이학석사학위논문

효모에서 *gas1* 돌연변이와 *rho*^{0} 돌연변이의 리보좀 DNA 안정성 조절

Regulation of ribosomal DNA stability by *gas1* mutant and *rho*^{0} mutant in *Saccharomyces cerevisiae*.

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ABSTRACT

In budding yeast, rDNA has a tandem repeat array of 9.1 kbp each rDNA unit by 100~200 copies. rDNA silencing process controls the stability of this highly repetitive region. Here, we report that Gas1 controls transcriptional silencing of rDNA by PKA signaling pathway. Gas1 is β-1,3-glucanosyltransferase and has a role in cell wall organization. Deletion of Gas1 decreases PKA activity, it causes dephosphorylation of two stress response transcription factors, Msn2 and Msn4. Dephosphorylated form of Msn2/4 can get into nucleus from the cytoplasm and promote the transcription of Pnc1, the activator of Sir2 which forms RENT complexes and silences rDNA. Moreover, cell wall damage reagent Congo red treated cell also shows similar pattern of increased Sir2-mediated rDNA silencing. Unlike in *gas1Δ*mutant, PKA activity is induced in *rho^0*mutant which is mitochondrial respiratory malfunction by mtDNA loss. Increased activity of PKA in *rho^0* mutant leads to decreased rDNA silencing. These findings suggest that *gas1Δ* mutant and *rho^0* mutant influence rDNA stability by controlling PKA pathway.

Keyward: rDNA Silencing, Replicative Life Span, Sir2, PKA, Gas1, *rho^0* Mutant.

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LIST OF ABBREVIASTIONS

Bcy1	Bypass of Cyclic-AMP requirement
Gas1	Glycophospholipid-Anchored Surface protein
Mpk1	MAP kinase
Msn2	Multicopy Suppressor of SNF mutation
NAD^+	Nicotinamide Adenine Dinucleotide
PKA	Protein Kinase A
Pnc1	Pyrazinamidase and NiCotinamidase
rDNA	Ribosomal DeoxyriboNucleic Acid
Sir2	Silent Information Regulator

Introduction

Saccharomyces cerevisiae, also known as budding yeast or baker's yeast, is a good model for research on aging process since factors related to aging are well conserved in yeast. Compared with other organisms, yeast grows and ages rapidly, which is advantageous for the research of aging. Replicative life span of yeast, which means how many times a mother cell can divide and make daughter cells before senescence, is heavily affected by the rDNA silencing process (1).

In budding yeast, ribosomal deoxyribonucleic acid (rDNA) has a tandem repeat structure of 100~200 copies (2). 9.1 kbp of each rDNA unit contains 35S rRNA gene, non-transcribed spacer (NTS) and 5S rRNA gene. Because of the highly repetitive structure, rDNA has intimate instability and homologous recombination happens frequently. That forms extrachromosomal rDNA circles (ERCs) which are accumulated in a mother cell and cause cell aging (1). In wild type cell, rDNA is stable to a certain degree by negative regulation of rDNA recombination. We call it as "rDNA silencing". rDNA silencing is performed by a histone deacetylase named Sir2 which forms RENT complex.

The sirtuins are large family of NAD^+-dependent protein deacetylases well conserved from prokaryote to mammal (3, 4). In human, Sirt1 deacetylates many proteins and regulates DNA damage, metabolism and longevity. Homolog of Sirt1 in budding yeast is Sir2. Sir2 acts as an important suppressor of rDNA

recombination and it causes extension of replicative life span.

The enzymatic activity of Sir2 is regulated by several factors such as nicotinamide adenine dinucleotide $(NAD)^+$ level and nicotinamide (NAM) level (5-7). Because Sir2 is NAD^+-dependent histone deacetylase, high level of NAD^+ activates Sir2. Sir2 converts NAD^+ to NAM via histone deacetylation process, and then NAM inhibits Sir2. The nicotinamidase named Pnc1 transforms NAM to nicotinic acid (NA) (8). This is a part of NAD salvage pathway so Pnc1 makes Sir2 more active by removing NAM. The promoter of PNC1 gene includes stress response promoter element (STRE), a binding site for the stress responsive transcription factors, Msn2 and Msn4 (9). When Msn2/4 imports into nucleus and binds to PNC1 promoter, expression of Pnc1 is induced. Msn2/4 is unable to get into nucleus when Msn2/4 is phosphorylated by several kinase, such as Protein kinase A (PKA).

Gas1 is β-1,3-glucanosyltransferase which forms β-1,3-glucan chains, has a key role in cell wall organization (10). β-1,3-glucan side chains have cross linking with β-1,6-glucan, chitin, proteins, and other components of cell wall. Gas1, a GPI anchored protein to the outer side of the plasma membrane, elongates β-1,3-glucan side chains(11, 12). When Gas1 loses its enzymatic activity, cell shows cell wall weakness and high sensitivity to cell wall affecting drug.

Surprisingly, it was reported that Gas1 regulates transcriptional silencing in *S. cerevisiae* (13). Gas1 induces telomeric silencing and reduces rDNA silencing. Those effects of silencing need enzymatic activity and interaction with Sir2.

However the signaling pathway from Gas1 to Sir2 is not clear until now.

rho^{0} mutant is complete loss of mitochondria DNA mutant. There are several abnormal phenotypes of *rho*^{0} mutant. Mitochondria malfunction is most important aspect of this mutant. In *rho*^{0} mutant, carbohydrate concentration is reduced and chronological life span is dramatically decreased (14). In 2011, Graef and Nunnari reported that *rho*^{0} mutant affects PKA signaling (15). They showed cells without mitochondrial DNA have increased PKA activity and related mitophagy. It was considered that mitochondrial respiratory deficiency causes activation of PKA and it makes several types of signaling like autophagy suppression.

In this study, we investigate a pathway from Gas1 to rDNA silencing. We confirmed rDNA stability change in *gas1Δ* cell, then checked NAD^{+} level and Pnc1 expression. After that, translocalization of Msn2, the stimulating transcription from cytosol to the nucleus, was observed. PKA which inhibit nuclear import of Msn2 and Msn4 by phosphorylation was down regulated without Gas1 protein. When Gas1 is deleted, cell has abnormal cell wall phenotype, similar to Congo red treated cell, so we tested rDNA silencing and PKA activity in Congo red condition. Since Congo red treatment to yeast cell leads to increased rDNA silencing and decreased PKA activity, we surmised that specific type of cell wall damage triggers transcription silencing of rDNA. Furthermore, we found that reduced PKA activity in *gas1Δ* cell and Congo red treated cell is mediated by the MAPK, Mpk1. Also we estimated that increased

activity of PKA leads to down-regulation of rDNA silencing. In *rho*^{0} mutant cell, rDNA silencing was decreased considerably. Our results suggest that loss of Gas1 encourages rDNA stability, On the other hand, mitochondrial malfunction by mtDNA loss discourages rDNA stability, while PKA mediates signaling pathway on both case.

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Materials and Methods

Yeast strains and growth conditions

The yeast strains used in this study are listed in Table 1. Yeast strains were genetically manipulated according to the one-step PCR-mediated gene targeting procedure, as previously described. Yeast transformation was performed using the lithium acetate method (16), and proper integration was confirmed by PCR. In all cases, deletion strains were constructed by the replacement of the corresponding open reading frames with a selectable marker. These substitutions were confirmed by sequencing with an appropriate primer. *rho*^{0} mutant derivatives of these strains were generated by several passages of wild-type cells in YPD medium supplemented with 25 μg/ml of ethidium bromide (15). pRS423-pr^{CUP}-*6×MYC-cki1*$^{2-200(S125/130A)}$ has been described previously (17).Rich medium (YPD; 1% yeast extract, 2% peptone, 2% glucose) and synthetic complete (SC) medium lacking appropriate amino acids for selection were prepared as previously described (18). Unless otherwise noted, cells were grown in YPD medium at 30°C.

Table 1. Yeast strains used in this study.

Strain	Genotype	Source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
DMY2798	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 leu2*::*mURA3*	(Huang *et al.*, 2006)
DMY2804	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 RDN1-NTS1*::*mURA3*	(Huang *et al.*, 2006)
HY1164	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 leu2*::*mURA3 gas1Δ*::*TRP1*	This study
HY1165	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 RDN1-NTS1*::*mURA3 gas1Δ*::*TRP1*	This study
HY1170	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MSN2-GFP-HIS3MX6*	(Huh *et al.*, 2003)
HY1171	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MSN2-GFP-HIS3MX6 gas1Δ*::*LEU2*	This study
HY1178	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SIR2-TAP-HIS3MX6*	(Ghaemmaghami *et al.*, 2003)
HY1509	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SIR2-TAP-HIS3MX6 rho^0*	This study
HY1497	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 HIS3MX6*::P_{RPL7B}-*HA-BCY1*	This study
HY1498	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 HIS3MX6*::P_{RPL7B}-*HA-BCY1 gas1Δ*::*URA3*	This study
HY1499	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 HIS3MX6*::P_{RPL7B}-*HA-BCY1 mpk1Δ*::*LEU2*	This study
HY1500	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 HIS3MX6*::P_{RPL7B}-*HA-BCY1 mpk1Δ*::*LEU2 gas1Δ*::*URA3*	This study
HY1406	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PNC1-TAP-HIS3MX6*	This study
HY1407	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PNC1-TAP-HIS3MX6 gas1Δ*::*URA3*	This study
HY1182	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gas1Δ*::*URA3*	This study
HY1501	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 HIS3MX6*::P_{HXK2}-*HA-BCY1*	This study
HY1502	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 HIS3MX6*::P_{HXK2}-*HA-BCY1 gas1Δ*::*URA3*	This study
HY1510	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* [pRS423 P_{CUP}-*6xMYC-cki1*$^{2\text{-}200(S125/130A)}$]	This study
HY1511	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* [pRS423 P_{CUP}-*6xMYC-cki1*$^{2\text{-}200(S125/130A)}$] *gas1Δ*::*URA3*	This study
HY1512	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* [pRS423 P_{CUP}-*6xMYC-cki1*$^{2\text{-}200(S125/130A)}$] *rho^0*	This study
HY1513	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rho^0*	This study

HY1514	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PNC1-TAP-HIS3MX6 rho^0*	This study
HY1515	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 leu2*::*mURA3 rho^0*	This study
HY1516	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 RDN1-NTS1*::*mURA3 rho^0*	This study
HY1517	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MSN2-GFP-HIS3MX6 rho^0*	This study

Silencing assay

Silencing at rDNA region was assayed as previously described (19,20). Yeast cells were grown to OD_{600} = 1.0, and 3 µl of 10-fold serial dilutions of the cell suspensions were spotted on the appropriate media. Plates were incubated at 30°C for 2-3 days.

Intracellular NAD^+ measurement

Intracellular NAD^+ measurement was performed as previously described with some modification (21). Yeast cells were grown in 50 ml YPD medium to OD_{600} ~ 1.4 and then harvested by centrifugation. Cell pellets were extracted for 30 min with 500 µl of ice-cold 1 M formic acid (saturated with butanol). 125 µl of ice-cold 100% trichloroacetic acid was added and incubated on ice for 15 min. The mixture was centrifuged at 4,000×g for 20 min at 4°C and the acid-soluble supernatant was saved. The pellet was re-extracted with 250µl of 20% trichloroacetic acid and pelleted again. The supernatants were combined and used for NAD^+ measurement. 150 µl of the acid extract was added to 850 µl of reaction buffer containing 300 mM Tris-HCl (pH 9.7), 200 mM lysine HCl, 0.2% ethanol, and 150 µg/ml alcohol dehydrogenase (A7011, Sigma). Reactions were incubated at 30°C for 20 min, and absorbance was measured at 340 nm. A base-line correction was made by subtracting the absorbance of a reaction without alcohol dehydrogenase. The intracellular NAD^+ concentration was calculated from the extinction coefficient as previously described (22). Each set

of experiments was performed at least three times. Statistical analysis was performed using Student's t-test.

Fluorescence microscopy

Fluorescence microscopy was performed on a Zeiss Axiovert 200M inverted microscope, as previously described (23). Strains expressing GFP-tagged protein have been described previously (24). Unless otherwise noted, cells were grown in SC medium at 30°C.

Western blot analysis

Whole-cell extracts were run on SDS-polyacrylamide gel electrophoresis. Western blot analysis was performed by standard methods using a mouse anti-GFP antibody (sc-9996, Santa Cruz Biotechnology) for detection of GFP-tagged proteins, an HRP-conjugated anti-mouse IgG antibody (A9044; Sigma) for detection of TAP-tagged proteins, an HRP-conjugated anti-HA antibody (sc-7392, Santa Cruz Biotechnology) for detection of HA-tagged proteins and a mouse anti-Myc antibody (sc-40, Santa Cruz Biotechnology) for the detection of Myc-tagged proteins. Hexokinase was used as a loading control and detected by anti-hexokinase antibody (H2035-02, United States Biological).

Results

1. The absence of Gas1 increases ribosomal DNA silencing.

First, we carried out a spot assay to confirm a role of Gas1 in ribosomal DNA silencing which was investigated previously (13). We used yeast strains carrying the mURA3 silencing reporter gene inserted either inside the NTS1 region of the rDNA locus (RDN1-NTS1::mURA3) or outside the rDNA array (leu2::mURA3) (19). As more silencing occurred, cells grew less in SC-Ura medium. Compared to wild type cell, *gas1Δ* cell shows increased silencing of mURA3 reporter gene at rDNA region. In addition, previous study of C. W. Ha confirmed that rDNA stability regulation by Gas1 is dependent on Sir2 and Congo red treated cell with similar cell wall damage has similar Sir2 dependent rDNA silencing. Also Sir2 expression level of *gas1Δ* cell and Congo red treated cell are same as that of wild type cell (data not shown).

Next, we measured intracellular NAD^+ level in *gas1Δ* cell. NAD^+ is a vital coenzyme connecting metabolism state to epigenetic system. Since Sir2 is NAD^+-dependent histone deactylatase, intracellular NAD^+ level is important for silencing function of Sir2. NAD^+ level of *gas1Δ* cell is higher than that of wild type cell.

These results suggest that loss of Gas1 up-regulates rDNA silencing by NAD^+-dependent histone deactylatase, Sir2 activity.

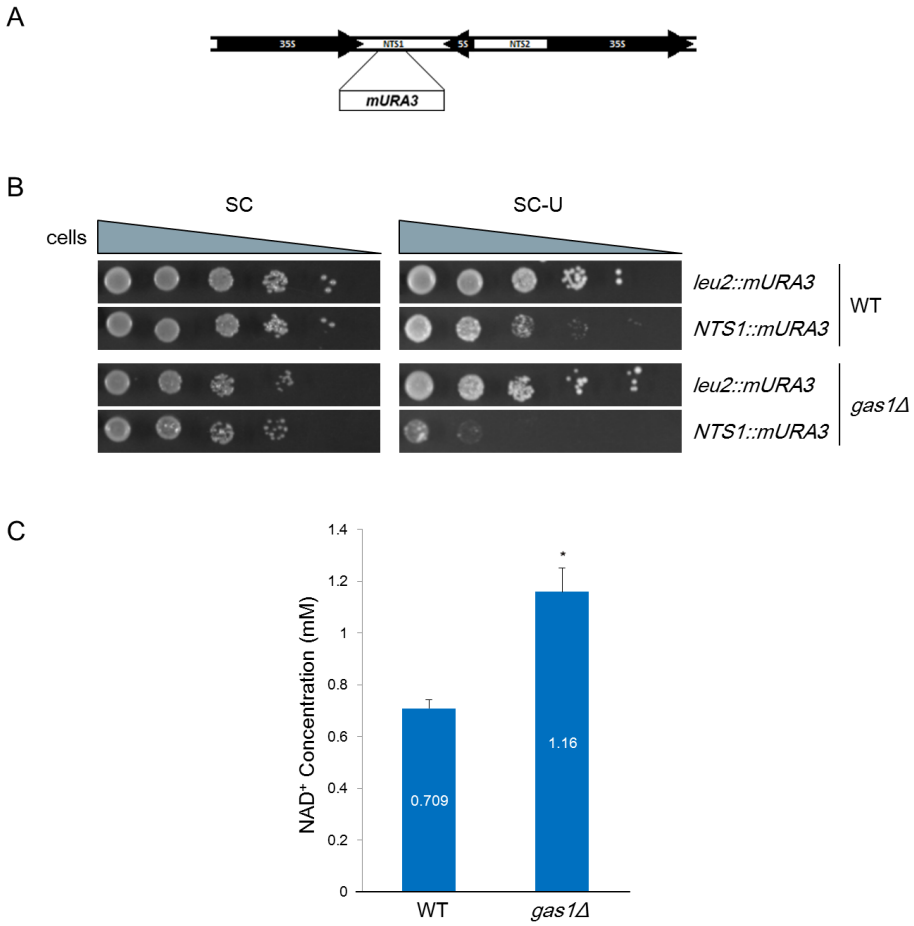


Figure 1. The loss of Gas1 enhances rDNA silencing. (A) The physical structure of the tandemly repeating RDN1 locus of *S. cerevisiae* is shown above, and a single 9.1-kb rDNA unit is shown expanded below. Each repeat yields a Pol I-transcribed 35S precursor rRNA and a Pol III-transcribed 5S rRNA. The 35S coding regions are separated by an NTS, which is divided by the 5S gene into NTS1 and NTS2. (B) The loss of Gas1 enhances rDNA silencing. Silencing within rDNA was assessed by monitoring the growth of cells (10-fold serial dilutions) plated on SC medium without uracil. SC medium was used as a plating control. (C) The loss of Gas1 raises the intracellular NAD^+ concentration. Values represent the average of three independent experiments, and error bars indicate the SEM. Asterisks indicate $P<0.05$, compared with WT cells (Student's t-test).

2. Loss of Gas1 gene induces the expression of Pnc1 in Msn2-dependent manner.

Gas1 regulates transcriptional silencing in rDNA locus by controlling Sir2 activity (data not shown). When Sir2 deacetylates histone, Sir2 converts NAD^+ to NAM, NAM interrupts Sir2 enzymatic activity (6). Nicotinamidase named Pnc1 eliminates NAM by converting to NA, and expression of Pnc1 is regulated by Msn2/4. Msn2 and Msn4 are transcription factors controlling the general stress response (9,25,26). They enter nucleus from cytoplasm when stimulus occurs, and activate transcription of several response genes. So, there is a possibility that Sir2 silencing in*gas1Δ* cell is affected by Pnc1-Msn2/4. We performed western blotting to check the cellular Pnc1 level. Expression of Pnc1 is increased in *gas1Δ* cell compared to wild type cell.

Phos-tag gel measurement of Msn2, the transcription factor of Pnc1 shows that Msn2 is less phosphorylated in case of *gas1Δ*, while Msn2 in wild type cell is highly phosphorylated. Msn2 phosphorylation pattern of *gas1Δ* is similar to that of glucose starvation, which is well known Msn2 nuclear accumulation condition. It means that Msn2 is capable to get inside the nucleus. Consistent with this data, translocalization of Msn2 is induced without Gas1. Through fluorescence microscopic analysis using C-terminal GFP fusion of Msn2, nuclear accumulation of Msn2 is observed in *gas1Δ* cell. These results suggest that the expression of Pnc1 is induced by Msn2 in the absence of GAS1 gene.

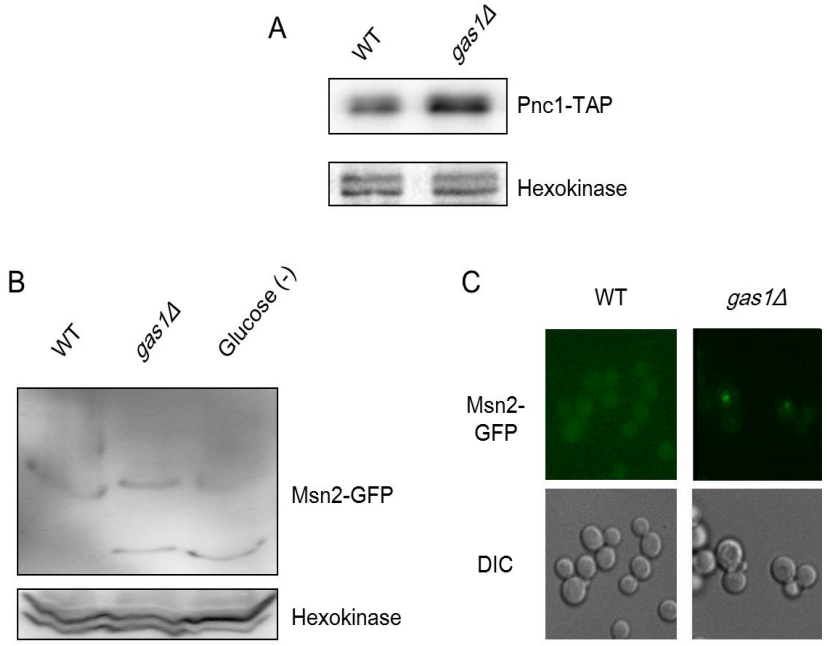


Figure 2. The loss of Gas1 induces the expression of Pnc1 and promotes rDNA stability in an Msn2-dependent manner. (A) The protein level of Pnc1 increases in the absence of Gas1. Total protein was extracted from WT and *gas1Δ*cells, and immunoblotting was performed using an HRP-conjugated anti mouse IgG antibody for the detection of TAP-tagged protein. Hexokinase was used as a loading control. The relative ratio of Pnc1 to hexokinase, normalized against that of WT cells, is shown below each lane. (B) The loss of Gas1 increases phosphorylation of Msn2. Total protein was extracted from WT and *gas1Δ* cells, and immunoblotting was performed using a mouse anti-GFP antibody for the detection of GFP-tagged protein. Hexokinase was used as a loading control. (C) The absence of Gas1 promotes nuclear accumulation of Msn2. WT and *gas1Δ*cells with chromosomally GFP-tagged Msn2 were grown to logarithmic phase in SC medium and analyzed by fluorescence microscopy.

3. PKA activity is reduced in the absence of Gas1.

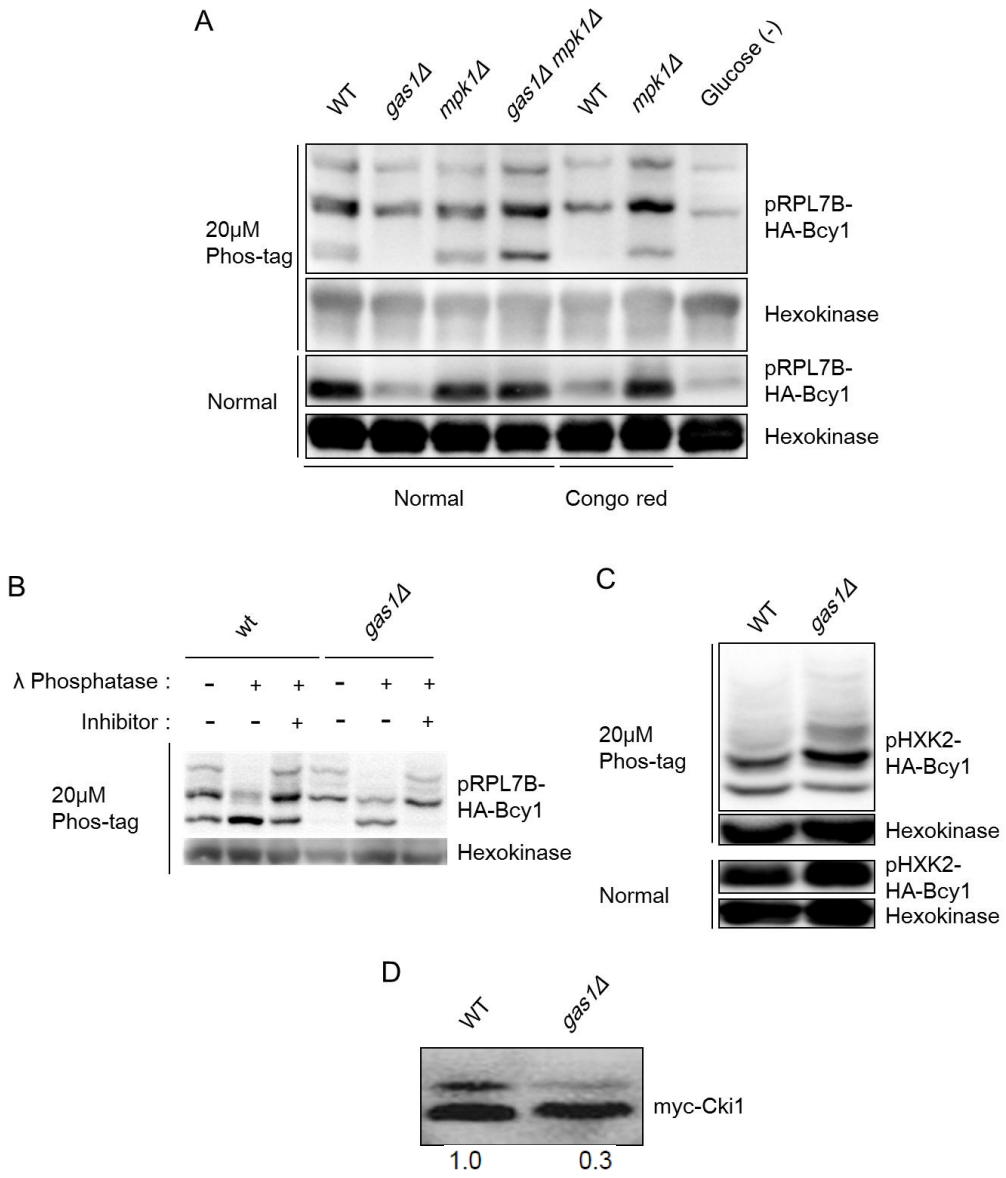
It is previously studied that Msn2 and Msn4 are inhibited to nuclear accumulation by phosphorylation (27, 28). One of the well-known kinase of Msn2/4 is PKA (27, 29, 30). When PKA activity is high, PKA phosphorylates Msn2 and inhibits import of Msn2 into the nucleus (29, 31, 32). On the other hands, when PKA activity is low, Msn2 exists in dephosphorylation form and is capable of nuclear import. PKA consists of two kinds of subunits (33, 34). Those are Tpk1, 2, 3 – the catalytic subunits and Bcy1 – the regulatory subunit. In low PKA activity, Tpk1 accumulates in nucleus and associates phosphorylated Bcy1. We thought that nuclear accumulation of Msn2 in *gas1Δ* cell is related with PKA activity, so we investigated PKA activity at the loss of Gas1. RPL-7B promoter N-terminal HA tagging Bcy1 is tested on 20μM Phos-tag PAGE. In the absence of Gas1, phosphorylation band shift occurs, similar to glucose starvation used as positive control. Ratio of phosphorylated Bcy1 is much higher in case of *gas1Δ* and Congo red treatment, compared with wild type. And the effects of *gas1Δ* and Congo red treatment disappeared when Mpk1, MAPK of cell wall integrity, is deleted. Phosphorylation patterns of *mpk1Δ* cell and *mpk1 gas1Δ* cell are similar to those of wild type cell. It implies that Gas1 signaling to Sir2 is mediated by PKA passing through Mpk1.

In addition, we confirmed that the mobility shift on Phos-tag gel is true phosphorylation. The shifted bands are vanished by λ phosphatase treatment and are recovered in presence of the phosphatase inhibitor, meaning that the

shift is real phosphorylation.

However, the expression level of Bcy1 is not equal between each condition, because RPL-7B promoter is sensitive to stress. Therefore there is a possibility of promoter-specific phosphorylation. To eliminate this possibility, we used HXK2 promoter which is a constant promoter. Within different promoter, Bcy1 is still highly phosphorylated in *gas1Δ* cell. It shows that increased phosphorylation of Bcy1 is not a promoter-specific effect.

Furthermore, we checked that increased Bcy1 phosphorylation really leads to decreased PKA activity. Using 6×myc-Cki1 as a PKA activity indicator, more phosphorylation shows more PKA activity. Without Gas1, phosphorylation is decreased in comparison to wild type. It shows that Bcy1 phosphorylation truly reflects PKA activity.


A
WT
gas1Δ
mpk1Δ
gas1Δ mpk1Δ
WT
mpk1Δ
Glucose (-)
20μM
Phos-tag
pRPL7B-
HA-Bcy1
Hexokinase
Normal
pRPL7B-
HA-Bcy1
Hexokinase
Normal
Congo red
B
wt
gas1Δ
λ Phosphatase :
Inhibitor :
20μM
Phos-tag
pRPL7B-
HA-Bcy1
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C
WT
gas1Δ
20μM
Phos-tag
pHXK2-
HA-Bcy1
Hexokinase
Normal
pHXK2-
HA-Bcy1
Hexokinase
D
WT
gas1Δ
myc-Cki1
1.0
0.3

Figure 3. Gas1 controls PKA activity in an Mpk1-dependent manner. (A) The loss of Gas1 and Congo red treatment increases phosphorylation of Bcy1 in an Mpk1-dependent manner. Total protein was extracted from WT, *gas1Δ*, *mpk1Δ*, and *gas1Δ mpk1Δ* cells with N-terminally HA-tagged Bcy1 and run on a normal SDS-polyacrylamide gel and an SDS-polyacrylamide gel containing 20 μM Phos-tag (lane 1-4). Protein extracts from WT and *mpk1Δ* cells treated with 100 μg/ml Congo red for 1 h were also run on the same gel (lane 5-6). Immunoblotting was performed using an HRP-conjugated anti-HA antibody for the detection of HA-Bcy1. Hexokinase was used as a loading control. (B) Upper bands of sample are confirmed as phosphorylation shift. Total protein was extracted from WT and *gas1Δ* cells with N-terminally HA-tagged Bcy1 and run on an SDS-polyacrylamide gel containing 20 μM Phos-tag. Immunoblotting was performed using an HRP-conjugated anti-HA antibody for the detection of HA-Bcy1. Hexokinase was used as a loading control. (C) Expression level of Bcy1 does not affect differential phosphorylation. Total protein was extracted from WT and *gas1Δ* cells with N-terminally HA-tagged Bcy1 and run on a normal SDS-polyacrylamide gel and an SDS-polyacrylamide gel containing 20 μM Phos-tag. Immunoblotting was performed using an HRP-conjugated anti-HA antibody for the detection of HA-Bcy1. Hexokinase was used as a loading control. (D) The absence of Gas1 decreases the *in vivo* activity of PKA. Total protein was extracted from WT and *gas1Δ* cells harboring pRS423-pr^{CUP}-*6×MYC-cki1*$^{2–200(S125/130A)}$, and immunoblotting was performed using a mouse anti-Myc antibody. The relative ratio of phosphorylated (Cki1-P) to non-phosphorylated (Cki1) forms of Cki1, normalized against that of WT cells, is shown below each lane.

4. Up-regulated PKA activity is occurred in *rho⁰* mutant.

rho⁰ mutant is the whole loss of mtDNA mutant. Complete absence of mtDNA causes some eccentric features like mitochondria dysfunction, swelled cell volume, and declined chronological life span to occur (14, 15). On the contrary to *gas1Δ* mutant, up-regulated PKA activity is previously discovered in *rho⁰* mutant. Intercellular concentration of carbohydrate is a convenient reference of PKA activity. High PKA activity promotes metabolism and it decreases carbohydrate storage, while low PKA activity impedes metabolism and it increases carbohydrate storage. Carbohydrate contents are decreased in *rho⁰* mutant, measured by iodine test. But there are several possibilities that decreased carbohydrate of *rho⁰* mutant is caused by other factors rather than PKA, considering mitochondrial malfunction of *rho⁰* mutant. Therefore, we confirmed the activity of PKA by checking 6×myc-Cki1 phosphorylation. Compared to wild type, phosphorylation is increased in *rho⁰* mutant. This data reaffirms up-regulated PKA activity in *rho⁰* mutant. In light of these results, we thought if rDNA silencing is induced when PKA activity is decreased, there is likelihood that increased PKA activity, such as *rho⁰* mutant, leads to reduced rDNA silencing.

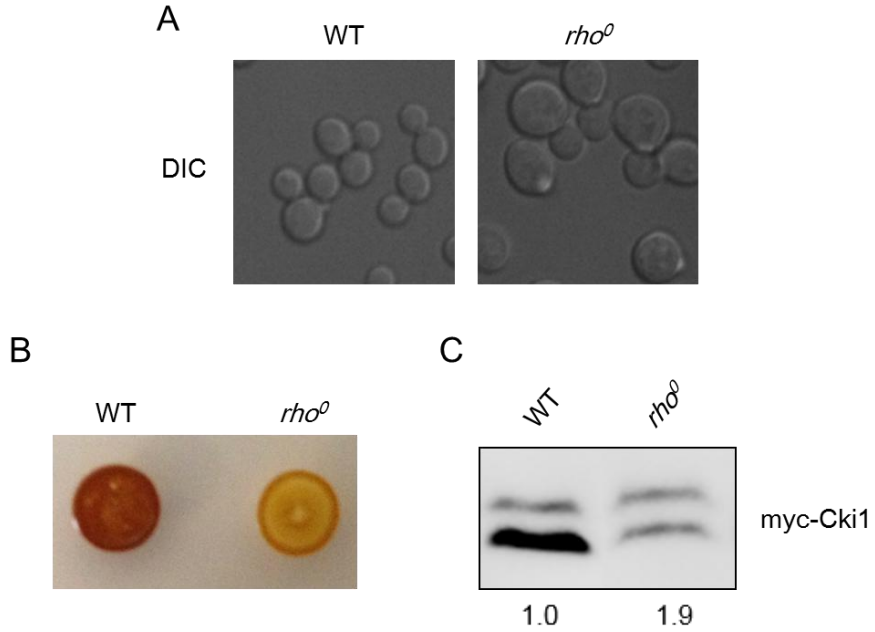

Figure 4. PKA activity is increased in *rho⁰* mutant. (A) Cells become swollen in *rho⁰* mutant. WT and *rho⁰* mutant cells were grown to logarithmic phase in SC medium and analyzed by fluorescence microscopy. (B) The absence of mitochondrial DNA increases carbohydrate concentration. Cells were grown to logarithmic phase in SC plate and analyzed by iodine test. (C) *rho⁰* mutant increases the *in vivo* activity of PKA. Total protein was extracted from WT and *rho⁰* mutant cells harboring pRS423-pr^{CUP}-*6×MYC-cki1*$^{2-200(S125/130A)}$, and immunoblotting was performed using a mouse anti-Myc antibody. The relative ratio of phosphorylated (Cki1-P) to non-phosphorylated (Cki1) forms of Cki1, normalized against that of WT cells, is shown below each lane.

5. Pnc1 expression by Msn2 is not increased when mitochondrial DNA is lost.

As mentioned earlier, Pnc1 is expressed by Msn2/4 nuclear import activation and the translocalization is affected by PKA (8, 9). It was ascertained that PKA is activated in *rho^0* mutant, so we performed fluorescence microscopic analysis using C-terminal GFP fusion Msn2 to check Msn2 localization. As expected, Msn2 nuclear accumulation is not observed in *rho^0* mutant cell, unlike *gas1Δ* or glucose starvation condition. Consistent with this, Pnc1 expression is not increased in *rho^0* mutant cell. It means that Msn2/4-Pnc1 cannot activate Sir2 when mtDNA is lost.

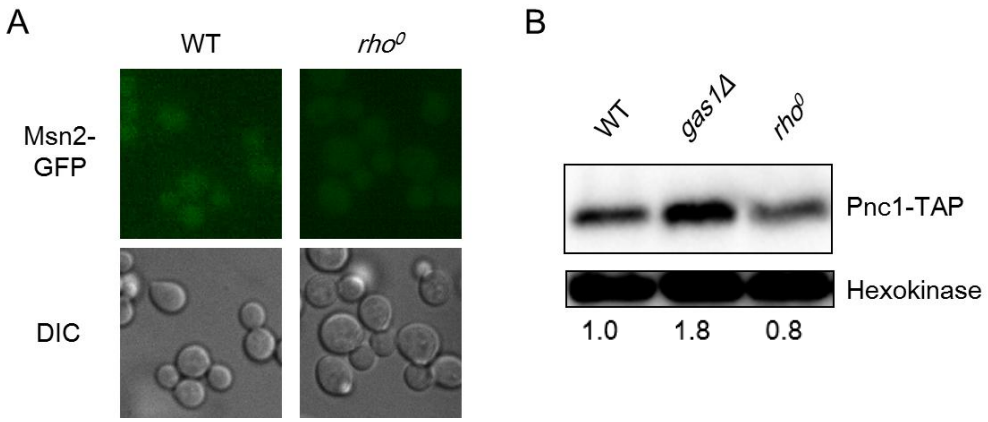

Figure 5. The loss of mitochondrial DNA reduces the expression of Pnc1 in an Msn2-dependent manner. (A) The absence of mitochondrial DNA does not promote nuclear accumulation of Msn2. WT and *rho*^{0} mutant cells with chromosomally GFP-tagged Msn2 were grown to logarithmic phase in SC medium and analyzed by fluorescence microscopy. (B) The protein level of Pnc1 decreases in the absence of mitochondrial DNA. Total protein was extracted from WT, *gas1Δ*and *rho*^{0} mutant cells, and immunoblotting was performed using an HRP-conjugated anti mouse IgG antibody for the detection of TAP-tagged protein. Hexokinase was used as a loading control. The relative ratio of Pnc1 to hexokinase, normalized against that of WT cells, is shown below each lane.

6. In *rho*^{0} mutant, ribosomal DNA silencing is decreased.

In case of PKA up-regulation by *rho*^{0} mutant, we thought that Sir2 might be affected. So, we first measured intracellular NAD^+ level in *rho*^{0} mutant cell. As, mentioned above, Sir2 needs NAD^+ to be activated. NAD^+ level of *rho*^{0} mutant cell is lower than that of wild type cell.

Next, we tested the expression of Sir2 to identify whether Sir2 expression level is changed. As a result, Sir2 expression level of *rho*^{0} mutant is same as that of wild type.

Then we finally performed a spot assay to find out rDNA silencing differences between wild type and *rho*^{0} mutant. We used previously mentioned strains, carrying the mURA3 silencing reporter gene inserted either inside the NTS1 region of the rDNA locus (RDN1-NTS1::mURA3) or outside the rDNA array (leu2::mURA3) (19). Compared to wild type cell, *rho*^{0} mutant cell displays dramatically decreased silencing of mURA3 reporter gene at rDNA region. It means that mitochondrial malfunction caused by mitochondrial DNA loss triggers declined rDNA stability and the signaling pathway is related with PKA pathway, not by controlling Sir2 expression level.

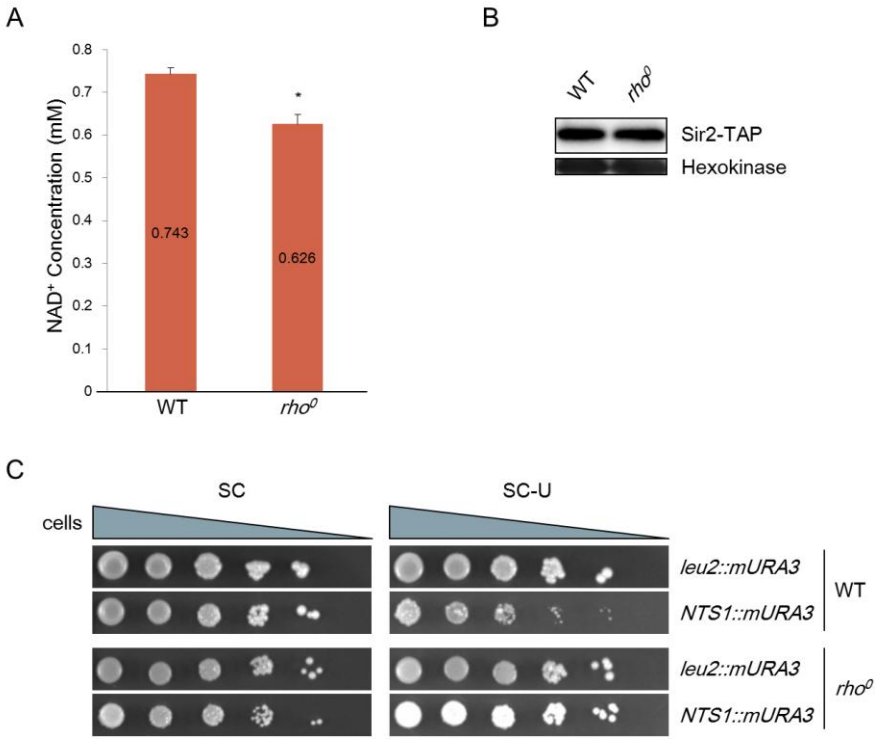

Figure 6. The loss of mitochondrial DNA reduces rDNA silencing. (A) *rho⁰* mutant decreases the intracellular NAD^+ concentration. Values represent the average of three independent experiments, and error bars indicate the SEM. Asterisks indicate $P<0.05$, compared with WT cells (Student's t-test). (B) The protein level of Sir2 maintain in the absence of mitochondrial DNA. Total protein was extracted from WT and *rho⁰* mutant cells, and immunoblotting was performed using an HRP-conjugated anti mouse IgG antibody for the detection of TAP-tagged protein. Hexokinase was used as a loading control. (C) *rho⁰* mutant diminishes rDNA silencing. Silencing within rDNA was assessed by monitoring the growth of cells (10-fold serial dilutions) plated on SC medium without uracil. SC medium was used as a plating control.

Discussion

In *Saccharomyces cerevisiae*, rDNA has a repetitive structure and it is a main reason of homologous recombination (1). ERCs formation by homologous recombination is repressed by Sir2 as a key component of RENT complex (3). Sir2 is affected by many factors. One of them is β-1,3-glucanosyltransferase Gas1 which has a role in cell wall organization by making crosslink of β-1,3-glucan (13). Previous study by Koch and Pillus shows that Gas1 controls rDNA stability through Sir2 (16). In our study, we demonstrated the pathway from Gas1 to rDNA silencing. When GAS1 gene is deleted, PKA activity becomes reduced and it causes induced Pnc1 expression by Msn2/4. Increased Pnc1 and NAD^+ level expedite rDNA silencing by Sir2. Also, there are significant possibilities that Mpk1 and cell wall MAPK signaling take part in signal transduction from Gas1 to PKA, which is a part of RAS-cAMP signaling cascade. Furthermore, Congo red treated cell shows similar rDNA silencing status. None of other cell wall organization enzymes or cell wall affecting agents relates with rDNA stability, only Gas1 and Congo red are linked with rDNA stability.

Gas1 is the important cell wall integrity protein due to its glucanosyltransferase activity, and there are sequential differences between Gas1 and other glucanosyltransferases (11). Although Bgl2 is also β-1,3-glucanosyltransferase, it plays a less important role (35). It has no sequence homologies with Gas1. GAS2, GAS3, GAS4 and GAS5 have high sequence homologies, but only Gas1 has both

the Cys Box and the Ser Box (36).

In *gas1Δ* cells, there are less glucan cross linkages and more chitin in cell wall. So they are more resistant to alkali and zymorase (37-39). However, they are sensitive to sodium dodecyl sulfate as osmotic destabilizing agent, nikkomycin as chitin synthesis inhibitor, and Calcofluor White as chitin antagonist. Intriguingly, the cell wall of *gas1Δ* cells shows dramatically decreased elasticity (40). The *gas1Δ* cells have significant differences, compared to other cell wall integrity genes deletion type. The cells have defection in cell separation and this defection is on account of dysfunction at bud growth and maturation (37). These are the reason of 'grape shape growth' of *gas1Δ* cells. Glucan fibers of the cell wall are weakened and it makes cell to swell. Interestingly, most of these features are common to Congo red treated cells. Congo red affects β-1,3-glucan, by forming thin fibrils and loosening their helical organization (41, 42). Therefore, it makes abnormal cell wall structure. In the presence of Congo red, cell volume is increased because of weakened cell wall. In addition, cells are forming specific chain connection. Both *gas1Δ* cells and Congo red treated cells are growing in clumped cell type and larger bulk, because of defective β-1,3-glucan cell wall. Therefore, we think that GAS1 deletion and Congo red treatment cause specific type of damage to β-1,3-glucan of yeast cell wall and it brings about rDNA silencing by Sir2 activation.

rho^{0} mutant cell has serious mitochondrial respiratory malfunction because mitochondrial DNA is completely missing (14). This type of mutant is well

known for mitophagy. In addition, PKA activity becomes induced and chronological life span (CLS) is collapsed in *rho*^{0} mutant cell (15). We found that rDNA stability is dropped in case of whole mtDNA loss. Also it is determined that Msn2/4 is not imported to nucleus and Pnc1 expression is slightly decreased in *rho*^{0} mutant. So we think that mitochondrial respiratory malfunction by mtDNA loss causes decreased rDNA silencing by controlling Sir2 activity. We presume that respiratory dysfunction of *rho*^{0} mutant triggers cAMP concentration change which leads to PKA activity change. In case of CYC3 gene deleted cell which shows respiratory dysfunction and decreased CLS like *rho*^{0} mutant cell, PKA activity increased.

Thus, we revealed a pathway, consisting of Gas1-Mpk1-PKA-Msn2/4-Pnc1-Sir2-rDNA, by which *gas1Δ* leads to suppression of PKA by Mpk1 route and promotes Sir2-mediated rDNA silencing. In addition, *rho*^{0} mutant signaling through PKA affects Msn2/4, Pnc1, Sir2 and rDNA silencing. These findings suggest the new insight for cell wall damage caused by *gas1Δ* and mitochondrial malfunction, which have effects on transcriptional silencing in rDNA region.

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국문초록

출아효모에서 rDNA는 9.1 kbp의 단위가 100~200회 직렬로 반복된 구조를 가지고 있다. 이렇듯 고도로 반복된 구역의 안정성은 rDNA silencing이라는 과정에 의해 조절이 된다. 우리는 이 연구에서 Gas1이 rDNA의 transcriptional silencing을 PKA 신호 전달 과정을 통해 조절한다는 점을 밝혔다. Gas1은 β-1,3-glucanosyltransferase로 세포벽을 형성하는데 기여한다. Gas1이 제거되었을 때 PKA 활성이 감소하였고 이는 두 종류의 스트레스 반응 전사인자인 Msn2와 Msn4의 탈인산화를 유발한다. 탈인산화된 Msn2/4는 세포질로부터 핵 안으로 들어올 수 있게 되고 Pnc1의 전사를 촉진시킨다. Pnc1은 RENT 복합체를 형성하여 rDNA를 silencing시키는 Sir2의 활성인자이다. 또한 세포벽 손상 물질인 Congo red를 세포에 처리할 경우에도 유사한 양상의, Sir2가 개입된 rDNA silencing의 증가 현상을 보여주었다.

반면, mtDNA의 상실로 인한 미토콘드리아 호흡 장애 상태인 *rho*^{0} 돌연변이에서는 Gas1이 제거된 상황과는 달리 PKA 활성이 증가되었다. PKA

활성의 증가는 rDNA silencing의 감소를 불러왔다. 이러한 발견들은 *gas1Δ* 돌연변이와 *rho*^{0} 돌연변이가 PKA 신호 전달 경로를 조절함으로 인해 각기 다른 방향으로 rDNA 안정성에 영향을 미친다는 것을 알려준다.

주요어: rDNA Silencing, 반복분열수명, Sir2, PKA, Gas1, *rho*^{0} 돌연변이.

감사의 글

예상보다 많이 힘들었지만, 값진 시간이었던 석사생활을 마무리하며, 기다림 끝에 소중한 분들께 이렇게 감사의 글을 쓰게 되어 정말 기쁩니다.

먼저 대학원 생활을 잘 할 수 있도록 지도해주신 허원기 교수님께 정말 감사 드립니다. 쉽지 않은 시간이었지만, 교수님께서 끊임없는 열의로 지도해주신 덕분에 석사 과정을 잘 마칠 수 있었습니다.

또한 제가 걱정 없이 공부만 할 수 있도록 지원해 주신 가족들에게 고마움을 전하고 싶습니다. 집에도 자주 내려가지 못하고 효도 한 번 제대로 하지 못하였는데도 항상 제가 공부하는 걸 응원해주시고 제 건강 걱정 하셨던 아버지, 어머니, 그리고 지후에게 정말 고맙고 사랑한다고 말하고 싶습니다.

공부하는 동안 가족 이상으로 긴 시간을 함께 했던 실험실 식구들에게도 고맙단 말을 전하고 싶습니다. 사수님이셨던 철웅이형께 제일 먼저 감사의 인사를 전합니다. 늘 인자한 웃음과 함께 조언해주셨던 용범이형께도 정말 많은 도움을 받았습니다. 멘토가 되어주셨던 대관이형, 방장이셨던 규범이형, 방장이신 연지누나께 많은 도움을 받아왔고 감사함이 이루 말할 수가 없네요. 조언과 격려를 아끼지 않으시던 봉근이형, 옆자리에서 많이 도와주셨던 연수누나께도 감사드립니다. 동기였던 철오형에게도 많은 도움을 받았습니다. 실험실 후배인 창수형에게도 고맙습니다. 실험실에서 가장 많이 대화를 나누었던 지원이형에게도 매우 감사드리며 앞으로 학위과정을 잘 해내길 기원합니다.

감사의 글을 쓰면서 석사과정 동안 있었던 일들이 주마등처럼 흘러가는 것은 누구나 비슷할 것 같습니다. 앞으로 이 순간을 떠올리며, 어떤 어려움에도 견뎌낼 수 있는 원동력이 되도록 하겠습니다. 항상 좋은 일이 가득하시길 바랍니다. 감사합니다.

2014년 2월 관악에서 김관태 드림