increased about 16-fold, and the transcription of *idn1* and *gnd1* increased 4-fold at the starvation condition. Therefore, when cells are starved, the influx to gluconate shunt pathway increases temporarily.

Judging from the fact that the gluconate shunt can make NADPH directly from glucose bypassing hexokinase and glucose 6-phosphate dehydrogenase (a rate-limiting enzyme), cells may have the reason for the additional activation of gluconate shunt pathway under glucose starvation. There can be some explanations. First, the gluconate shunt can produce the same amount of NADPH with less enzymes. For pentose phosphate pathway, cells need three enzymes; hexokinase (Hxk2), glucose 6-phosphate dehydrogenase (Zwf1), and 6-phosphogluconolactonase (SPCC16C4.10). But for the gluconate shunt, cells need two enzymes; glucose dehydrogenase (Gcd1) and glucokinase (Idn1), as gluconolactone can be converted to gluconate by a chemical equilibrium (Pocker and Green, 1973). So it is supposed that gluconate shunt pathway can be more beneficial to cells because the pathway produces the same amount of NADPH with fewer enzymes under starvation condition. It would be necessary to check the kinetics of pentose phosphate pathway and gluconate shunt. If the whole reactions of the gluconate shunt are more effective than the pentose phosphate pathway, cells necessarily use the gluconate shunt under starvation condition. Recently, the accumulating evidences show that hexokinase can function as a molecular switch as well as a glycolysis enzyme (Roberts and Miyamoto, 2015; Tan and Miyamoto, 2015). In mammalian cells, hexokinase II functions as a protective molecule. Hexokinase II is phosphorylated by Akt, and binds to mitochondria for mitochondrial protection. Also, hexokinase II binds and blocks TORC1, facilitating autophagy under glucose depletion condition (Roberts and Miyamoto, 2015). Although these processes may differ in *S. pombe*, it is possible that hexokinase is involved in other protective functions. So, under starvation condition, the enzymatic activity of hexokinase would be replaced by other activities. This may be the reason why cells need the gluconate shunt pathway without hexokinase under starvation condition. Therefore, cells need to upregulate the gluconate shunt

pathway for generating NADPH, together with pentose phosphate pathway. But when glucose becomes depleted completely, cells should shut-down both pentose phosphate pathway and gluconate shunt pathway because the substrate (glucose) for NADPH is absent. Also, cells need to reduce the synthesis of 5-carbon sugars for the nucleotides. Rsv1 may be involved in this process for blocking those pathways by repressing the related enzymes under glucose starvation. This process may be important for cells to save energy.

Meanwhile, the glycerol 3-phosphate shuttle pathway was activated by Rsv1, as the transcripts of cytosolic glycerol 3-phosphate dehydrogenase *gpd1* gene was reduced in *Δrsv1* cells. And the mitochondrial glycerol 3-phosphate dehydrogenase *gut2* was directly up-regulated by Rsv1, resulting in the activated glycerol 3-phosphate shuttle pathway (Matsuzawa et al., 2010). The glycerol 3-phosphate shuttle was suggested to play a role in the condition of low growth rate and to be important at the starvation condition in yeast, as the shuttle can activate the process of oxidative phosphorylation (Rodrigues et al., 2006). So cells activate the glycerol 3-phosphate shuttle pathway for facilitating the respiration under low energy condition. Thus, Rsv1 may be necessary for the activation of glycerol 3-phosphate shuttle under energy-limited condition, to help the respiration process.

And *dak2* gene, encoding a dihydroxyacetone kinase, was directly repressed by Rsv1. The *dak2* gene is involved in the process of glycerol assimilation, together with *gld1* encoding a glycerol dehydrogenase (Matsuzawa et al., 2010). These genes were found to be up-regulated in wild-type cells when cells were starved for glucose, when we analyzed the WT RNA-seq results of exponential phase and starvation condition (data not shown). Also, *gld1* was reported to have defect in glucose-derepression in *Δssp2* cells, showing that *gld1* pathway is regulated by glucose signal (Matsuzawa et al., 2011). So the repression of *dak2* by Rsv1 was quite unexpected, as the process of glycerol assimilation is

actually activated when cells are starved of glucose. One explanation is that Rsv1 can regulate the rate of glycerol assimilation under glucose starved-condition, although experimental proof would be necessary. Otherwise, as Dak2 is less effective than Dak1 for glycerol assimilation (Matsuzawa et al., 2010), Rsv1 may repress the induction of *dak2* under starvation condition as for energy costs.

Taken together, Rsv1 is important for regulating various carbon metabolic processes like NADPH-generating pathway, glycerol 3-phosphate shuttle pathway, and glycerol usage pathway. These metabolic pathways should be controlled carefully to maintain cellular energy homeostasis under nutrient starvation condition.

Gene	Systematic ID	Description	ChIPseq-FC	RNAseq-FC
	SPCC191.10	Schizosaccharomyces pombe specific protein	9.23	5.93
	SPAC1039.02	extracellular 5'-nucleotidase, human NT5E family (predicted)	7.13	4.80
gcd1	SPCC794.01c	glucose dehydrogenase	45.38	4.10
dak2	SPAC977.16c	dihydroxyacetone kinase	4.38	3.08
	SPCC1529.01	transmembrane transporter (predicted)	52.03	3.03
	SPAC17A2.11	Schizosaccharomyces pombe specific protein	18.75	2.69
mug146	SPCC1235.12c	Schizosaccharomyces specific protein	8.37	2.19
gnd1	SPBC660.16	phosphogluconate dehydrogenase, decarboxylating	20.07	2.16
map2	SPCC1795.06	P-factor pheromone	9.54	2.09
gsf2	SPCC1742.01	cell surface glycoprotein, galactose-specific flocculin	6.27	2.08
	SPAC683.03	Schizosaccharomyces pombe specific protein	6.1	1.80
amt1	SPCPB1C11.01	plasma membrane ammonium transmembrane transporter	5.1	1.69
erg11	SPAC13A11.02c	sterol 14-demethylase	9.03	1.63
pyp2	SPAC19D5.01	tyrosine phosphatase	4.96	0.61
	SPCC417.16	mitochondrial protein (predicted)	15.46	0.59
gut2	SPCC1223.03c	glycerol-3-phosphate dehydrogenase (predicted)	7.29	0.52
hsp9	SPAP8A3.04c	heat shock protein	5.67	0.47
	SPAC29A4.22	dubious	6.88	0.44
	SPAC26F1.11	dubious	6.68	0.28
	SPCC794.16	Putative uncharacterized protein	44.97	0.21
rsv1	SPBP4H10.09	transcription factor	13.24	0.01

Figure III-17. List of 21 direct target genes of Rsv1.

List of 21 direct target genes of Rsv1 obtained from the ChIP-seq and RNA-seq results. Predicted functions of the genes, the fold change value from ChIP-seq result, and the fold change value of RNA-seq result are presented together. Among 21 genes, 13 genes were up-regulated in *Δrsv1* cells and 8 genes were down-regulated in *Δrsv1* cells.

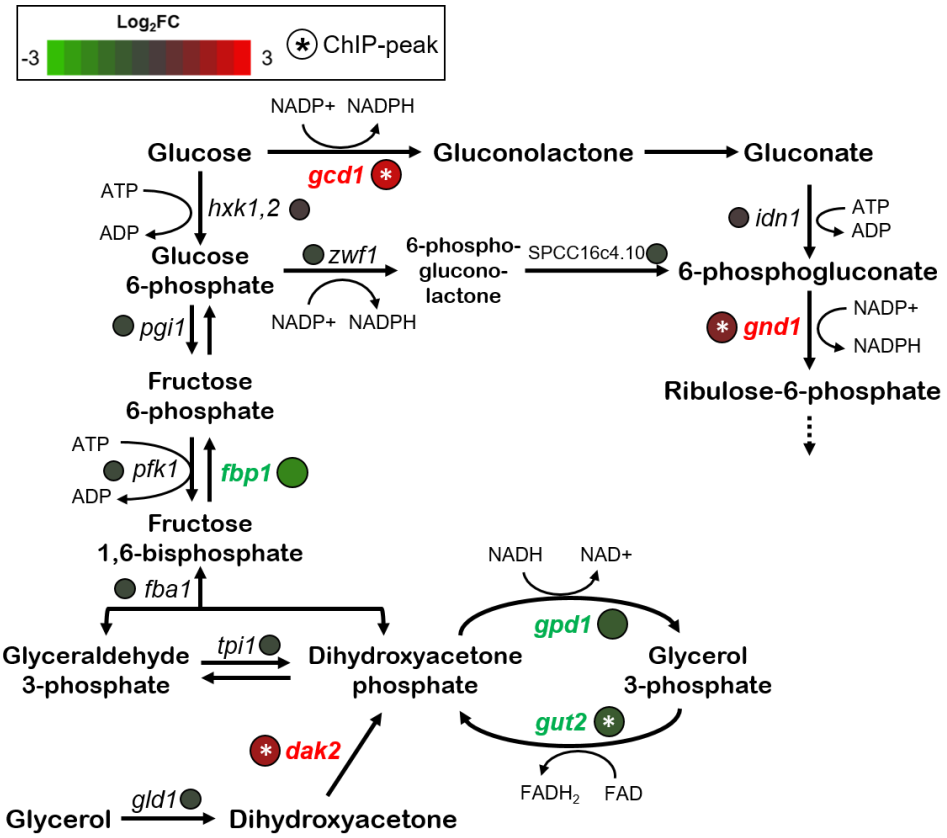


Figure III-18. Carbon metabolism processes regulated by Rsv1.

Carbon metabolism processes like glycolysis, pentose phosphate pathway, gluconate shunt pathway, and glycerol metabolism are briefly described. Significant target genes of Rsv1 (*gcd1*, *gnd1*, *fbp1*, *dak2*, *gpd1*, and *gut2*) involved in these processes are marked with their expression levels (the ratio of *Δrsv1* cells to wild-type cells). Red-colored genes are repressed by Rsv1, and green-colored genes are activated by Rsv1. Asterisks mean the presence of Rsv1 ChIP-peak.

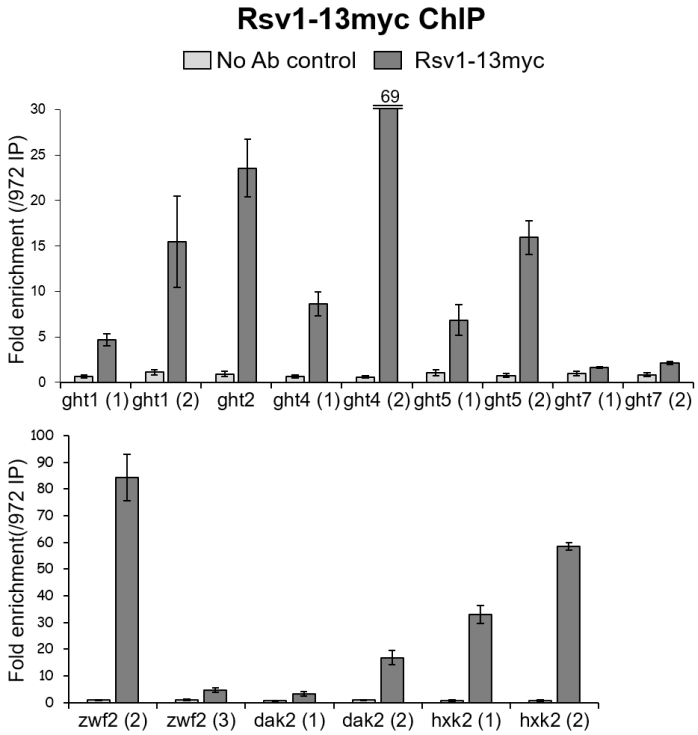


Figure III-19. Recruitment of Rsv1 to the genes related to glucose usage.
Wild-type cells and cells expressing Rsv1-13myc were grown in YE media, and transferred to YED for 2 hours. Cells were fixed with formaldehyde, and cells were lysed to be immunoprecipitated with an anti-myc antibody. The level of Rsv1-13myc binding was defined as 'fold enrichment' over a 972 no-tag control. No-antibody immunoprecipitation assay was also conducted as a control. Numbers of 1, 2, and 3 mean the different primer sets for the same genes. Data are means ± SEM from three independent experiments.

III. 3. 4. Rsv1 may work with other proteins for target regulation

When the ChIP-seq result was analyzed with HOMER peak calling with the option for transcription factor, the one significant motif 'CCCCNC' was obtained (Fig. III-16B). But when the ChIP-seq result was analyzed with MACS2 for peak calling, two consensus motifs were found in the target promoters of Rsv1 (Fig. III-20A). The first one is 'CCCCNC', which has been picked up by HOMER, and the second one is 'CCAAT'. And when SpaMo analysis was conducted with 'CCCCNC' as the primary motif sequence, the 'CCAAT' sequence was also obtained (Fig. III-20B). In addition to the finding of the second motif, the analysis with MACS2 suggested the more target genes of Rsv1 which were omitted when analyzed with HOMER. And these genes were found to be the actual target genes of Rsv1 via ChIP-qPCR assay (Fig. III-19). Thus, both programs for peak calling should be considered and used appropriately for purposes.

The 'CCAAT' sequence is well studied to be conserved in the promoters of many eukaryotic genes. In budding yeast, 'CCAAT' sequence is sensed by HAP complex, which consists of Hap2/Hap3/Hap5 proteins with DNA-binding capacity. And the fourth subunit Hap4 acts as an activator through its activation domain (Forsburg and Guarente, 1989; McNabb et al., 1995). Yeast *HAP4* gene transcription is repressed in glucose-rich media, while it is derepressed in non-fermentable carbon source media. And this *HAP4* expression mainly regulates the transcriptional activation capacity of HAP complex (Forsburg and Guarente, 1989). In *S. pombe*, Php2/Php3/Php4/Php5, the CBP (CCAAT-binding protein) complex, have the conserved short regions with budding yeast counterparts, and they can also recognize the 'CCAAT' motif (Mercier et al., 2006).

To find out whether the CBP complex can actually recognize the 'CCAAT'-containing sequences in the target genes of Rsv1, we constructed the strain

expressing Php2-5flag fusion protein. And when we performed the ChIP-qPCR assay of Php2-5flag, we could find that Php2 protein was enriched to the promoters of target genes of Rsv1, *gcd1* and *ght1*, which have 'CCAAT' sequence (Fig. III-20C, D). And the enrichment of Php2-5flag increased under glucose starvation condition. This leads to the conclusion that the 'CCAAT' motif of CBP complex is indeed distributed to the target promoters of Rsv1 under the condition of glucose limitation.

In regulating *fbp1* gene, Php2/3/5 complex is involved in the formation of open chromatin structure contributing the transcription at the precise TSS (transcription start site), which is antagonized by Tup11/12 corepressors (Asada et al., 2014). So it can be assumed that the CBP complex may help to modulate the chromatin configuration around the target genes of Rsv1 under the condition of glucose starvation. Further research would be necessary for demonstrating the action of CBP complex to modulate the transcription of the target genes together with Rsv1. Also, it would be interesting to study the enrichment and role of CBP complex under starvation condition at the genome-wide level. Besides the CBP complex, there may be other proteins that can work with Rsv1 under glucose starvation. When the distances between Rsv1 peak sites and TSSs of target genes were checked, Rsv1 was shown to locate far from the TSSs of genes. So there could be other mediator proteins working with Rsv1 for the regulation of target genes. Meanwhile, Rsv1 and Rst2 proteins recognize the same motif sequence, and both localize in the nucleus under glucose starvation. Although we could not compare the target genes of Rsv1 and Rst2, there may be some distinct target genes of Rsv1 and Rst2 under glucose starvation. And they would have the different binding partners for the regulation of the distinct target genes.

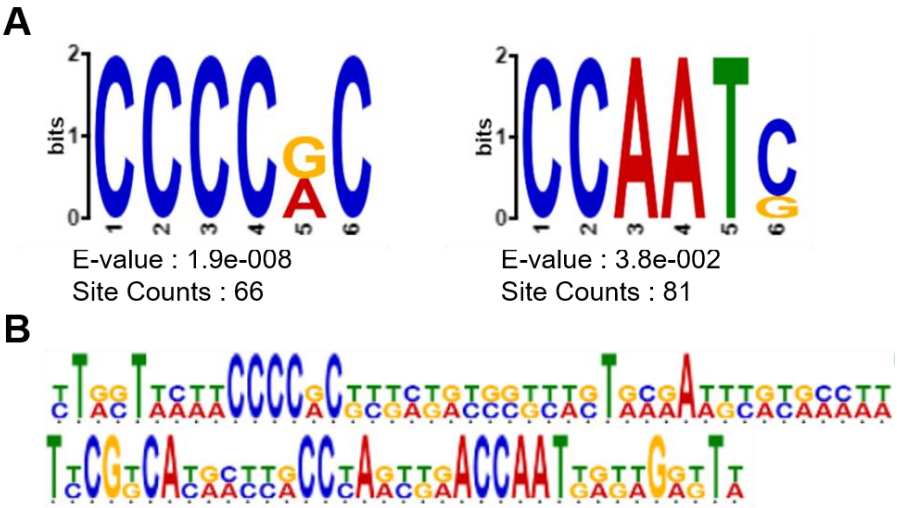

A
bits
E-value : 1.9e-008
Site Counts : 66
E-value : 3.8e-002
Site Counts : 81
B
C
972
No-tag
Rsv1-5flag
primer-3
primer-2
gcd1
primer-1
ght1
D
Php2-5flag ChIP
Php2-5flag (Exponential)
Php2-5flag (-C 2hr)
Fold Enrichment/972 no-tag
gcd1-primer-2
gcd1-primer-3
ght1-primer-1
ght1-primer-2

Figure III-20. The second consensus motif of Rsv1 obtained from ChIP-seq analysis.

(A) MEME analysis of Rsv1 ChIP-seq peaks. ChIP-seq peaks were obtained with MACS2, and the 170 targets which have the value of '-LOG_{10}(qvalue)>200' were selected. The FASTA file of peak summit +/- 100 bp sequences was analyzed for MEME. Two significant motifs obtained by MEME are shown with *E*-values and site-counts.

(B) SpaMo analysis of Rsv1 ChIP-seq peaks. The 'CCCCNC' sequence from (A) was used for the primary motif sequence. For input sequences, FASTA file of Rsv1 peak summit +/- 200 bp was used. CIS-BP Single Species DNA (*S. pombe*) was used as a motif database.

(C) IGV view of Rsv1-5flag ChIP-seq peaks around *gcd1* and *ght1* genes. Arrows show the ORF regions of the genes. Primers used for ChIP-qPCR assays were marked with boxes, indicating the primer location around the gene.

(D) ChIP-qPCR assay of Php2-5flag protein. Cells were grown in complex media to OD_{600} 0.7~0.8 (exponential), and cells were moved to starvation media for 2 hours (-C 2hr). Gene-specific primers were used for detection of the immunoprecipitated DNA. Php2-5flag binding was defined as 'fold enrichment' over 972 no-tag control.

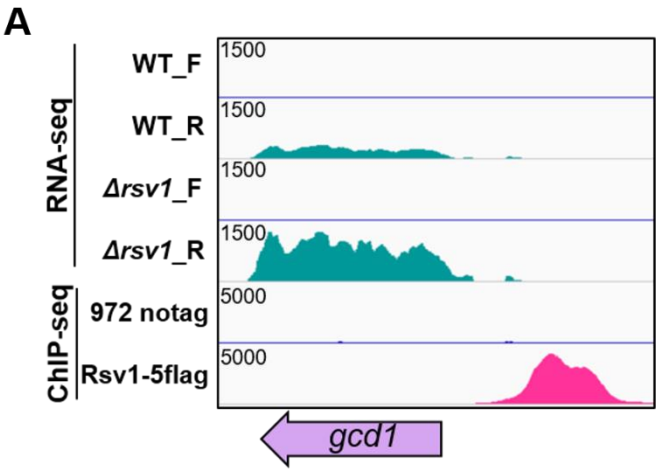
III. 3. 5. Gcd1 is an important factor for the function of Rsv1

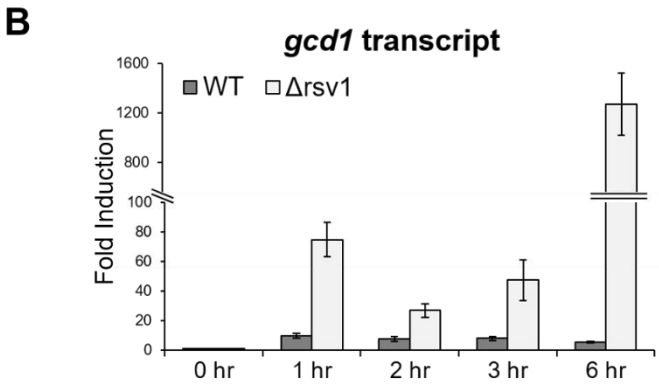
Gcd1 (SPCC794.01c) is a novel NADP+-dependent glucose dehydrogenase which acts in the gluconate shunt pathway with Idn1 (Corkins et al., 2017). At first, Gcd1 was supposed to be a glucose-6-phosphate dehydrogenase, but Corkins *et al*. reported that Gcd1 uses glucose as a substrate rather than glucose-6-phosphate, proving its role as a glucose dehydrogenase. And *gcd1* gene was the most prominent target gene of Rsv1 obtained from genome-wide sequencing. When analyzed through ChIP-seq and RNA-seq, Rsv1 was enriched to *gcd1* gene promoter with highest score, and the amount of *gcd1* gene product increased in *Δrsv1* mutant cells in glucose-starved media (Fig. III-21A). Also, we have found that *gcd1* transcript was also accumulated much in *Δrsv1* mutant cells at stationary phase (in RNA-seq analysis, data not shown).

We extracted total RNA from wild-type cells and *Δrsv1* mutant cells for detecting *gcd1* transcript under the condition of extended starvation. As shown in Fig. III-21B, *gcd1* transcript was accumulated much over time in *Δrsv1* mutant cells. The *gcd1* transcript also increased in wild-type cells in 30 minutes, but the increase disappeared in long-term starvation. Like the transcript level, Gcd1 protein was also accumulated much in *Δrsv1* mutant cells (Fig. III-21C). We prepared total protein extracts for electrophoresis both in wild-type cells and *Δrsv1* mutant cells at 6 hours of glucose starvation, and could detect a different band pattern (Fig. III-21C, arrow). The band was identified as Gcd1 protein via LC-MS/MS. Thus, both *gcd1* transcript and Gcd1 protein were accumulated in *Δrsv1* mutant cells, and the degree of the accumulation was larger under the condition of long-term starvation.

We checked the enrichment of Rsv1 to *gcd1* promoter region through ChIP-qPCR assay. Rsv1 protein remained enriched to *gcd1* promoter until 8 hours after starvation (Fig. III-22A). Next, to find out the relevance of *gcd1* in maintaining cell survival of *Δrsv1* cells, we examined the cellular viabilities of

wild-type cells, *Δrsv1* cells, *Δgcd1* cells, and *Δrsv1Δgcd1* double deletion cells. As presented in Fig. III-22B, *Δrsv1Δgcd1* double deletion cells showed the prolonged viability in comparison to *Δrsv1* single mutant cells. This means that the deletion of *gcd1* can suppress the phenotype of *Δrsv1* mutant cells, although full recovery of cell viability was not accomplished. Therefore, our analysis revealed that *gcd1* is one of the main effector genes of Rsv1, and that the repression of *gcd1* expression by Rsv1 is important for maintaining cell viability in glucose-depleted condition.


A
RNA-seq
WT_F
WT_R
Δrsv1_F
Δrsv1_R
ChIP-seq
972 notag
Rsv1-5flag
1500
1500
1500
1500
5000
5000
gcd1


B
gcd1 transcript
WT
Δrsv1
Fold Induction
1600
1200
800
100
80
60
40
20
0
0 hr
1 hr
2 hr
3 hr
6 hr

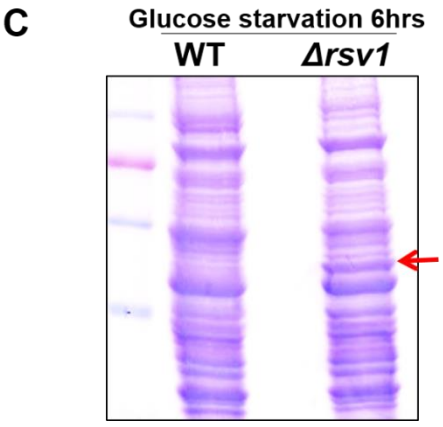

C
Glucose starvation 6hrs
WT
Δrsv1

Figure III-21. Overexpressed *gcd1* transcript and Gcd1 protein in *Δrsv1* cells.
(A) Representative IGV view of RNA-seq and ChIP-seq results around *gcd1* gene locus. Strand-specific RNA-seq result is presented with one of two sets of sequencing assays (F: forward, R: reverse), and the location of Rsv1 ChIP-peak around *gcd1* gene locus is depicted.
(B) *gcd1* transcript levels of wild-type cells and *Δrsv1* deletion cells in glucose-starved condition. After cDNA synthesis, *gcd1*-specific primer set was used for RT-qPCR analysis. Results are presented as means ± SEM from three independent experiments.
(C) Overexpressed Gcd1 protein in *Δrsv1* deletion cells. Cells were grown to exponential phase in YE media, and transferred to YED starvation media to be incubated for 6 hours. Total protein extracts were prepared from wild-type cells and *Δrsv1* deletion cells. Protein extracts were separated in SDS-PAGE gel. Differentially overexpressed protein band in *Δrsv1* deletion cells (arrow) was isolated and identified with LC-MS/MS.

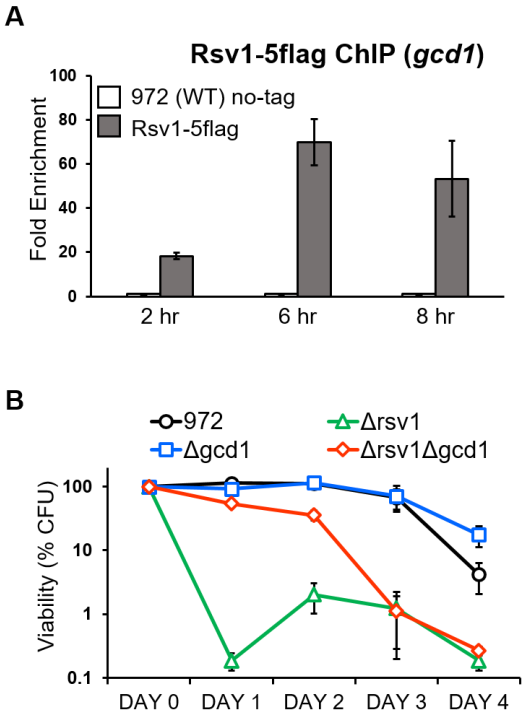


Figure III-22. *gcd1* is important for regulating the viability of *Δrsv1* cells.

(A) Long-term binding of Rsv1 protein to *gcd1* upstream. Wild-type cells and cells expressing Rsv1-5flag were grown to OD_{595} 0.7~0.8 in YE media, and moved to YED media. After the transfer, cells were collected as indicated times for ChIP-qPCR assay. *gcd1* upstream region-specific primer set (*gcd1*-up) was used for qPCR. Data means ± SEM from three independent experiments.

(B) Viability of WT, *Δrsv1*, *Δgcd1*, and *Δrsv1Δgcd1* cells in glucose-starved condition. Cells were prepared as in Fig. III-1B in YE complex media. Glucose-starved cells were daily collected and spotted onto YE solid media. The number of colonies was counted, and the colony number at day 0 was regarded as 100% survival rate. Error bars show SEM from three biological independent results.

III. 3. 6. Expression of the genes involved in pentose phosphate pathway and gluconate shunt pathway in *Δrsv1* cells

In addition to pentose phosphate pathway (PPP), gluconate shunt pathway is another pathway to generate NADPH and 6-phosphogluconate in *S. pombe*. Gluconate shunt can bypass hexokinase as well as glucose 6-phosphate dehydrogenase, which is a rate-limiting step of PPP. When glucose enters the cell, glucose is rapidly phosphorylated to glucose 6-phosphate by hexokinase. And glucose 6-phosphate is delivered to either glycolysis or PPP. When glucose concentration becomes low, both glycolysis influx and PPP influx must be regulated as they are tightly connected. Under glucose-limited condition, glycolysis rate becomes low and cells produce energy by mitochondrial respiration. Thus, cells need more NADPH for scavenging the ROS generated by respiration through promoting PPP activity (Zhao et al., 2017).

Gcd1, the direct target of Rsv1, is involved in the gluconate shunt of pentose phosphate pathway, and is derepressed in *Δrsv1* cells. Besides Gcd1, many other enzymes are known to support gluconate shunt and PPP. Hexokinase (Hxk2) can convert glucose to glucose 6-phosphate, and glucose 6-phoshpate dehydrogenase (Zwf1) generates NADPH and 6-phosphogluconolactone by oxidizing glucose 6-phosphate. 6-phosphogluconolactone is converted to 6-phosphogluconate by 6-phosphogluconate dehydrogenase (SPCC16c4.10) via oxidative decarboxylation. In gluconate shunt, glucose is directly oxidized to gluconolactone by glucose dehydrogenase (Gcd1). In aqueous solution, gluconate exists in equilibrium with the cyclic ester gluconolactone, and gluconate is phosphorylated to 6-phosphogluconate by glucokinase (Idn1). 6-phosphogluconate generated by PPP and gluconate shunt can be converted to ribulose 5-phosphate by phosphogluconate dehydrogenase (Gnd1) (Fig. I-6).

To investigate whether other gluconate shunt genes are also regulated by Rsv1, the enrichment of Rsv1 to these genes was examined. To detect the

recruitment of Rsv1 in prolonged starvation condition, cells were glucose-starved for 2 hours, 6 hours, and 8 hours. As shown in Fig. III-23, Rsv1 was bound to *hxk2* and *zwf1* of PPP, and to *idn1* of gluconate shunt. Also, Rsv1 was recruited to *gnd1* upstream region. Thus, Rsv1 seems to be enriched to gluconate shunt and PPP genes under glucose starvation, and Rsv1 becomes more enriched to these genes under prolonged starvation condition. And the expressions of these genes were investigated in wild-type cells and *Δrsv1* cells (Fig. III-24). As we could not detect these genes in the gene list of RNA-seq result of 2 hours of glucose starvation condition, we predicted that the expressions of these genes are not changed in *Δrsv1* cells under short-term starvation of glucose. As predicted, the genes of gluconate shunt and PPP were similarly expressed in wild-type and *Δrsv1* cells under 1 or 2 hours of glucose starvation. But when cells were starved for prolonged time, *idn1*, *hxk2*, *zwf1*, and *gnd1* genes were highly expressed in *Δrsv1* cells. And SPCC16c4.10 gene was also induced twice in *Δrsv1* cells after 10 hours of starvation (Fig. III-24). Taken together, Rsv1 is responsible for repressing the genes of gluconate shunt and PPP under the prolonged starvation of glucose (Fig. III-25).

Then, why is Rsv1 repressing the genes of gluconate shunt and PPP under glucose starvation? Given the fact that gluconate shunt also produces NADPH like PPP, it is supposed that gluconate shunt plays a role in generating reducing power together with PPP. Under low glucose level, cells activate the mitochondrial respiration, resulting in ROS production. So NADPH must be generated via gluconate shunt or PPP upon glucose reduction. When glucose becomes depleted completely, cells cannot use glucose, and should stop activating both gluconate shunt and PPP. In this condition, the generation of NADPH and the activation of PPP and gluconate shunt are not necessary for cells. So the expression of the genes involved in gluconate shunt and PPP should be shut down under the prolonged starvation condition. And Rsv1 is

supposed to regulate this process. Given that Rsv1 is more enriched to those genes in the prolonged starvation, Rsv1 seems to be involved in the repression of both gluconate shunt and PPP genes when glucose becomes depleted. And as shown in the case of *gcd1*, this repressive function of Rsv1 may be important for maintaining cell survival under glucose-starved condition. Therefore, Rsv1 may be involved in modulating the metabolic pathways for adapting cells to starvation condition.

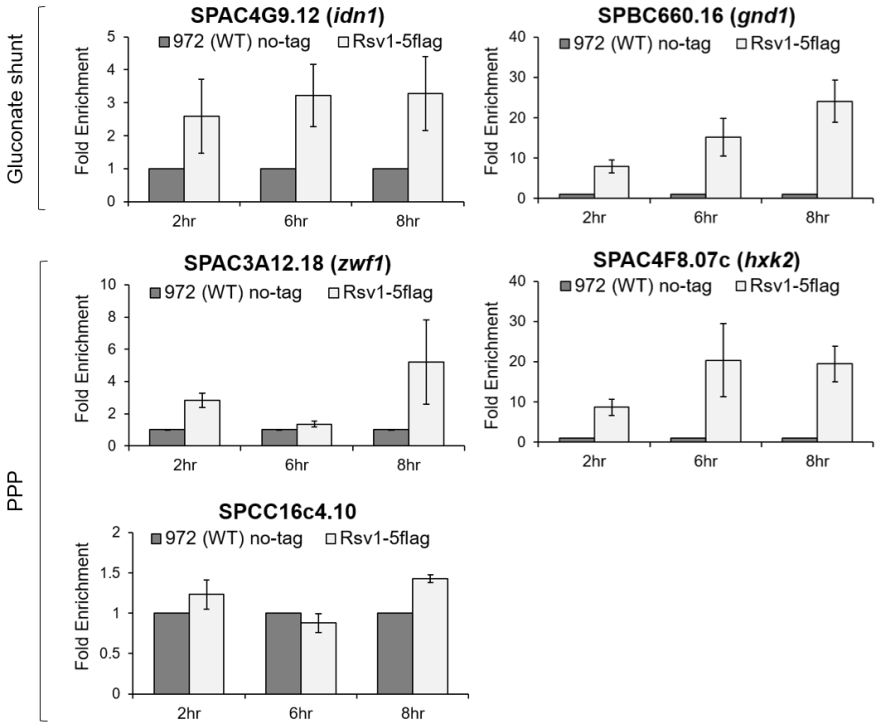


Figure III-23. Recruitment of Rsv1 to gluconate shunt genes and PPP genes. Enrichment of Rsv1-5flag protein to the genes of gluconate shunt pathway and PPP was explored (Fig. I-6). Cells were grown in YE complex media, and transferred to YED media and incubated as indicated times (2 hr, 6 hr, and 8 hr). Cells were collected and cell extracts were immunoprecipitated with an anti-flag antibody. Immunoprecipitated DNA fragments were analyzed with gene-specific primers (Table II-4). Results were presented with the value of the fold enrichment over 972 no-tag cells. Error bars mean the SEM from three independent assays.

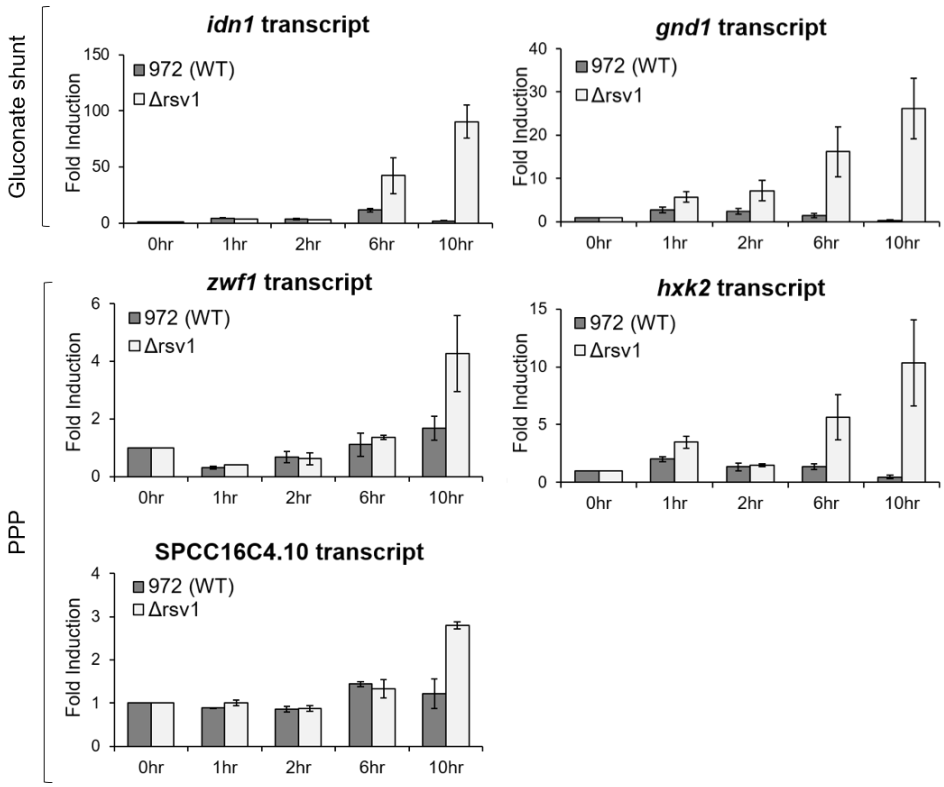


Figure III-24. Expression of gluconate shunt and PPP genes in Δ*rsv1* cells under glucose-starved condition.

Expression of the genes of gluconate shunt and PPP was explored in wild-type cells and Δ*rsv1* cells. Exponentially-grown cells were transferred to glucose-starved YED media, and further incubated as indicated times (0 hr, 1 hr, 2 hr, 6 hr, and 10 hr). Total RNA extracts were analyzed with RT-qPCR. Gene-specific primer sets were used for the detection of gene induction (Table II-3). Error bars indicate the SEM from three independent assays.

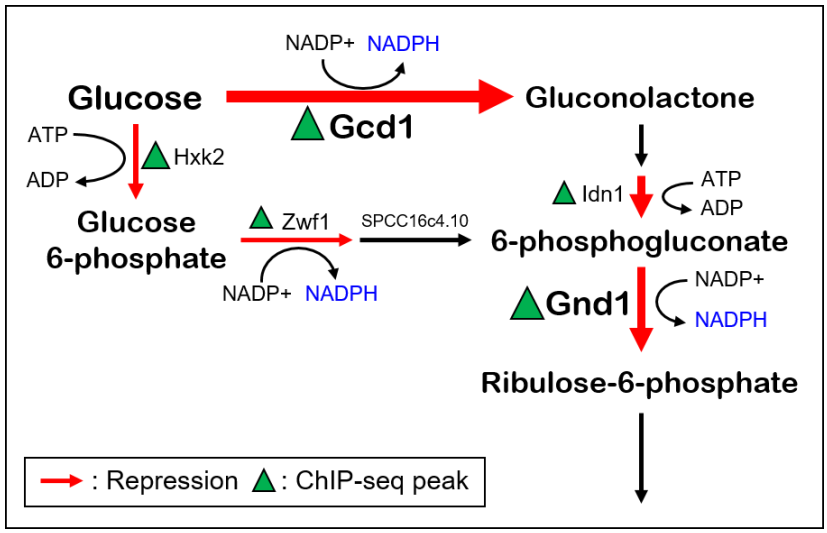


Figure III-25. Model for the regulation of PPP and gluconate shunt genes by Rsv1 under long-term glucose starvation condition.

The regulatory function of Rsv1 for PPP and gluconate shunt genes is described. Under the condition of prolonged starvation of glucose, Rsv1 represses many of PPP and gluconate shunt genes. Especially, Rsv1 inhibits the influx of PPP and gluconate shunt by repressing the expression of *gcd1*, *hxk2*, and *zwf1*, which regulate the flow of these pathways. The red arrows show the function of Rsv1 as a repressor. The green triangle is marked when the gene has the peak of Rsv1 ChIP-seq result.

III. 3. 7. Rsv1 may be involved in maintaining zinc homeostasis under glucose starvation

Gcd1 was first identified to be responsible for accumulation of gluconate in *Δloz1* deletion cells, proving its involvement in gluconate shunt pathway. Loz1 (SPAC25B8.19c; for *loss of zinc sensing*) is the main regulator of cellular zinc level in *S. pombe*. The transcription of *loz1* decreases at high zinc level, but Loz1 gains its repressor activity under high zinc condition. It was revealed that the induction of *gcd1* is repressed under zinc-replete condition, which is dependent on Loz1 repressor. The Loz1-dependent repression of *gcd1* under zinc-rich condition implies that the regulation of gluconate shunt influx may be involved in zinc homeostasis and cell survival (Corkins et al., 2017). So the finding that the induction of *gcd1* is also repressed by Rsv1 led us to investigate the relevance of Rsv1 to zinc homeostasis.

In addition to *gcd1*, there are other zinc-related genes that are regulated by Rsv1 (Fig. III-26). Zrt1 is a zinc-regulated transporter, and can uptake zinc under zinc-limited condition (Corkins et al., 2013; Dainty et al., 2008). And Zym1 is a metallothionein, which is required for capturing excess zinc through the thiol group of its cysteine residues (Borrelly et al., 2002; Corkins et al., 2013). Free zinc ion is highly deleterious to cells, so the cellular zinc ion should be monitored and regulated deliberately. As shown in Fig. III-26, *zrt1* and *zym1* genes were directly regulated by Rsv1. Rsv1 can be recruited to the promoters of both *zrt1* and *zym1* under glucose starvation condition. Moreover, the binding of Rsv1 became stronger under longer starvation condition (8 hr). When cells were starved for glucose, *zrt1* gene was repressed and *zym1* was highly up-regulated in wild-type cells. But in *Δrsv1* cells, *zrt1* was not repressed and *zym1* was not fully activated (Fig. III-26). Thus, Rsv1 seems to be responsible for regulation of the expressions of *zrt1* and *zym1* under glucose starvation, proving its involvement in maintaining zinc homeostasis.

It is unclear how the cellular zinc level changes upon glucose starvation. But in the light of the transcriptional changes of wild-type cells, it is supposed that cellular zinc level becomes higher upon glucose starvation, because *zrt1* level becomes lower and *zym1* level becomes higher under glucose starvation condition. So it may be important for cells to adapt to the changes of cellular zinc level after glucose becomes depleted. And Rsv1 may play a role in responding to zinc levels under glucose starvation.

To find out the relevance of Rsv1 to cellular zinc status, we checked the responses of *Δrsv1* cells to various zinc levels (Fig. III-27). As the cellular zinc level seems to be higher under glucose starvation, TPEN (*N,N,N',N'*-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine), the chelator for zinc with high affinity, was first treated to wild-type cells and *Δrsv1* cells. But we could not find any effect of TPEN (data not shown). Next, we treated various concentrations of zinc to wild-type cells and *Δrsv1* cells, and it was revealed that high concentrations of zinc can help *Δrsv1* cells to recover their viability under glucose starvation (Fig. III-27A). *Δrsv1* cells showed the prolonged viability when treated with 800 μM $ZnSO_4$ after glucose starvation. To test whether this phenomenon can happen with another metal, $FeCl_2$ of 800 μM was also treated to cells (Fig. III-27A). But when supplemented with $FeCl_2$, *Δrsv1* cells could not recover their viability. Thus, only zinc can suppress the viability defect of *Δrsv1* cells under glucose starvation, when treated at high concentration. Also, the high zinc could suppress the viability defect of *Δrsv1* cells even at stationary phase (Fig. III-27B). Taken together, the high concentration of zinc can help to recover the viability of *Δrsv1* cells under glucose-deprived condition, suggesting the relationship between Rsv1 and zinc homeostasis.

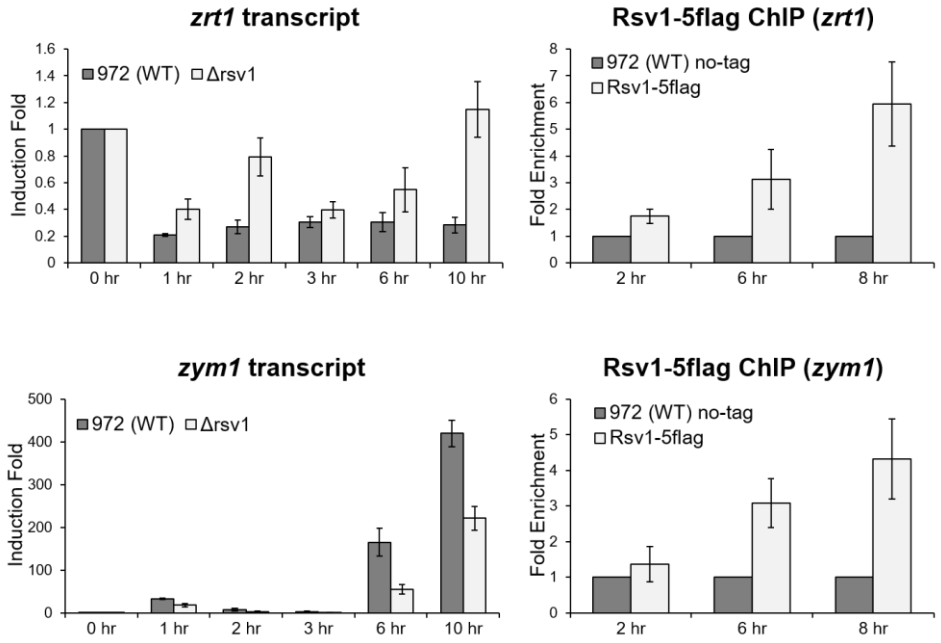


Figure III-26. Rsv1 can regulate zinc-related genes under glucose starvation. For RT-qPCR assays, wild-type cells and *Δrsv1* cells were prepared in complex media. Cell aliquots were collected upon glucose starvation at indicated times for total RNA preparation. Gene-specific primer sets were used for RT-qPCR. Results were presented as means ± SEM from three independent assays. For ChIP-qPCR assays, wild-type cells and cells expressing Rsv1-5flag were glucose-starved for indicated times in complex media. Gene promoter-specific primer sets were used for ChIP-qPCR. Error bars indicate the SEM from three independent assays.

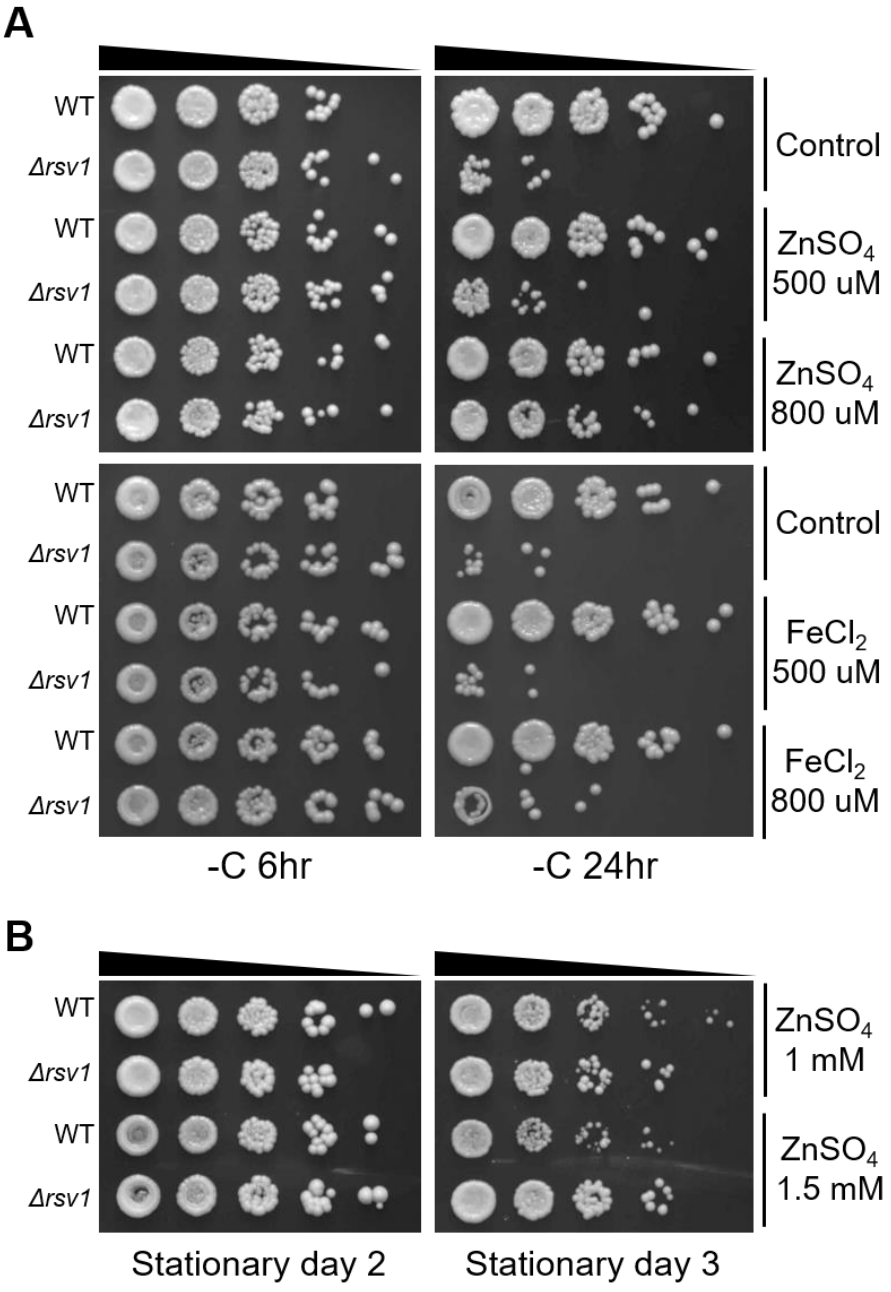

A
WT
Δrsv1
WT
Δrsv1
WT
Δrsv1
Control
ZnSO4
500 uM
ZnSO4
800 uM
WT
Δrsv1
WT
Δrsv1
WT
Δrsv1
Control
FeCl2
500 uM
FeCl2
800 uM
-C 6hr
-C 24hr
B
WT
Δrsv1
WT
Δrsv1
ZnSO4
1 mM
ZnSO4
1.5 mM
Stationary day 2
Stationary day 3

Figure III-27. Effect of high concentrations of zinc on *Δrsv1* cells.

(A) The effect of high concentrations of zinc and iron on wild-type cells and *Δrsv1* cells after glucose starvation. Cells were grown in YE complex media and moved to YED media at exponential phase for glucose starvation. Soon after the media change, $ZnSO_4$ or $FeCl_2$ were treated to YED media as indicated concentrations. After 6 and 24 hours of incubation, the same numbers of cells were spotted onto YE solid media.

(B) The effect of high concentrations of zinc on wild-type cells and *Δrsv1* cells at stationary phase. Cells were grown to stationary phase in YE media. When cells reached the plateau phase, $ZnSO_4$ of 1 mM and 1.5 mM was treated to the cells. Cells were diluted and spotted onto YE solid media after 48 (day 2) and 72 (day 3) hours.

III. 3. 8. Rsv1 may function in maintaining cell viability by repressing *loz1* induction under long-term starvation of glucose

Repressor Loz1 was reported to regulate target genes at the condition of excess zinc for zinc homeostasis in *S. pombe.* But the expression of *loz1* gene is activated under the condition of low zinc (Corkins et al., 2013). When cells are treated with excess zinc, Loz1 directly represses the *zrt1* gene, encoding the high affinity zinc transporter, and indirectly activates the *zym1* gene, encoding the metallothionein. As Rsv1 also regulates the expression of *zrt1* and *zym1*, the relationship between Rsv1 and Loz1 was explored (Fig. III-28). The *loz1* gene has the consensus motif sequence of Rsv1 at upstream region. Although there is no exact 'CCCCNC' sequence, *loz1* has four 'CCCC' sequences at is upstream. There is also the ChIP-peak of Rsv1 at *loz1* upstream region (Fig. III-28A). And when the expression of *loz1* was monitored, the transcription of *loz1* was derepressed in *Δrsv1* cells only under the long-term starvation condition (from 6 hours of starvation) (Fig. III-28B). Therefore, Rsv1 is involved in the direct repression of *loz1* transcription upon prolonged starvation of glucose.

To find out the phenotypic relationship between Rsv1 and Loz1, the viability test was conducted in WT, *Δrsv1*, *Δloz1*, and *Δrsv1Δloz1* cells (Fig. III-28C). And it was revealed that *Δrsv1Δloz1* cells recovered the viability defect of *Δrsv1* single mutant cells. *Δloz1* cells showed the similar viability with wild-type cells. Thus, it seems that the repressive function of Rsv1 on *loz1* transcription has an important role in regulating cell survival, which may be associated with zinc homeostasis. As shown in Fig. III-27, high concentrations of zinc can help *Δrsv1* cells to recover their survival. Since the transcription of *loz1* is repressed by high zinc, the treatment of high zinc may reflect the repression of *loz1* transcription. Thus, the effect of high zinc described in Fig. III-27 may be caused by the repression of *loz1* transcription, which should be proven experimentally. But still, the reason why the expression of *loz1* should be

repressed under the condition of prolonged starvation of glucose remains elusive.

Taken together, Rsv1 has two important repressive roles, repression of *gcd1* (gluconate shunt) and repression of *loz1*, which are important for maintaining cell viability (Fig. III-29). Rsv1 represses the induction of *gcd1* much stronger under the condition of prolonged starvation of glucose. Moreover, in this condition, Rsv1 is also involved in the repression of other genes of gluconate shunt and PPP. At the same time, Rsv1 represses *loz1*, which is also important for maintaining cell survival. Interestingly, *gcd1* was also found to be regulated by Loz1 under zinc-replete condition. So it would be interesting research to find out whether the repressive role of Rsv1 for *loz1* is associated with Gcd1 and zinc homeostasis. And the reason why Rsv1 represses *loz1* expression under glucose starvation remains to be examined in the future.

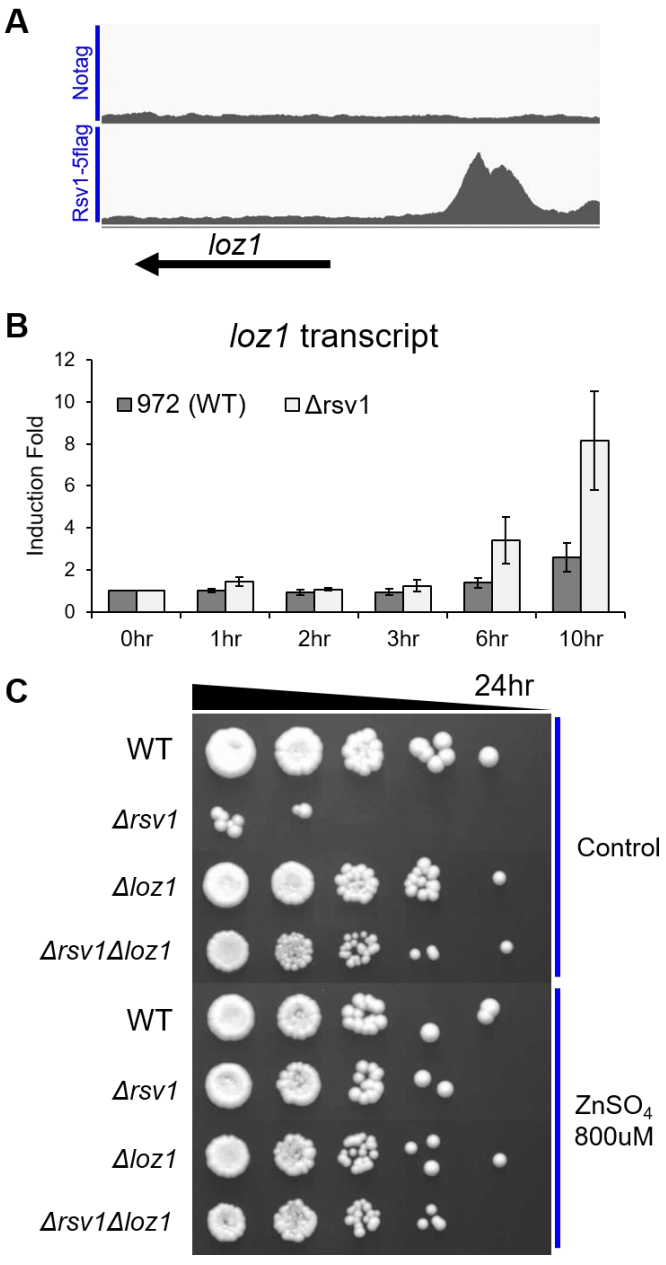

A
Notag
Rsv1-5flag
loz1
B
loz1 transcript
Induction Fold
12
10
8
6
4
2
0
972 (WT)
Δrsv1
0hr
1hr
2hr
3hr
6hr
10hr
C
24hr
WT
Δrsv1
Δloz1
Δrsv1Δloz1
Control
WT
Δrsv1
Δloz1
Δrsv1Δloz1
ZnSO4
800uM

Figure III-28. Rsv1 represses the *loz1* transcription for cell survival.

(A) Rsv1-5flag ChIP-seq result for *loz1* gene region. IGV picture was obtained around *loz1* gene (reverse). The location of *loz1* CDS is marked with an arrow.

(B) Induction of *loz1* in wild-type and *Δrsv1* cells under glucose-starved condition. Cells were glucose-starved in YE complex media for indicated times. Total RNA extracts were obtained from each sample, and synthesized to cDNAs for RT-qPCR. The *loz1* upstream-specific primer set was used for qPCR. Error bars indicate the SEM from three independent assays.

(C) Viability spotting assay of wild-type, *Δrsv1*, *Δloz1*, and *Δrsv1Δloz1* cells. Cells were grown in YE complex media to exponential phase, and transferred to YED media supplemented with 800 μM $ZnSO_4$. Control samples were not treated with $ZnSO_4$. After 24 hours of further incubation, cell aliquots were spotted onto YE solid media.

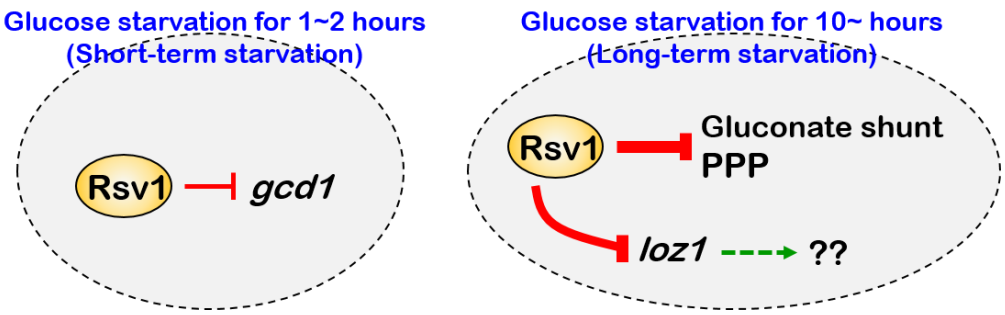


Figure III-29. Model for the function of Rsv1 for cell survival.

There are two strategies for Rsv1 to regulate cell survival. First, Rsv1 represses the expression of *gcd1*, the gluconate shunt gene. And when cells are starved for glucose for a long time, Rsv1 helps to repress gluconate shunt pathway and pentose phosphate pathway. This process may be important for maintaining cell energy status. Second, Rsv1 represses the expression of *loz1*. It may be important to repress the induction of *loz1* under glucose starvation condition, as shown in *Δrsv1Δloz1* cell viability. This function may be related to zinc homeostasis, as Loz1 is an important zinc-sensing regulator.

III. 4. Perspectives for Future Studies

III. 4. 1. Distinct roles of the homologous proteins; Rsv1, Rst2, and Scr1

Fission yeast has three homologous zinc finger proteins regulated by glucose; Rsv1, Rst2, and Scr1. They can recognize and bind to the same target DNA sequence. Especially, both Rsv1 and Rst2 are localized in the nucleus under glucose-reduced condition, where they can bind to the same DNA sequences of some genes. Presently, as there are the genome-wide data only from Rsv1, we do not know how many common genes Rsv1 and Rst2 can regulate, or how many distinct target genes they have. But in the light of phenotypic difference of *Δrsv1* and *Δrst2* cells, it is clear that Rsv1 and Rst2 have distinct target genes. There is a report that the N-terminal amino acid stretch can affect the specificity of target DNA recognition, and Rst2 has relatively longer N-terminal sequences than Rsv1 (Siggers et al., 2014). So Rsv1 and Rst2 may have different preference to bind the C-rich target sequence. Also, it is possible that Rsv1 and Rst2 have the distinct partner proteins to regulate target genes. It would be interesting to define the target specificity of Rsv1 and Rst2, and to find how these two proteins cooperate to help cells to cope with the glucose starvation condition. Also, it would be interesting to investigate that Rsv1 and Rst2 can be recruited to the sites preoccupied with Scr1 to play a role in target gene regulation.

III. 4. 2. Rsv1 may interact with other partner proteins for cell survival under prolonged glucose starvation

As mentioned above, there is a possibility of the interacting partners of Rsv1 to recognize and regulate the target genes specifically. And via ChIP-qPCR experiment, Php2 protein of CBP complex was found to bind to the target genes of Rsv1, maybe facilitating the reorganization of the chromatin structure. The majority of peak sites of Rsv1 was located far from the TSS sites when ChIP-

seq result was analyzed, suggesting there may be some mediator proteins which can work with Rsv1. Also, when the long-term binding of Rsv1 was monitored, Rsv1 showed the more enriched binding to the some target genes. So Rsv1 was assumed to interact with other proteins under long-term starvation, leading to the enriched recruitment to some target genes, where Rsv1 was not enriched at short-term starvation. It would be interesting to find out the binding partners of Rsv1, and to explore the meaning of their interaction to regulate target genes.

III. 4. 3. Specific involvement of Rsv1 in relation to zinc homeostasis

It has been known that zinc is important for the regulation of apoptosis, cell cycle, and autophagy. Recently, zinc was shown to be a positive regulator of autophagy, both for basal autophagy and induced autophagy in mammalian cells (Hung et al., 2013; Lee and Koh, 2010; Liuzzi and Yoo, 2013; Liuzzi et al., 2014). And TPEN, the zinc chelator, inhibits the autophagy induced by H_2O_2 and ethanol in mammalian cells. But, surprisingly, the autophagy was induced by zinc limitation in *S. cerevisiae* (Kawamata et al., 2017). Also, it was revealed that the metallothionein is important for regulating cell defense mechanisms. When cells are exposed to oxidative stress condition, the metallothionein releases zinc to activate autophagy process (Liuzzi et al., 2014). Thus, zinc is important for regulating cell survival in many ways.

As shown in Fig. III-27, *Δrsv1* cells showed the recovery of cell survival when they were treated with high concentrations of zinc under glucose starvation. And it was also confirmed that this phenomenon happens only when cells were supplemented with high zinc, as *Δrsv1* cells did not recover the viability when treated with high iron, another cation. And Rsv1 was involved in regulating the expression of *zrt1* and *zym1*, which are mainly regulated by Loz1 under zinc-excess condition. Interestingly, Rsv1 was also bound to *loz1* upstream,

affecting the *loz1* transcription under long-term glucose starvation condition. Presently, it is unclear how the excess zinc can recover the viability of *Δrsv1* cells, and what the function of Rsv1 is in relation to Loz1 and zinc homeostasis. But seen from the evidences, there is a possibility that Rsv1 may be involved in maintaining cell survival by repressing *loz1* transcription. The reason why Rsv1 regulates *loz1* under long-term starvation of glucose remains elusive. As zinc is involved in many cellular functions and the function of zinc is revised in many ways recently, it would be appealing to investigate the function of Rsv1 regarding Loz1 and zinc homeostasis.

III. 4. 4. Closing remarks

For cell survival, the multiple and complicated biological processes must be fine-tuned, as one dysfunctional process can have the deleterious effects on cells. So the maintenance of cell survival can be challenged at any point of cell lifespan. Thus, it should be considered in long-term when investigating the mechanisms of cell survival. But because of the experimental limitations, the continuous monitoring of molecular mechanisms is restricted. Nevertheless, it would be important to contemplate the molecular processes depending on time, to understand the mechanisms of cell survival. Rsv1 is also thought to have some distinct roles under long-term starvation condition, and Rsv1 may have more target genes in this condition. It would be interesting to study the long-term function of Rsv1, which help to understand the strategy of Rsv1 to maintain cell viability under starvation condition.

CHAPTER IV.
REFERENCES

Alfa, C., and Laboratory, C.S.H. (1993). Experiments with Fission Yeast: A Laboratory Course Manual (Cold Spring Harbor Laboratory Press).

Asada, R., Takemata, N., Hoffman, C.S., Ohta, K., and Hirota, K. (2014). Antagonistic controls of chromatin and mRNA start site selection by Tup family corepressors and the CCAAT-binding factor. Molecular and cellular biology, MCB. 00924-00914.

Borrelly, G.P., Harrison, M.D., Robinson, A.K., Cox, S.G., Robinson, N.J., and Whitehall, S.K. (2002). Surplus zinc is handled by Zym1 metallothionein and Zhf endoplasmic reticulum transporter in Schizosaccharomyces pombe. Journal of Biological Chemistry.

Buck, M.J., and Lieb, J.D. (2006). A chromatin-mediated mechanism for specification of conditional transcription factor targets. Nature genetics *38*, 1446.

Byrne, S.M., and Hoffman, C.S. (1993). Six git genes encode a glucose-induced adenylate cyclase activation pathway in the fission yeast Schizosaccharomyces pombe. Journal of cell science *105*, 1095-1100.

Chen, K., Wilson, M.A., Hirsch, C., Watson, A., Liang, S., Lu, Y., Li, W., and Dent, S.Y. (2012). Stabilization of the promoter nucleosomes in nucleosome-free regions by the yeast Cyc8–Tup1 corepressor. Genome research.

Chen, X., Schreiber, K., Appel, J., Makowka, A., Fähnrich, B., Roettger, M., Hajirezaei, M.R., Sönnichsen, F.D., Schönheit, P., and Martin, W.F. (2016). The Entner–Doudoroff pathway is an overlooked glycolytic route in cyanobacteria and plants. Proceedings of the National Academy of Sciences *113*, 5441-5446.

Choi, S., Hu, Y.-M., Corkins, M.E., Palmer, A.E., and Bird, A.J. (2018). Zinc transporters belonging to the Cation Diffusion Facilitator (CDF) family have complementary roles in transporting zinc out of the cytosol. PLoS genetics *14*, e1007262.

Coleman, J.E. (1992). Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. Annual review of biochemistry *61*, 897-946.

Conrad, M., Schothorst, J., Kankipati, H.N., Van Zeebroeck, G., Rubio-Texeira, M., and Thevelein, J.M. (2014). Nutrient sensing and signaling in the yeast Saccharomyces cerevisiae. FEMS microbiology reviews *38*, 254-299.

Cooper, J.P., Roth, S.Y., and Simpson, R.T. (1994). The global transcriptional regulators, SSN6 and TUP1, play distinct roles in the establishment of a repressive chromatin structure. Genes & Development *8*, 1400-1410.

Corkins, M.E., May, M., Ehrensberger, K.M., Hu, Y.-M., Liu, Y.-H., Bloor, S.D., Jenkins, B., Runge, K.W., and Bird, A.J. (2013). Zinc finger protein Loz1 is required for zinc-responsive regulation of gene expression in fission yeast.

Proceedings of the National Academy of Sciences, 201300853.
Corkins, M.E., Wilson, S., Cocuron, J.-C., Alonso, A.P., and Bird, A.J. (2017). The gluconate shunt is an alternative route for directing glucose into the pentose phosphate pathway in fission yeast. Journal of Biological Chemistry, jbc. M117. 798488.
Dainty, S.J., Kennedy, C.A., Watt, S., Bähler, J., and Whitehall, S.K. (2008). Response of Schizosaccharomyces pombe to zinc deficiency. Eukaryotic cell *7*, 454-464.
Davie, J.K., Trumbly, R.J., and Dent, S.Y. (2002). Histone-dependent association of Tup1-Ssn6 with repressed genes in vivo. Molecular and cellular biology *22*, 693-703.
Eshaghi, M., Lee, J.H., Zhu, L., Poon, S.Y., Li, J., Cho, K.-H., Chu, Z., Karuturi, R.K.M., and Liu, J. (2010). Genomic binding profiling of the fission yeast stress-activated MAPK Sty1 and the bZIP transcriptional activator Atf1 in response to H2O2. PloS one *5*, e11620.
Fagerström-Billai, F., Durand-Dubief, M., Ekwall, K., and Wright, A.P. (2007). Individual subunits of the Ssn6-Tup11/12 corepressor are selectively required for repression of different target genes. Molecular and cellular biology *27*, 1069-1082.
Fantes, P.A., and Hoffman, C.S. (2016). A brief history of Schizosaccharomyces pombe research: a perspective over the past 70 years. Genetics *203*, 621-629.
Flamholz, A., Noor, E., Bar-Even, A., Liebermeister, W., and Milo, R. (2013). Glycolytic strategy as a tradeoff between energy yield and protein cost. Proceedings of the National Academy of Sciences, 201215283.
Forsburg, S.L., and Guarente, L. (1989). Identification and characterization of HAP4: a third component of the CCAAT-bound HAP2/HAP3 heteromer. Genes & development *3*, 1166-1178.
Gaither, L.A., and Eide, D.J. (2001). Eukaryotic zinc transporters and their regulation. In Zinc Biochemistry, Physiology, and Homeostasis (Springer), pp. 65-84.
Graham, N.A., Tahmasian, M., Kohli, B., Komisopoulou, E., Zhu, M., Vivanco, I., Teitell, M.A., Wu, H., Ribas, A., and Lo, R.S. (2012). Glucose deprivation activates a metabolic and signaling amplification loop leading to cell death. Molecular systems biology *8*, 589.
Gromöller, A., and Lehming, N. (2000). Srb7p is a physical and physiological target of Tup1p. The EMBO journal *19*, 6845-6852.
Hao, Z., Furunobu, A., Nagata, A., and Okayama, H. (1997). A zinc finger protein required for stationary phase viability in fission yeast. Journal of cell science *110*, 2557-2566.
Hatano, T., Morigasaki, S., Tatebe, H., Ikeda, K., and Shiozaki, K. (2015). Fission

yeast Ryh1 GTPase activates TOR Complex 2 in response to glucose. Cell Cycle *14*, 848-856.

Higuchi, T., Watanabe, Y., and Yamamoto, M. (2002). Protein kinase A regulates sexual development and gluconeogenesis through phosphorylation of the Zn finger transcriptional activator Rst2p in fission yeast. Molecular and cellular biology *22*, 1-11.

Hirota, K., Hoffman, C.S., and Ohta, K. (2006). Reciprocal nuclear shuttling of two antagonizing Zn finger proteins modulates Tup family corepressor function to repress chromatin remodeling. Eukaryotic cell *5*, 1980-1989.

Hirota, K., Hoffman, C.S., Shibata, T., and Ohta, K. (2003). Fission yeast Tup1-like repressors repress chromatin remodeling at the fbp1+ promoter and the ade6-M26 recombination hotspot. Genetics *165*, 505-515.

Hoffman, C. (2005). Glucose sensing via the protein kinase A pathway in Schizosaccharomyces pombe (Portland Press Limited).

Hoffman, C.S., and Winston, F. (1990). Isolation and characterization of mutants constitutive for expression of the fbp1 gene of Schizosaccharomyces pombe. Genetics *124*, 807-816.

Hoffman, C.S., and Winston, F. (1991). Glucose repression of transcription of the Schizosaccharomyces pombe fbp1 gene occurs by a cAMP signaling pathway. Genes & Development *5*, 561-571.

Hung, H.-H., Huang, W.-P., and Pan, C.-Y. (2013). Dopamine-and zinc-induced autophagosome formation facilitates PC12 cell survival. Cell biology and toxicology *29*, 415-429.

Ikeda, K., Morigasaki, S., Tatebe, H., Tamanoi, F., and Shiozaki, K. (2008). Fission yeast TOR complex 2 activates the AGC-family Gad8 kinase essential for stress resistance and cell cycle control. Cell cycle *7*, 358-364.

Janoo, R.T., Neely, L.A., Braun, B.R., Whitehall, S.K., and Hoffman, C.S. (2001). Transcriptional regulators of the Schizosaccharomyces pombe fbp1 gene include two redundant Tup1p-like corepressors and the CCAAT binding factor activation complex. Genetics *157*, 1205-1215.

Kambe, T., Tsuji, T., Hashimoto, A., and Itsumura, N. (2015). The physiological, biochemical, and molecular roles of zinc transporters in zinc homeostasis and metabolism. Physiological reviews *95*, 749-784.

Kaniak, A., Xue, Z., Macool, D., Kim, J.-H., and Johnston, M. (2004). Regulatory network connecting two glucose signal transduction pathways in Saccharomyces cerevisiae. Eukaryotic cell *3*, 221-231.

Kapahi, P., Chen, D., Rogers, A.N., Katewa, S.D., Li, P.W.-L., Thomas, E.L., and Kockel, L. (2010). With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. Cell metabolism *11*, 453-465.

Kato, H., Kira, S., and Kawamukai, M. (2013). The transcription factors Atf1 and Pcr1 are essential for transcriptional induction of the extracellular

maltase Agl1 in fission yeast. PloS one *8*, e80572.
Kawai, M., Nakashima, A., Ueno, M., Ushimaru, T., Aiba, K., and Uritani, M. (2001). Fission yeast tor1 functions in response to various stresses including nitrogen starvation, high osmolarity, and high temperature. Current genetics *39*.
Kawamata, T., Horie, T., Matsunami, M., Sasaki, M., and Ohsumi, Y. (2017). Zinc starvation induces autophagy in yeast. Journal of Biological Chemistry, jbc. M116. 762948.
Kim, D.-U., Hayles, J., Kim, D., Wood, V., Park, H.-O., Won, M., Yoo, H.-S., Duhig, T., Nam, M., and Palmer, G. (2010). Analysis of a genome-wide set of gene deletions in the fission yeast Schizosaccharomyces pombe. Nature biotechnology *28*, 617.
Kunitomo, H., Higuchi, T., Iino, Y., and Yamamoto, M. (2000). A zinc-finger protein, Rst2p, regulates transcription of the fission yeast ste11+ gene, which encodes a pivotal transcription factor for sexual development. Molecular biology of the cell *11*, 3205-3217.
Lawrence, C.L., Maekawa, H., Worthington, J.L., Reiter, W., Wilkinson, C.R., and Jones, N. (2007). Regulation of Schizosaccharomyces pombe Atf1 protein levels by Sty1-mediated phosphorylation and heterodimerization with Pcr1. Journal of Biological Chemistry *282*, 5160-5170.
Lee, M., Chatterjee, S., and Struhl, K. (2000). Genetic analysis of the role of Pol II holoenzyme components in repression by the Cyc8-Tup1 corepressor in yeast. Genetics *155*, 1535-1542.
Lee, S.-J., and Koh, J.-Y. (2010). Roles of zinc and metallothionein-3 in oxidative stress-induced lysosomal dysfunction, cell death, and autophagy in neurons and astrocytes. Molecular brain *3*, 30.
Liu, Y., Song, X.D., Liu, W., Zhang, T.Y., and Zuo, J. (2003). Glucose deprivation induces mitochondrial dysfunction and oxidative stress in PC12 cell line. Journal of cellular and molecular medicine *7*, 49-56.
Liuzzi, J., and Yoo, C. (2013). Role of zinc in the regulation of autophagy during ethanol exposure in human hepatoma cells. Biological trace element research *156*, 350-356.
Liuzzi, J.P., Guo, L., Yoo, C., and Stewart, T.S. (2014). Zinc and autophagy. Biometals *27*, 1087-1096.
Madrid, M., Soto, T., Franco, A., Paredes, V., Vicente, J., Hidalgo, E., Gacto, M., and Cansado, J. (2004). A cooperative role for Atf1 and Pap1 in the detoxification of the oxidative stress induced by glucose deprivation in Schizosaccharomyces pombe. Journal of Biological Chemistry *279*, 41594-41602.
Madrid, M., Vázquez-Marín, B., Franco, A., Soto, T., Vicente-Soler, J., Gacto, M., and Cansado, J. (2016). Multiple crosstalk between TOR and the cell

integrity MAPK signaling pathway in fission yeast. Scientific reports *6*, 37515.

Magasanik, B. (1961). Catabolite repression. Paper presented at: Cold Spring Harbor symposia on quantitative biology (Cold Spring Harbor Laboratory Press).

Marambio, P., Toro, B., Sanhueza, C., Troncoso, R., Parra, V., Verdejo, H., García, L., Quiroga, C., Munafo, D., and Díaz-Elizondo, J. (2010). Glucose deprivation causes oxidative stress and stimulates aggresome formation and autophagy in cultured cardiac myocytes. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease *1802*, 509-518.

Matsuzawa, T., Fujita, Y., Tohda, H., and Takegawa, K. (2011). Snf1-like protein kinase Ssp2 regulates glucose derepression in Schizosaccharomyces pombe. Eukaryotic cell, EC. 05268-05211.

Matsuzawa, T., Fujita, Y., Tohda, H., and Takegawa, K. (2012). Snf1-like protein kinase Ssp2 regulates glucose derepression in Schizosaccharomyces pombe. Eukaryotic cell *11*, 159-167.

Matsuzawa, T., Ohashi, T., Hosomi, A., Tanaka, N., Tohda, H., and Takegawa, K. (2010). The gld1+ gene encoding glycerol dehydrogenase is required for glycerol metabolism in Schizosaccharomyces pombe. Applied microbiology and biotechnology *87*, 715-727.

McDowall, M.D., Harris, M.A., Lock, A., Rutherford, K., Staines, D.M., Bähler, J., Kersey, P.J., Oliver, S.G., and Wood, V. (2014). PomBase 2015: updates to the fission yeast database. Nucleic acids research *43*, D656-D661.

Mcnabb, D.S., Tseng, K., and Guarente, L. (1997). The Saccharomyces cerevisiae Hap5p homolog from fission yeast reveals two conserved domains that are essential for assembly of heterotetrameric CCAAT-binding factor. Molecular and cellular biology *17*, 7008-7018.

McNabb, D.S., Xing, Y., and Guarente, L. (1995). Cloning of yeast HAP5: a novel subunit of a heterotrimeric complex required for CCAAT binding. Genes & development *9*, 47-58.

Mennella, T.A., Klinkenberg, L.G., and Zitomer, R.S. (2003). Recruitment of Tup1-Ssn6 by yeast hypoxic genes and chromatin-independent exclusion of TATA binding protein. Eukaryotic cell *2*, 1288-1303.

Mercier, A., Pelletier, B., and Labbé, S. (2006). A transcription factor cascade involving Fep1 and the CCAAT-binding factor Php4 regulates gene expression in response to iron deficiency in the fission yeast Schizosaccharomyces pombe. Eukaryotic cell *5*, 1866-1881.

Moreno, S., Klar, A., and Nurse, P. (1991). [56] Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. In Methods in enzymology (Elsevier), pp. 795-823.

Mukai, Y., Matsuo, E., Roth, S.Y., and Harashima, S. (1999). Conservation of

Histone Binding and Transcriptional Repressor Functions in a Schizosaccharomyces pombeTup1p Homolog. Molecular and cellular biology *19*, 8461-8468.

Neely, L.A., and Hoffman, C.S. (2000). Protein kinase A and mitogen-activated protein kinase pathways antagonistically regulate fission yeast fbp1transcription by employing different modes of action at two upstream activation sites. Molecular and cellular biology *20*, 6426-6434.

Nehlin, J.O., Carlberg, M., and Ronne, H. (1991). Control of yeast GAL genes by MIG1 repressor: a transcriptional cascade in the glucose response. The EMBO journal *10*, 3373-3377.

Ohtsuka, H., Mita, S., Ogawa, Y., Azuma, K., Ito, H., and Aiba, H. (2008). A novel gene, ecl1+, extends the chronological lifespan in fission yeast. FEMS yeast research *8*, 520-530.

Oliva, A., Rosebrock, A., Ferrezuelo, F., Pyne, S., Chen, H., Skiena, S., Futcher, B., and Leatherwood, J. (2005). The cell cycle–regulated genes of Schizosaccharomyces pombe. PLoS biology *3*, e225.

Otsubo, Y., and Yamamato, M. (2008). TOR signaling in fission yeast. Critical reviews in biochemistry and molecular biology *43*, 277-283.

Papamichos-Chronakis, M., Conlan, R.S., Gounalaki, N., Copf, T., and Tzamarias, D. (2000). Hrs1/Med3 is a Cyc8-Tup1 corepressor target in the RNA polymerase II holoenzyme. Journal of Biological Chemistry *275*, 8397-8403.

Papamichos-Chronakis, M., Petrakis, T., Ktistaki, E., Topalidou, I., and Tzamarias, D. (2002). Cti6, a PHD domain protein, bridges the Cyc8-Tup1 corepressor and the SAGA coactivator to overcome repression at GAL1. Molecular cell *9*, 1297-1305.

Papamichos-Chronakis, M., Gligoris, T., and Tzamarias, D. (2004). The Snf1 kinase controls glucose repression in yeast by modulating interactions between the Mig1 repressor and the Cyc8-Tup1 co-repressor. EMBO reports *5*, 368-372.

Pataki, E., Sipiczki, M., and Miklos, I. (2017). Schizosaccharomyces pombe rsv1 Transcription Factor and its Putative Homologues Preserved their Functional Homology and are Evolutionarily Conserved. Current microbiology *74*, 710-717.

Peekhaus, N., and Conway, T. (1998). What's for dinner?: Entner-Doudoroff metabolism inEscherichia coli. Journal of bacteriology *180*, 3495-3502.

Pocker, Y., and Green, E. (1973). Hydrolysis of D-glucono-. delta.-lactone. I. General acid-base catalysis, solvent deuterium isotope effects, and transition state characterization. Journal of the American Chemical Society *95*, 113-119.

Proft, M., and Struhl, K. (2002). Hog1 kinase converts the Sko1-Cyc8-Tup1

repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. Molecular cell *9*, 1307-1317.

Reiter, W., Watt, S., Dawson, K., Lawrence, C.L., Bähler, J., Jones, N., and Wilkinson, C.R. (2008). Fission yeast MAP kinase Sty1 is recruited to stress-induced genes. Journal of Biological Chemistry *283*, 9945-9956.

Roberts, D., and Miyamoto, S. (2015). Hexokinase II integrates energy metabolism and cellular protection: Akting on mitochondria and TORCing to autophagy. Cell death and differentiation *22*, 248.

Rodrigues, F., Ludovico, P., and Leão, C. (2006). Sugar metabolism in yeasts: an overview of aerobic and anaerobic glucose catabolism. In Biodiversity and ecophysiology of yeasts (Springer), pp. 101-121.

Roux, A.E., Quissac, A., Chartrand, P., Ferbeyre, G., and Rokeach, L.A. (2006). Regulation of chronological aging in Schizosaccharomyces pombe by the protein kinases Pka1 and Sck2. Aging cell *5*, 345-357.

Roux, P.P., and Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiology and molecular biology reviews *68*, 320-344.

Rubenstein, E.M., McCartney, R.R., Zhang, C., Shokat, K.M., Shirra, M.K., Arndt, K.M., and Schmidt, M.C. (2008). Access denied: Snf1 activation loop phosphorylation is controlled by availability of the phosphorylated threonine 210 to the PP1 phosphatase. Journal of Biological Chemistry *283*, 222-230.

Sabatinos, S.A., and Forsburg, S.L. (2010). Molecular genetics of Schizosaccharomyces pombe. In Methods in enzymology (Elsevier), pp. 759-795.

Salat-Canela, C., Paulo, E., Sánchez-Mir, L., Carmona, M., Ayté, J., Oliva, B., and Hidalgo, E. (2017). Deciphering the role of the signal-and Sty1 kinase-dependent phosphorylation of the stress-responsive transcription factor Atf1 on gene activation. Journal of Biological Chemistry, jbc. M117. 794339.

Sánchez-Mir, L., Salat-Canela, C., Paulo, E., Carmona, M., Ayté, J., Oliva, B., and Hidalgo, E. (2017). Phospho-mimicking Atf1 mutants bypass the transcription activating function of the MAP kinase Sty1 of fission yeast. Current genetics, 1-6.

Sansó, M., Gogol, M., Ayté, J., Seidel, C., and Hidalgo, E. (2008). Transcription factors Pcr1 and Atf1 have distinct roles in stress-and Sty1-dependent gene regulation. Eukaryotic cell *7*, 826-835.

Santangelo, G.M. (2006). Glucose signaling in Saccharomyces cerevisiae. Microbiology and Molecular Biology Reviews *70*, 253-282.

Schothorst, J., Zeebroeck, G.V., and Thevelein, J.M. (2017). Identification of Ftr1 and Zrt1 as iron and zinc micronutrient transceptors for activation of the PKA pathway in Saccharomyces cerevisiae. Microbial Cell *4*, 74.

Shiozaki, K., and Russell, P. (1996). Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. Genes & development *10*, 2276-2288.

Siggers, T., Reddy, J., Barron, B., and Bulyk, M.L. (2014). Diversification of transcription factor paralogs via noncanonical modularity in C2H2 zinc finger DNA binding. Molecular cell *55*, 640-648.

Spivey, E.C., Jones Jr, S.K., Rybarski, J.R., Saifuddin, F.A., and Finkelstein, I.J. (2017). An aging-independent replicative lifespan in a symmetrically dividing eukaryote. Elife *6*, e20340.

Steinkraus, K., Kaeberlein, M., and Kennedy, B. (2008). Replicative aging in yeast: the means to the end. Annual review of cell and developmental biology *24*, 29-54.

Stettler, S., Warbrick, E., Prochnik, S., Mackie, S., and Fantes, P. (1996). The wis1 signal transduction pathway is required for expression of cAMP-repressed genes in fission yeast. Journal of Cell Science *109*, 1927-1935.

Takemata, N., Oda, A., Yamada, T., Galipon, J., Miyoshi, T., Suzuki, Y., Sugano, S., Hoffman, C.S., Hirota, K., and Ohta, K. (2016). Local potentiation of stress-responsive genes by upstream noncoding transcription. Nucleic acids research *44*, 5174-5189.

Tan, V.P., and Miyamoto, S. (2015). HK2/hexokinase-II integrates glycolysis and autophagy to confer cellular protection. Autophagy *11*, 963-964.

Tatebe, H., Morigasaki, S., Murayama, S., Zeng, C.T., and Shiozaki, K. (2010). Rab-family GTPase regulates TOR complex 2 signaling in fission yeast. Current Biology *20*, 1975-1982.

Townley, R., and Shapiro, L. (2007). Crystal structures of the adenylate sensor from fission yeast AMP-activated protein kinase. Science *315*, 1726-1729.

Tzamarias, D., and Struhl, K. (1994). Functional dissection of the yeast Cyc8–Tupl transcriptional co-repressor complex. Nature *369*, 758.

Wahi, M., Komachi, K., and Johnson, A. (1998). Gene regulation by the yeast Ssn6-Tup1 corepressor. Paper presented at: Cold Spring Harbor symposia on quantitative biology (Cold Spring Harbor Laboratory Press).

Wahls, W.P., and Smith, G.R. (1994). A heteromeric protein that binds to a meiotic homologous recombination hot spot: correlation of binding and hot spot activity. Genes & development *8*, 1693-1702.

Wamelink, M., Struys, E., and Jakobs, C. (2008). The biochemistry, metabolism and inherited defects of the pentose phosphate pathway: a review. Journal of inherited metabolic disease *31*, 703-717.

Watanabe, Y., and Yamamoto, M. (1996). Schizosaccharomyces pombe pcr1+ encodes a CREB/ATF protein involved in regulation of gene expression for sexual development. Molecular and Cellular Biology *16*, 704-711.

Watson, A.D., Edmondson, D.G., Bone, J.R., Mukai, Y., Yu, Y., Du, W., Stillman,

D.J., and Roth, S.Y. (2000). Ssn6–Tup1 interacts with class I histone deacetylases required for repression. Genes & development *14*, 2737-2744.
Weisman, R., and Choder, M. (2001). The fission yeast TOR homolog, tor1+, is required for the response to starvation and other stresses via a conserved serine. Journal of Biological Chemistry *276*, 7027-7032.
Wilkinson, M.G., Samuels, M., Takeda, T., Toone, W.M., Shieh, J.-C., Toda, T., Millar, J., and Jones, N. (1996). The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. Genes & development *10*, 2289-2301.
Wong, K.H., and Struhl, K. (2011). The Cyc8–Tup1 complex inhibits transcription primarily by masking the activation domain of the recruiting protein. Genes & development *25*, 2525-2539.
Wood, V., Gwilliam, R., Rajandream, M.-A., Lyne, M., Lyne, R., Stewart, A., Sgouros, J., Peat, N., Hayles, J., and Baker, S. (2002). The genome sequence of Schizosaccharomyces pombe. nature *415*, 871.
Zhang, Z., and Reese, J.C. (2004). Redundant mechanisms are used by Ssn6-Tup1 in repressing chromosomal gene transcription in Saccharomyces cerevisiae. Journal of Biological Chemistry *279*, 39240-39250.
Zhao, Y., Hu, X., Liu, Y., Dong, S., Wen, Z., He, W., Zhang, S., Huang, Q., and Shi, M. (2017). ROS signaling under metabolic stress: cross-talk between AMPK and AKT pathway. Molecular cancer *16*, 79.
Zhu, Z., Frey, O., Haandbaek, N., Franke, F., Rudolf, F., and Hierlemann, A. (2015). Time-lapse electrical impedance spectroscopy for monitoring the cell cycle of single immobilized S. pombe cells. Scientific reports *5*, 17180.
Zorov, D.B., Juhaszova, M., and Sollott, S.J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiological reviews *94*, 909-950.

국문초록

세포들은 생애 동안 여러 스트레스 상황에 노출되는데, 특히 영양소 결핍은 주요한 스트레스 상황 중 하나이다. 포도당은 대부분의 생물들이 선호하는 탄소원이므로 세포 내 포도당 농도를 인지하고 조절하는 것은 세포생장에 매우 중요하다. 포도당이 고갈되면 세포들은 대사활동을 재설계하고 영양소 결핍에 의한 스트레스에 대응하기 시작한다. 분열성 효모에서, Rsv1은 징크 핑거 도메인을 가진 전사인자로서 정지상에서 세포 생존에 중요하다고 알려져 있다. *rsv1* 유전자가 제거된 세포들은 에탄올, 열, 그리고 zymolase에 민감하게 반응하며, cAMP-PKA 신호 전달 경로가 *rsv1* 유전자 발현에 관여한다는 사실이 알려져 있다. 그러나 포도당 고갈 상황에서 *rsv1* 유전자를 발현시키는 정확한 신호 전달 메커니즘과 이에 관여하는 전사인자들에 대한 연구는 미비하다. 또한 Rsv1가 전사인자로서 하는 역할, 즉 세포생존을 위해 조절하는 유전자들에 대해서는 알려져 있지 않다.

포도당이 고갈 되었을 때 *rsv1*의 발현을 조절하는 메커니즘을 알아보기 위하여 어떠한 신호전달이 관여되어 있는지 알아보았다. MAPK와 PKA 신호전달은 포도당 농도에 따라 하위 유전자를 조절하는데 중요하다. MAPK 유전자인 *sty1*이 결손 된 세포에서 *rsv1* 유전자 발현이 저해되었고, Sty1의 주요한 작용자인 *atf1*이 결손 되어도 비슷한 정도로 *rsv1* 유전자 발현이 줄어드는 것을 확인하였다. 또 다른 중요한 신호전달경로인 cAMP-PKA는 촉매소단위인 Pka1와 조절소단위인 Cgs1으로 이루어져있다. Pka1이 지속적으로 활성을 지닌 *cgs1* 결핍 세포에서 *rsv1* 유전자 발현은 크게 줄어들었다. 따라서, MAPK와 PKA 신호전달경로가 *rsv1* 유전자 발현에 중요함을 알 수 있었다.

이들 신호전달경로의 어떤 전사인자들이 *rsv1* 유전자를 직접 조절하는지

알아보기 위하여 포도당 고갈 상황에서 유전자들을 조절하는데 중요한 역할을 하는 MAPK 경로의 Atf1과 PKA 경로의 Rst2 전사인자들에 대하여 ChIP 실험을 수행하였다. *rsv1* 유전자는 상부에 Atf1과 Rst2가 인지하는 consensus motif를 지니고 있다. 포도당 고갈 조건과 포도당이 풍부한 조건에서 이들 단백질의 *rsv1* 상부 결합을 확인 한 결과, Atf1과 Rst2가 포도당 고갈 조건에서만 *rsv1* 유전자 상부에 직접 결합함을 보았다. 또한, Rst2와 같은 motif를 인지하는 Scr1 전사인자는 포도당이 풍부한 환경에서만 *rsv1* 유전자 상부를 인지함을 보여, Rst2와 Scr1이 서로 다른 포도당 환경에서 *rsv1* 발현에 관여함을 알 수 있었다. 따라서, Atf1과 Rst2는 촉진인자로서, Scr1은 억제인자로서 *rsv1* 발현을 조절한다.

Rsv1에 의해 조절되는 유전자들을 알아보기 위하여 ChIP-seq과 RNA-seq을 진행하였다. ChIP-seq 결과, 71개의 Rsv1 peak을 얻을 수 있었고 Rsv1이 'CCCCNC' motif를 인지함을 알게 되었다. 또한 RNA-seq을 통하여 232개의 유전자가 Rsv1에 의해 발현 양이 변하는 것을 알았고 그 중 21개가 Rsv1 peak을 지니고 있었다. 따라서 Rsv1이 이들 21개의 유전자를 직접적으로 조절함을 밝혔으며, 이들 중 13개 유전자는 Rsv1에 의해 억제되고 8개 유전자는 Rsv1에 의해 활성화 되었다. 이들은 대개 탄소 대사와 연관된 유전자들이었다. 이들 유전자 중에서, *gcd1*은 Rsv1의 가장 강한 ChIP-seq peak을 지니고 있었고 발현 양이 *rsv1* 유전자 결손 세포에서 크게 derepression 되었다. 또한 *rsv1*과 *gcd1*이 모두 결손 된 세포는 *rsv1* 하나에 대해 결손 된 세포보다 세포생존이 오래 지속되었다. Gcd1은 오탄당 인산염 경로의 글루콘산 분로에 관여하는 포도당 탈수소 효소이다. 흥미롭게도, Rsv1은 포도당 고갈 상황이 계속되면 다른 글루콘산 분로 유전자들의 발현도 억제하였다. 글루콘산 분로는 NADPH를 생성하는데 중요하며, 포도당이 고갈되면 짧은 기간 내에 관련 유전자들 발현이 증가하게 된다. 그리고 아마도 포도당고갈이 지속되는 상황에서 이들 유전자들을 다시 억제하는 데에 Rsv1이 역할을 할 것으로 예상된다.

또한, 위의 전장 유전체 해독 결과를 통하여 Rsv1이 *gcd1*, *gnd1*, *gut2* 등의 유전자를 조절함으로써 오탄당 인산염 경로를 억제하고 글리세롤 인산 경로를 통한 에너지 생성 경로를 활성화하여 세포 생존에 관여할 것임을 확인할 수 있었다.

주요 단어:

분열성 효모, 세포생존, 포도당 고갈, Rsv1, MAPK 경로, Sty1, Atf1, PKA 경로, Cgs1, Rst2, Scr1, ChIP-seq, RNA-seq, 탄소 대사, Gcd1, 글루콘산 분로, 아연.