Analysis of inducers and suppressors controlling cell lysis in *S. pombe ura4* deletion strains

（分裂酵母 *ura4* 破壊株の細胞溶解を制御する促進及び抑圧因子の解析）

KOHEI NISHINO

西野　耕平

2017

Abbreviations

2DG	2-deoxyglucose
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
DMF	N,N-DiMethyl Formamide
DMSO	DiMethyl SulfOxide
DNA	Deoxiribo Nucleic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
EMM	Edinburgh Minimal Medium
5-FOA	5-Fluoro Orotic Acid
5-FU	5-Fluoro Uracil
GC-MS	Gas-Chromatography-Mass spectrometry
GFP	Green fluorescent protein
kan	Kanamycin
LC-MS	Liquid-Chromatography-Mass spectrometry
MRM	Multiple Reaction Monitoring
MVB	MultiVesicular Body
nat	Clone NAT
nmt	no message in thiamine
ODCase	OMP Decarboxylase
OMP	Orotidine 5'-Mono Phosphate
PCR	Polymerase Chain Reaction
PDA	PhotoDiode Array
RT	Room Temperature
UDP	Uridine Di Phoshate
UDPG	Uridine Di Phoshate-Glucose
UMP	Uridine Mono Phosphate
UTP	Uridine Tri Phosphate
WT	Wild Type
YE	Yeast Extract
YES	Yeast Extract with Supplements
YPD	Yeast extract peptone dextrose

Chapter 1

Introduction

Fission yeast as eukaryotic model organism

Fission yeast has been used for genetics, molecular biology and bio-production studies by many researchers around the world[1]. Four fission yeast species are currently known namely, *Schizosaccharomyces pombe*, *S. japonicus*, *S octosporus* and *S. cryophilus*. Among them *S. pombe* is most extensively investigated. *S. pombe* is a rod shape, with the length of 10-12μm and the width of 3-4μm. *S.pombe* has the following advantages as a model organism. 1) The genome of *S. pombe* has been determined with 5118 encoded genes in 12.57Mbp of DNA on three chromosomes and the essential genes as estimated to be 2,235. 2) Because non-essential gene deletion library of *S. pombe* is available, it is easy to reverse genetic screening. 3)Because *S. pombe* forms both haploid and diploid, it is easy to construct a gene deletion strains and methodology of gene manipulation in well established. 4)Because *S. pombe* is rod shape,it is used to study cell polarity not like *S. cerevisiae*. 5) Because its cytokinesis is remarkably similar to that of animal cells, *S. pombe* is beneficial to study cell division.

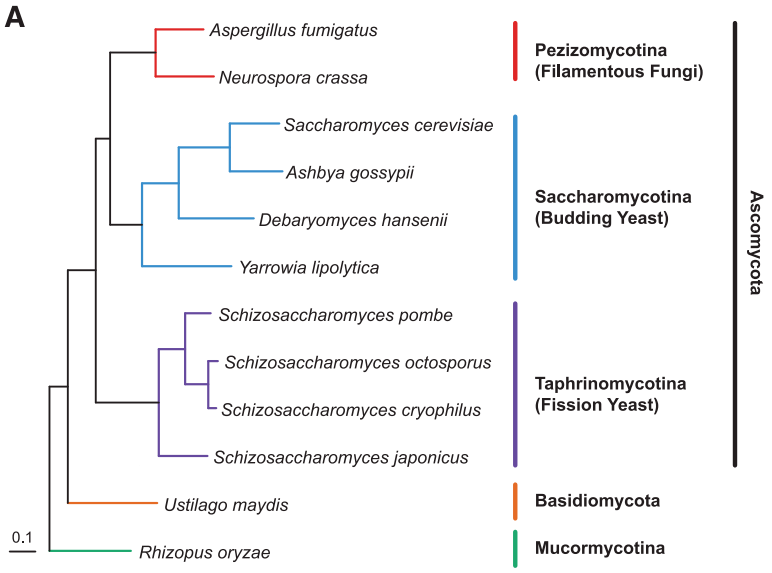


Figure 1-1 *Schizosaccharomyces* phylogeny[2]

A maximum-likelihood phylogeny of 12 fungal species from 440 core orthologs (each occurring once in each of the genomes) from fly to yeast. A maximum-parsimony analysis produces the same topology. Both approaches have 100% bootstrap support for all nodes.

Cell lysis of *S.pombe* and cell wall

When part of the cell wall or septa is broken and cellular components are leaked by either gene deletion or drug, it is called cell lysis. The cell lysis of *S. pombe* has been observed under seneral condition. For example, aculeacin A, a peptide antibiotic product, induced cell lysis of *S. pombe*[3]. In this case, the cell lysis is induced by inhibiting the synthesis of β(1,3)-glucan which is a major component of cell wall in *S. pombe*. 2-deoxyglucose (2DG) is also known as cell lysis inducer in *S. pombe*[4]. 2DG induces cell lysis by inhibiting the synthesis of glucan and mannan which are components of cell wall. In this case, cell lysis occurs at the site of glucan synthesis of cell wall.

Cell lysis is also observed as one of the phenotype among *S. pombe* mutants. Bgs1, Ags1/Mok1 and Bgs4 involve β or α-glucan synthesis in the formation cell wall or septa in *S. pombe*[5-7]. Therefore, such mutant or conditional knockout strains in *bgs1, bgs4* or *ags1/mok1* resulted in cell lysis in S. pombe. In this case, cell lysis is induced by decrease in cell wall integrity. For this reason, cell lysis is suppressed by osmotic stabilization, such as sorbitol. Furthermore, cell lysis is induced also in the hyperactivation of Nak1-Mor2 or overexpression of *Sjace2*, both are involved in cytokinesis. In this case, cell lysis starts at septa during cytokinesis[8,9].

A major component of cell wall in *S. pombe* is β-glucan, which is classified into β-1,3glucan (48~54%) and β-1,6glucan (4~7%) by binding mode of glucan. The cell wall of *S. pombe* consists of also, which α-glucan is classified into α-1,3 glucan (28~32%) and α-1,4 glucan (7%) by binding mode of glucan. On the outside of the cell wall are present galactomannan binding protein (9~14%. These components form a network in a mesh shape on the cell wall (Fig1-2) [10-12].

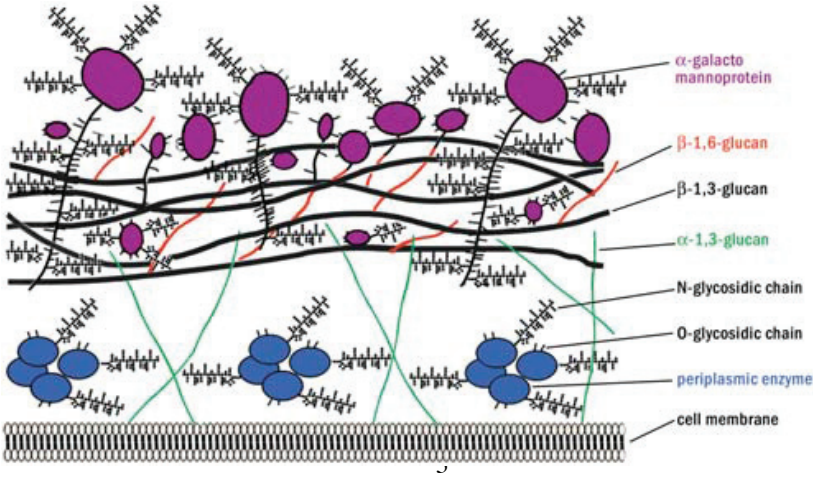


Figure 1-2 Model of molecular composition and structure of cell wall of *S. pombe*.

Cell lysis of *S. pombe ura4* gene deletion mutants[13]

Cell lysis in *ura4* gene deletion mutant is induced when cells are grown in YPD media. The *ura4* gene deletion mutant is sensitive to zymolyase, which induces β-glucanase when cells are grown in YPD media. Cell lysis of *ura4* mutant is suppressed when sorbitol was added in YPD media. Ura4 protein is orotidine-5-monophoaphate (OMP) decarboxylase which catalyses the last step of *de novo* UMP synthesis pathway. Ura1, Ura2, Ura3, Ura4, and Ura5 proteins are involved in UMP synthesis (Fig 1-3). The cell lysis of the *ura4* mutants is suppressed by concomitant deletion of *ura1*, *ura2, ura3* or *ura5* genes. Accumulation of OMP, on intermediate in *de novo* UMP synthesis pathway, is observed. But, the detailed mechanism how OMP accumulation affects cell lysis is unclear. Cell lysis of *ura4* gene mutants is induced in YPD media but note in YE medium. Therefore polypeptone must contain inducer(s) of cell lysis in *ura4* gene mutants. Polypeptone is made of low molecular weight peptide and free amino acids. Polypeptone has been used in the medium as a nitrogen source or amino acids source. However, cell lysis of *ura4* gene mutants was not induced by addition of single amino acid in YE medium.

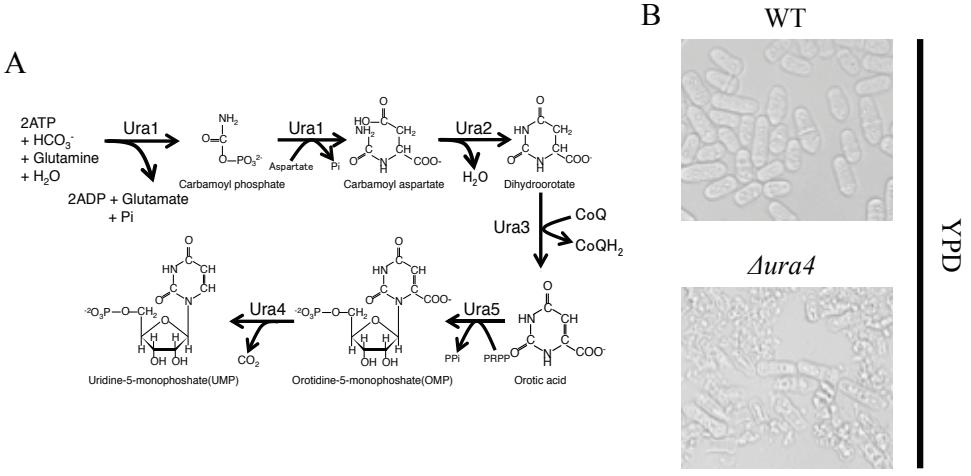


Figure 1-3. Ura1 is a bifunctional enzyme of carbamoyl phosphate synthetase I and aspartate transcarbamoylase, Ura2 is dihydroorotase, Ura3 is dihydroorotate dehydrogenase which requires quinone as a cofactor and localizes in mitochondria, Ura5 is orotate phosphoribosyl transferase, and Ura4 is OMP decarboxylase. (B) Microscopic images of *S. pombe* WT and *Δura4* cells. Cells were grown on YPD medium.

Ubiquitination of protein

Ubiquitin is a low molecular weight protein consisting of 76 amino acids. Ubiquitin is conserved in almost all eukaryotic organisms but not present in prokaryotes. This protein has a role as a post-translational modification molecule by covalent bonding to a lysine residue of the target protein. Modification is either by polyubiquitin or monoubiquitin, the chain length of polyubiquitin is important. The primary role of ubiquitin is protein degradation by ubiquitin-proteasome system[14,15]. However, other roles are found in and DNA repair, cell cycle, signal, autophagy or endocytosis without degradation[16,17]. Ubiquitination to target proteins is conducted by a series of reaction through E1(ubiquitin activating enzyme), E2(ubiquitin-conjugating enzyme) and E3(ubiquitin ligase). Generally ubiquitin is activated with ATP by E1. Activated ubiquitin is transferred from E1 to E2. Then E2 bounds to E3, and E3 bounds to the target protein. Finally ubiquitin is transferred from E2 to the target protein, and ubiquitination of the target protein is completed. Only one type (Uba1) E1 presents in *S. pombe*, while E2 are 11 kinds and E3 present more than 100 kinds. Therefore, selectivity of the target protein is determined by combination of E2-E3 (Fig 1-4).

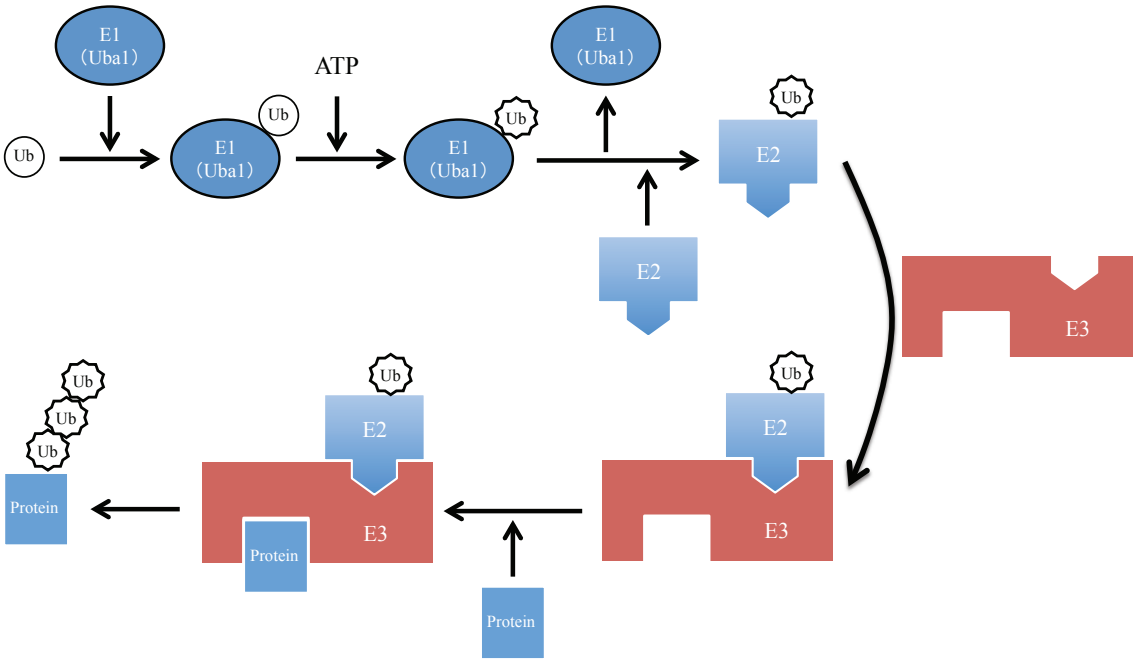
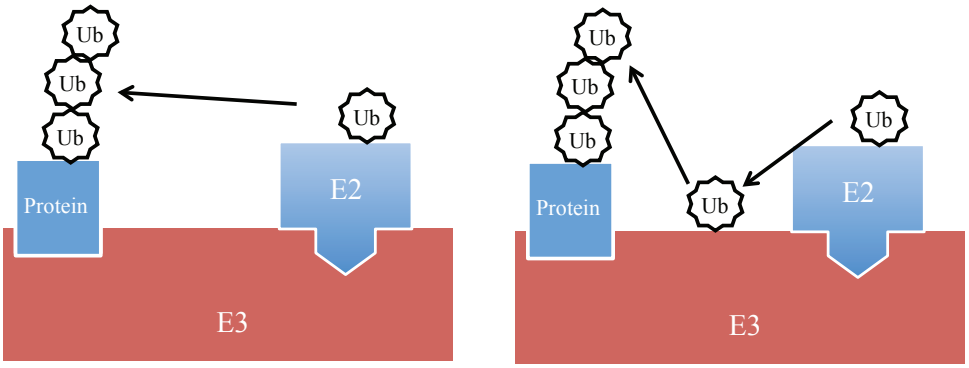


Figure 1-4. Major enzymatic components and reaction of the ubiquitination. E1:ubiquitin activating enzyme, E2:ubiquitin-conjugating enzyme and E3:ubiquitin ligase.

E3 ubiquitin ligase Pub1

S. pombe Pub1 is a HECT-type E3 ubiquitin ligase and is a homolog to Rsp5 of *Saccharomyces cerevisiae*. HECT type E3 ligase has the characteristic in that ubiquitin transfer to target protein from E2 via E3. Ubiquitin is transferred directly to target protein by RING type E3 ligase (Fig 1-5). Pub1 and Rsp5 are known to be involved in localization control of membrane proteins through ubiquitination[18-21]. Aat1 is ubiquitinated and localized to the Golgi apparatus by Pub1 under nitrogen-rich condition. It is known that physical interaction of Pub1 and Arrestin protein Any1 is increased in *tsc1* or *tsc2* gene deletion strains. Therefore, membrane localization of Aat1 decreased in *tsc1* gene deletion strains. Aat1 is deubiquitinated and localization to plasma membrane increased in nitrogen depletion condition. *S. cerevisiae* Rsp5 is essential gene, *S. pombe* but *pub1* gene is non-essential gene, because there are *pub2* and *pub3* genes as other HECT type E3 ligases in *S. pombe*. Three WW domains existing in RSP5 are important to bind to target protein. There are also WW domains in Pub1, Pub2 and Pub3 in *S. pombe*. *pub1* and *pub3* genes are highly homologous to each other and double gene deletion strains shows synthetic lethality[22]. *S. cerevisiae* Rsp5

is involved in the quality control mechanism of membrane protein[17,23,24]. For example, localization of uracil transporter Fur4 is under the control of ubiquitination. LID domain of Fur4 is exposed away from the membrane by heat or oxidative stress. The RSP5 and arrestin proteins (ATRs) cooperatively recognize target protein[23,25]. Fur4 that is ubiquitinated is degraded by endocytosis. Further, exposure of LID domain takes place by uracil incorporation from membrane. Fur4 is degraded through ubiquitination by excess substrate.



RING type HECT type

Figure 1-5. Characteristic of two type E3 ubiquitin ligase. Two major type of E3 are illustrated.

Quality control of the plasma membrane[26]

When membrane proteins are damaged by temperature or drug stress are recognized, it is quickly degraded. Then, there is a change in the localization of membrane proteins from Golgi to plasma membrane under vegetative conditions. E3 ubiquitin ligase such as Pub1 and RSP5 are involved in recognition of membrane proteins (Fig 1-6).
Such a mechanism is called a membrane protein quality control mechanism. The membrane quality control system of eukaryotic cells is very important for proper cellular function.

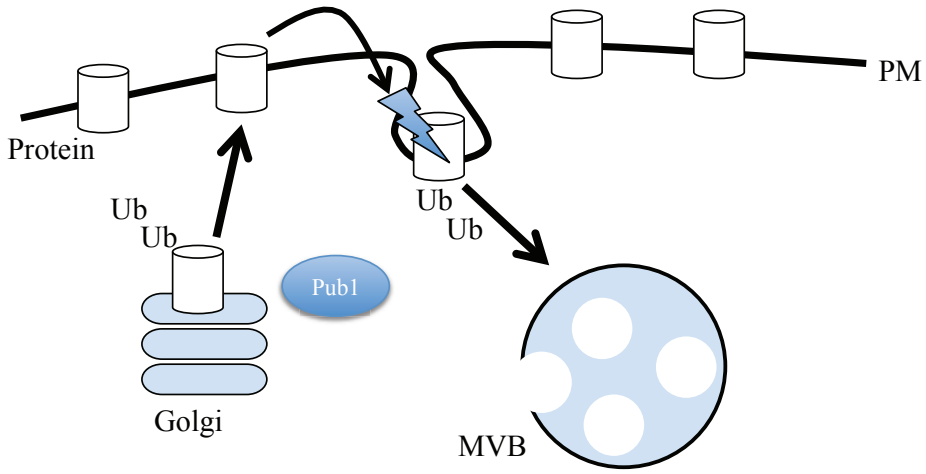


Figure 1-6. Trafficking pathways of cell surface proteins in eukaryotic cells. Ub : ubiquitin, PM : plasma membrane MVB : multivesicular body

Marker gene for gene deletion or transformation

Marker genes are important for genetic analysis. For example *leu1, ade6, his1* and *ura4* genes are widely used in *S. pombe* as auxotrophic marks.

These genes mutants show nutrient auxotrophic such as leucine, adenine, histidine and uracil. Moreover, *ura4* mutants show uracil auxotrophic and 5-Fluoroorotic acid (5-FOA) resistance. 5-FOA is converted to 5-fluorouridine-5-monophosphate (5-FUMP) by Ura5 and Ura4 in *S. pombe*, and it shows the cell toxicity. The *ura4* and *ura5* gene mutant cells shows 5-FOA resistance[13], because the conversion to 5-FUMP does not occur. 5-FOA is used for selection of *URA3* in *S. cerevisiae* on well[27]. Such phenotype is used for the loss of the plasmid and construction of strains.

Medium of growing fission yeast.

Nutrients in the culture medium are important factors in growing microorganisms. This is because the essential nutrients are different between microorganisms. Generally, complete medium (YE, YES or YPD) and synthetic medium (EMM or SD) are used for growing yeast. Different media are used in the budding yeast and fission yeast. YES media and EMM media are used for culture of *S. pombe*, YPD media and SD media are used for culture of budding yeast, but YPD media is not recommended for growing fission yeast. Complete medium is made from the decomposition product of proteins and yeast extract, that contains many nutrients necessary for the growth of yeast. The degradation of casein protein is called polypeptone and used for nitrogen source or amino acids source. Yeast extract is used for vitamin, mineral source and various

nutrients. Therefore, complete medium promotes the growth of yeast and has been used to bio-production. The detailed components of complete medium are not well studied. In addition, the difference of components of medium occurs among the manufacturers or luen within lot.

The detailed components of synthetic medium are clear, and used for the selection of auxotrophic strains. Further, in some cases yeast phenotype is difference in synthetic medium and complete medium. Auxotrophy and media sometimes affect phenotype: in *S. cerevisiae*, amino acid, inositol, and uracil concentrations influence growth of BY family strains[28,29], whereas in *S. pombe*, G418 can be used to select on YE or EMM containing glutamate, but not on EMM medium containing ammonium chloride as nitrogen source[30].

Thus, components in the medium are important factor in experimental and production process.

Table1-1 component of YES media

YES(1L)	
Yeast Extract	5g
D-glucose	30g
Supplement (uracil, adenine, leusine, hisitidine, lysine)	each 225mg

Table1-2 component of YPD media

YPD(1L)	
Yeast Extract	10g
D-glucose	20g
polypeptone	20g

Table1-3 components of EMM media

EMM(1L)	
KH phthalate	3g
Na_2HPO_4	5.56g
D-glucose	20g
NH_4Cl	5g
100×salt stock	10ml
1000×vitamin stock	1ml
10000×mineral stock	100μl

Table1-4 component of SD media

SD(1L)	
Yeast Nitrogen base w/o Amino acid	1.6g
D-Glucose	20g
$(NH_4)_2SO_4$	5g
drop out mix	1.6g

Aim of this research

Fission yeast *S. pombe* is one of useful model organisms to understand basic mechanism of cells. In this thesis, I found on cell lysis mechanism of *S. pombe*.

Cell lysis of *ura4* gene deletion strains induced in YPD media, but the details mechanism is not known. With the aim to identify suppressor or inducer involved in cell lysis of *ura4* gene deletion strains. First, I used non-essential gene deletion library screen to identify suppressors of cell lysis. All 3,400 strains were spotted onto YPD medium and severity of cell lysis was measured using a BCIP assay. Second, I investigated inducer and suppressor in yeast extract or polypeptone by GC-MS and LC-MS. To identify these compounds, I measured five yeast extract and polypeptone using GC-MS analysis. This paper aims to elucidate the mechanism of inducer and suppressor of cell lysis in *ura4* gene deletion strains.

Chapter 2

Cell lysis in *S. pombe ura4* mutants is suppressed by loss of functional Pub1, which regulates the uracil transporter Fur4.

Introduction

The fission yeast *Schizosaccharomyces pombe* is a popular model organism due to its amenability to genetic, molecular, and cell biological analysis. *S. pombe* has been used for the study of numerous biological processes such as the cell cycle, signal transduction, cell morphogenesis, chromatin structure, and metabolism[31-34]. The *S. pombe ura4* gene is particularly valuable as a marker for gene disruption or as a reporter to monitor gene expression at a locus of insertion. The *ura4* gene encodes orotidine-5-monophosphate (OMP) decarboxylase in the *de novo* uridine-5-monophosphate (UMP) synthesis pathway[35,36]. It was shown previously that *S. pombe ura4* mutant cells underwent lysis when grown in a medium containing polypeptone, such as YPD[13]. YPD is widely used for growth of *Saccharomyces cerevisiae*. However, YPD is not commonly used for *S. pombe*, because cultivated lines often exhibit unexpected and unwanted alterations in their phenotypes of interest. This is particularly apparent in *S. pombe ura4* mutants; however, similar effects have not been observed in mutants of the *S. cerevisiae* counterpart, *URA3*.

Cell lysis is observed specifically in *S. pombe ura4* mutants and is not observed in other uracil auxotrophs (*ura1*, *ura2*, *ura3*, and *ura5* mutants)[13]. A precursor molecule, OMP, accumulates substantially in *ura4* mutants, and this might act as a trigger for cell lysis[13]. Cell lysis is suppressed by the addition of uracil. This suppresses the accumulation of OMP, probably because uracil inhibits the *de novo* UMP pathway at a point upstream of the Ura4 reaction. Addition of sorbitol to growth media maintains high osmolarity and, consequently, also suppresses cell lysis.

To further understand the mechanisms underlying cell lysis in *S. pombe ura4* mutants, the author performed a suppressor screen using a *S. pombe* gene deletion library obtained from Bioneer Corp.[37] Screening of 3,400 disruptants revealed several putative suppressors including Δ*pub1*, which strongly suppressed cell lysis in *ura4* mutants. The *pub1* gene encodes a HECT-type E3 ubiqutin ligase[38]. Pub1 is associated with low pH tolerance, regulation of leucine uptake in response to NH^{4+}, and the cell cycle. Pub1 is also required for membrane localization of some membrane proteins, including the amino acid and peptide transporters Aat1, Cat1, and Ptr2, and the GPI anchored protein Ecm33[18,20,21,39]. Ubiquitination of Aat1 or Cat1 alters their localization.

In this study, the author screened a *S. pombe* mutant library for suppression of cell lysis in *ura4* mutants and analyzed the mechanism underlying *pub1* suppression. The

results showed that Pub1 altered the localization of the uracil transporter Fur4 from the Golgi locus and vacuoles to the plasma membrane. When Fur4 was predominantly localized at the membrane, uracil uptake increased and cell lysis was suppressed. A novel regulatory mechanism regarding Fur4 and its relationship with cell lysis is proposed.

Materials and methods

Strains and media

The *S. pombe* strains used in this study are listed in Table 2-1. Standard yeast culture media and genetic manipulations were used[40]. *S. pombe* strains were grown in complete YES medium (0.5% yeast extract (Oxoid Ltd.), 3% glucose, and 225 mg/l each of adenine, leucine, uracil, histidine, and lysine hydrochloride), in YPD medium (1% yeast extract, 2% glucose, and 2% polypeptone (Nihon Pharmaceuticals Co. Ltd.)), YE medium(1% yeast extract, and 2% glucose), or in EMM medium (0.3% potassium hydrogen phthalate, 0.56% sodium phosphate, 0.5% ammonium chloride, 2% glucose, vitamins, minerals, and salts)[40]. EMM(-N) medium lacked NH_4Cl. The appropriate auxotrophic supplements were added as necessary (225 mg/l of leucine and/or uracil) to EMM or EMM(-N). 5-Fluorouracil (5-FU) was added to a final concentration of 1, 3, 5, 50, 200, or 500 μM.

Table 2-1: Strains used in this chapter

Strain	Genotype	Reference
L972	*h⁻*	Lab stock
PR109	*h⁻ ura4-D18 leu1-32*	Lab stock
UMP31	*h⁻ ura4::kanMX6*	[13]
KNP16	*h⁻ fur4::hphMX6*	This study
KNP25	*h⁻ pub1::natMX6*	This study
KNP27	*h⁻ ura4::kanMX6 fur4::hphMX6*	This study
KNP32	*h⁻ ura4::kanMX6 pub1::natMX6*	This study
KNP38	*h⁻ ura4::kanMx6 fur4::hphMX6 pub1::natMX6*	This study
KNP63	*h⁻ ura4-D18 leu1-32 pub1::natMX6*	This study
KNP76	*h⁻ fur4-GFP-hphMX6*	This study
KNP83	*h⁻ pub1::natMX6 fur4-GFP-hphMX6*	This study

KNP87	*h⁻ pub1::natMX6 fur4::hphMX6*	This study
KNP95	*h⁻ ura4::kanMX6 fur4-GFP-hphMX6*	This study

Construction of expression plasmid

To construct the plasmid expressing Fur4-GFP, the *fur4-GFP* fusion gene was amplified by PCR from the genomic DNA of KNP76 using a set of primers of fur4P-NdeI and pFA6a-d-SalISmaIR or pFA6a-d-SalISmaIF and 13MYC-SR (Table 2-2). Two pieces of the fragment were combined by PCR using primers of fur4P-NdeI and 13MYC-SR. After digesting with NdeI and SmaI, the fragment was cloned into the same sites of pREP42. The sequence of pREP42-Fur4-GFP plasmid was confirmed by sequence analysis.

Gene disruption

Chromosomal genes were disrupted using PCR generated fragments[41]. The 1.5 kb *kanMX6*, 1.7 kb *hphMX6*, and 1.2 kb *natMX6* modules were amplified with flanking homology sequences corresponding to the target genes[42]. Correct disruption of the gene of interest was verified by colony PCR using appropriate primers[43]. GFP C-terminal tagged gene was also generated using PCR. Tag incorporation was confirmed by colony PCR and by immunoblotting with specific antibodies. The primers used in this study are listed in Table 2-2.

Table 2-2: Primer used in this chapter

Primer	Sequence
fur4-d-A	5′-CATTGAGGGAATTGAAAGGCG-3′
fur4-d-B	5′-GGGGATCCGTCGACCTGCAGCGTACGACAAGACTTTTGTAGGGAAAG-3′
fur4-d-C	5′-GTTTAAACGAGCTCGAATTCATCGATGGTTTGATCTCGTCTTAATAG-3′
fur4-d-D	5′-CTGTGTTCAATAAGGCGT-3′
fur4-d-chk1	5′-TTCCCGAAGAAGCTGATGAC-3′
fur4-d-chk2	5′-CAAGTCCATACCGTGACATTC-3′
fur4-tag-A	5′-GCAGCCCCTATTACTTTTGG-3′
fur4-tag-B	5′-GGGGATCCGTCGACCTGCAGCGTACGAAGGAAAACAACCCGATAATTTTTTGC-3′
fur4-tag-C	5′-GTTTAAACGAGCTCGAATTCATCGATCATCAAATGGAAATAACACGCC-3′

fur4-tag-D	5′- CCGCTACTTTGGCACTTG-3′
fur4-tag-chk1	5′-GCGTTTAGACAGTAAGCG-3′
fur4-tag-chk2	5′-TTACAGCGTCTATTGATCCCG-3′
pub1-d-A	5′-CGTATTTTCTGTACGCC-3′
pub1-d-B	5′-GGGGATCCGTCGACCTGCAGCGTACGAGGTAAAATTTCAAACCACGC-3′
pub1-d-C	5′-GTTTAAACGAGCTCGAATTCATCGATGGATAGCTAGCTATTGATTAC-3′
pub1-d-D	5′-CACATGTATTGAAGCGGCTATTG-3′
pub1-d-chk1	5′-GTCCATAGCTGTTGAAGAG-3′
pub1-d-chk2	5′-TCATAGCTCTGCGGTCTG-3′
fur4 P-NdeI	5'-ATTCATATGATGGAGTCTGTGGATAATAATTC-3'
pFA6a-d-sal 1smal F	5’-CGCTGCAGGTCGCCGGATCCCAGGGTTAATTAAC-3’
pFA6a-d-sal1 sma1 R	5’-GTTAATTAACCCTGGGATCCGGCGACCTGCAGCG-3’
13MYC-SR	5’-ACACCCGGGAGATCTATATTACCCTGTTA-3’

Screening for extragenic suppressors of *ura4-D18*

For primary screening of the gene deletion library (Ver. 4) from Bioneer Corp., cells were pre-cultured on YES medium and subsequently spotted onto YPD medium for incubation at 30°C for 3 days. For the alkaline phosphatase assay, plates were overlaid with 1% agar containing 0.05 M glycine-NaOH (pH 9.8) and 2.5 mg/ml of 5-bromo-4-chloro-3-indorylphosphate (BCIP), and incubated for 10, 30, and 60 min. After this primary screen, putative suppressor mutants were re-suspended in water to a density of 4×10^6 cells/ml. The cell suspensions were serially diluted (1:10), spotted on YPD plates, and incubated for 3 days at 30°C. A further BCIP assay was also performed. Putative suppressor mutant cells were also observed by microscopy.

Spot assay

Cells were grown on YES plates for 3 days at 30°C and were then re-suspended in water to a density of 2×10^6 cells/ml. Cell suspensions were serially diluted (1:10), spotted onto YES, YPD, or EMM plates, and incubated for 4 days at 30°C. The alkaline phosphatase assay was performed as described above.

Fluorescence microscopy of GFP and RFP

Fur4-GFP tagged *S. pombe* cells were pre-grown to log-phase in YES medium. Cells were then washed twice in MilliQ water, transferred to YES, YPD, EMM, EMMU (EMM+Uracil), EMM(-N), or EMMU(-N) media, and grown at 30°C for 12 h. Fur4-GFP fluorescence or Gms1-RFP images of living cells were observed using a BX51 fluorescent miscroscope (Olympus Corp.) connected to a digital camera DP70 (Olympus Corp.). The author used imageJ software (NIH, Bethesda, MD) to merge two images. Vacuolar membrane was visualized with FM4-64. Cells were incubated with 8 μ M FM4-64 for 1h at 30˚C and then washed twice with dH_2O to remove free FM4-64.

Detection of ubiquitinated Fur4 protein

Polyubiquitination analysis was performed as described previously[44]. For detection of the polyubiquitinated Fur4 protein, wild-type or mutant *S. pombe* cells were transformed with plasmid pREP42 or pREP42-Fur4-GFP and pREP1 or pREP1-6His-AtUbiquitn (provided by Dr. H. Seino), which contained a gene encoding 6×His tagged ubiquitin under the *nmt1* promoter. Cells were cultured at 30°C in EMM medium for 18 h. Cells were then harvested by centrifugation and washed once with ice-cold stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, and 1 mM NaN_3, pH 8.0). Whole cell extracts were prepared in denaturing Buffer U (8 M Urea, 100 mM sodium phosphate, and 50 mM Tris-HCl, pH 8.0) and incubated for 1 h with Ni^2-NTA agarose beads (Qiagen Corp.) at room temperature. The beads were then washed four times with Buffer U. Precipitated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were immunoblotted with anti-GFP antibody (Roche Ltd.) at a 1/1,000 dilution. Detection of proteins was performed using the ECL system (GE Healthcare UK, Ltd.). Membranes were also probed using an anti-ubiquitin antibody (U5379; Sigma-Aldrich Co.), at a 1/100 dilution, as a loading control.

Measurement of uracil in medium by LC-MS

Exponentially growing cells in YES medium were transferred to fresh YPD medium at time point 0 and cultured at 30°C. Initial cell concentrations in YPD were 5×10^5, 5×10^6, or 5×10^7 cells/ml. Culture aliquots were taken at each desired time point, and after removal of cells by centrifugation and addition of acetonitrile to a final concentration of 50%, supernatants were stored at 4°C. LC-MS data were obtained using a MassLynx system (Waters) coupled to a Xexo-TQ mass spectrometer (Waters). LC separation was performed on an ACQUITY UPLC BEH Amide column (Merck SeQuant; 2.1×100 mm, 1.7 μm particle size). Buffer A (acetonitrile + 0.1% formic acid) and buffer B (H_2O + 0.1% formic acid) were used as the mobile phase, with gradient elution from 80% A (20% B) to 62% A (38% B) in 3.5 min at a

0.4 ml/min flow rate. The initial conditions were restored after 4 min and maintained for 7 min at a flow rate of 0.4 ml/min. Uracil was detected using MRM mode (ESI (+) 113>96)

Uracil transport assay

The efficiency of cellular uracil uptake was assessed as described previously[45,46]. Cells were cultivated in YES medium at approximate densities of 4×10^6–1×10^7 cells/ml. Cells were collected, and 2×10^7 cells were washed twice with sterile water, suspended in 4 μM [U-^{14}C]uracil (Moravek Biochemicals Inc.) for 1 min at room temperature, then quickly filtered using an Omnipore membrane filter (Millipore Corp., 47 mm diameter and 1 μm pore size). Filters were washed twice with sterile water, and radioactivity was counted using a Beckmann LS6000TA scintillation counter.

Results

Screen for suppressors of YPD-induced cell lysis in *ura4* mutants

It was shown previously that cell lysis was induced by polypeptone in *S. pombe ura4* mutants [13]. Approximately 90% of cells burst when *S. pombe ura4* mutants were grown in YPD medium until the stationary phase. To further understand the mechanisms governing this cell lysis, the author screened a 3,400 non-essential gene deletion library (Bioneer Corp.) for lysis suppressors. The deletion library contained *leu1-32*, *ura4-D18*, and *ade6-M210* (or *ade6-M216*) mutations as well as individually disrupted genes (replaced with a kanamycin resistance gene). It was expected that this library would include some strains in which polypeptone-induced cell lysis was suppressed. The presence of the *ura4-D18* mutation in the library facilitated screening. All 3,400 strains were pre-cultured on YES medium, and then spotted onto YPD medium and incubated at 30°C for 3 days. Severity of cell lysis was measured using a BCIP assay. In the assay, lysed cells turned blue as a result of alkaline phosphatase release from burst cells. Unlysed cells remained white (Fig. 2-1A). Of the 3,400 strains, 125 putative suppressor mutants were identified in the primary screening that were less blue than the parental strain. For secondary screening, the 125 putative suppressor strains were grown in YES medium, appropriately diluted, and spotted on YPD medium before verification of cell lysis intensity by microscopic examination (Fig. 2-1B). The secondary screening identified 123 suppressor strains, some of which are shown in Figure 2-1. The Δ*ura1*, Δ*ura2*, Δ*ura3*, and Δ*ura5* strains, which the author previously identified as Δ*ura4* lysis suppressors[13], were identified, lending confidence to the validity of the aurhor's screening approach. Of the remaining suppressor strains, the author observed that lysis was particularly strongly suppressed in the Δ*pub1* strain, and the author investigated this further. The *pub1* gene encodes a HECT-type E3 ubiqutin ligase, which is homologous to *S. cerevisiae RSP5*. The *ΔSPAC11D3.15* strain was selected as a good suppressor (Fig.

2-1A), but the later analysis turned out that this strain contains an additional mutation that affects cell lysis.

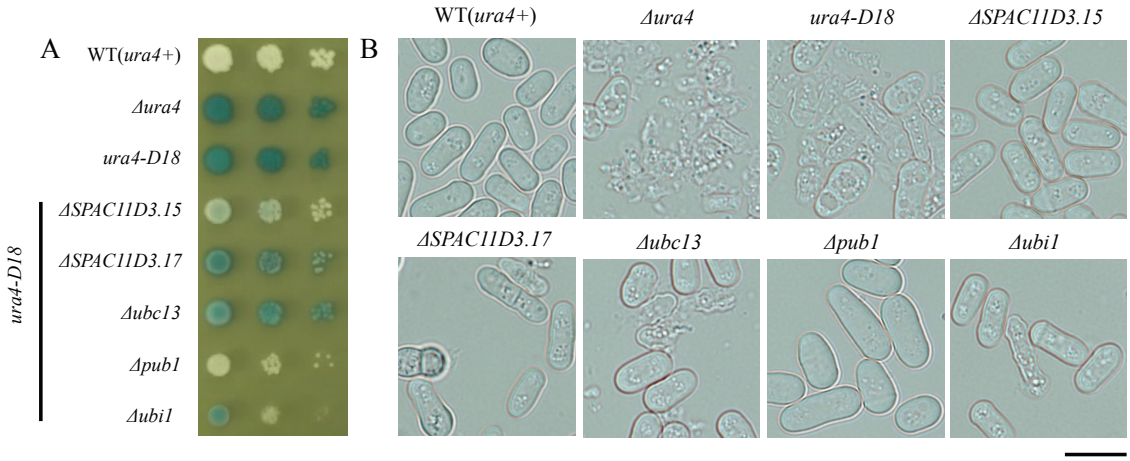


Figure 2-1. Deletions suppressing cell lysis in Δ*ura4* strains grown on YPD medium.
(A) L972 (*ura4*$^{+}$), UMP31 (Δ*ura4*), KT35 (*ura4-D18*), and indicated deletion mutants (Bioneer deletion library ver4.0) were grown for 12 h, then spotted onto YPD medium and incubated for 3 days at 30°C. For the alkaline phosphatase assay, YPD plates were overlaid with a phosphatase assay solution containing 50 mM glycine-NaOH (pH 9.8), 1% agar, and 2.5 mg/ml of BCIP for 30 min. (B) Cells were also observed by microscopy after 3 days of growth on YPD. Five examples of mutants exhibiting different levels of lysis suppression are shown. Bar: 10 μm.

Pub1 is involved in uracil uptake

A new Δ*ura4* Δ*pub1* double mutant was constructed to further examine the effect of *pub1* deletion on the suppression of cell lysis. Cell lysis in the Δ*ura4* cells was significantly suppressed by deletion of the *pub1* gene when cells were grown on YPD medium (Fig. 2-2A). Next, the author wished to determine the role of Pub1 in cell lysis. Because strong suppression was observed in uracil-auxotrophic *ura1*, *ura2*, *ura3*, and *ura5* mutants [13], the author first asked whether Δ*pub1* was also a uracil auxotroph. However, Δ*pub1* cells did not display uracil auxotrophy (Fig. 2-2B), indicating that Pub1 was unlikely to be involved in the uracil metabolic pathway. Next, to ask whether Pub1 was involved in uracil uptake, Δ*pub1* cells were tested for sensitivity to 5-FU, a toxic uracil analog. When grown on EMM or YPD, Δ*pub1* cells exhibited higher sensitivity to 5-FU than wild-type cells or strains carrying other mutations (Figs 2-2B and C). This suggested that the uracil uptake system was up-regulated in Δ*pub1* cells. The author observed that deletion of the gene encoding Fur4, a uracil transporter, conferred resistance to 5-FU,

as previously shown[46] (Fig. 2-2B). The author reasoned that Pub1 might affect Fur4, and created a Δ*fur4* Δ*pub1* double deletion mutant to examine this interaction. The high sensitivity of Δ*pub1* cells to 5-FU was suppressed by *fur4* deletion (Figs 2-2B and C), which suggested that Pub1 had a role in the uracil transport pathway involving Fur4.

Next, the author created the Δ*ura4* Δ*fur4* and Δ*ura4* Δ*fur4* Δ*pub1* mutants to assess the effect of *fur4* deletion on cell lysis in a Δ*ura4* background. As described above, cell lysis was suppressed by Δ*pub1*; however, deletion of *fur4* reversed this suppressive effect (Fig. 2-2D). This provided further support for the hypothesis that Pub1 affected the function of the uracil transporter Fur4.

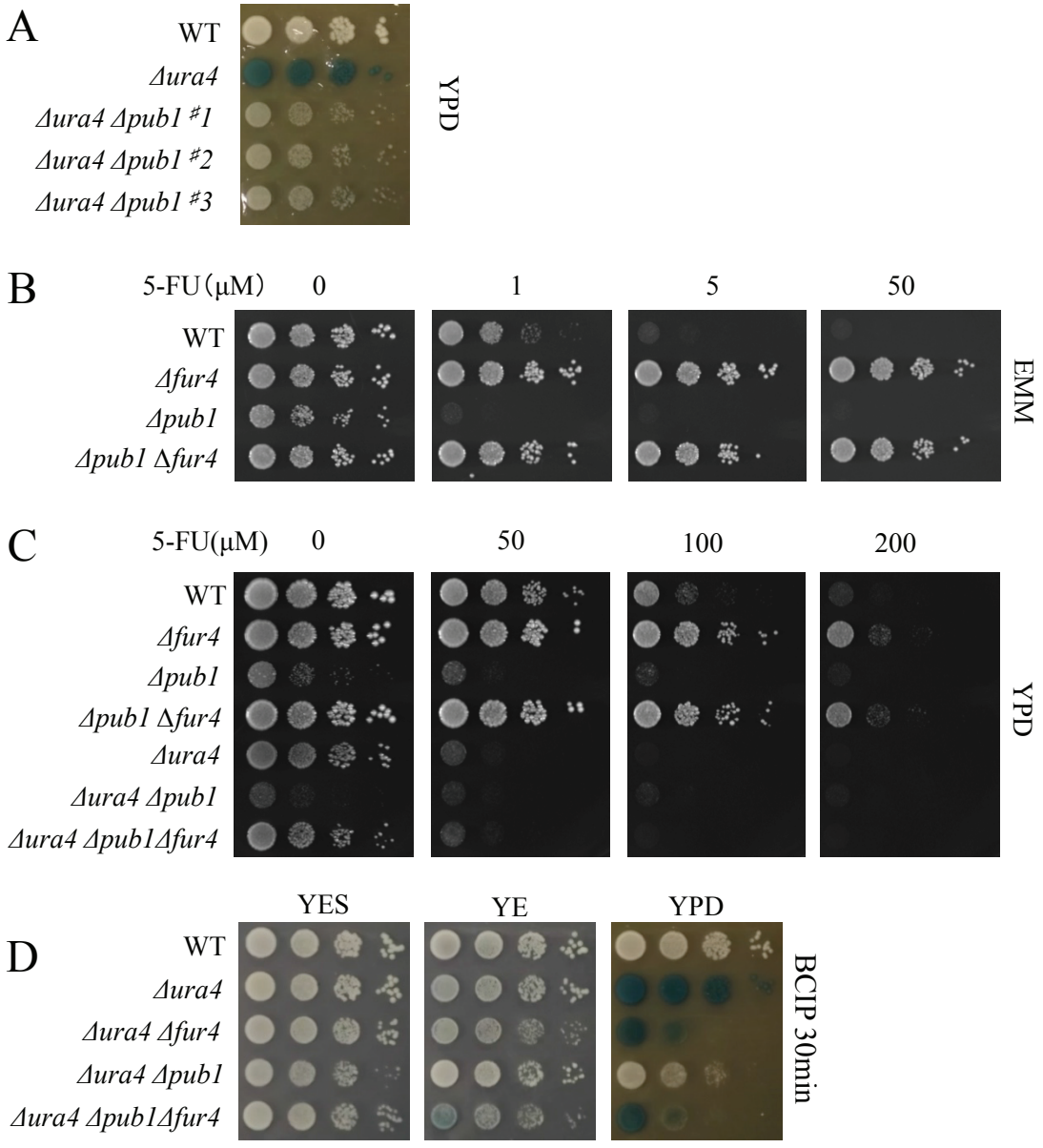


Figure 2-2. Suppression of cell lysis and 5-FU sensitivity in Δ*pub1* cells.

(A) L972, UMP31 (Δ*ura4*), and KNP32 (Δ*ura4* Δ*pub1*) were grown for 12 h in YES liquid medium. These cells were spotted on YPD or YPD+Ade+Leu medium and incubated for 3 days. For the alkaline phosphatase assay, BCIP was used as described in Figure 2-1. (B and C) L972 (*ura4*$^{+}$), KNP16 (Δ*fur4*), KNP25 (Δ*pub1*), KNP87 (Δ*pub1*Δ*fur4*), UMP31 (Δ*ura4*), KNP32 (Δ*ura4* Δ*pub1*), and KNP38 (Δ*ura4* Δ*pub1* Δ*fur4*) cells were grown in YES liquid medium for 12 h. The indicated cells were spotted on EMM (B) or YPD (C) in the presence or absence of 5-FU (1, 5, 50, 100, or 200 μM) and then incubated at 30°C for 3 days. (D) L972 (*ura4*$^{+}$), UMP31 (Δ*ura4*), KNP32 (Δ*ura4* Δ*pub1*), and KNP38 (Δ*ura4* Δ*pub1* Δ*fur4*) cells were grown in YES liquid medium for 12 h. These cells were spotted on YES, YE, and YPD plates and incubated at 30°C for 3 days. For the alkaline phosphatase assay, BCIP was used as described in Figure 2-1.

Fur4 plays a role in the uptake of uracil

One possible explanation for the mutant data described above is that Pub1 down-regulates Fur4. This might be achieved by altering the localization of Fur4 or, as Pub1 has ubiquitin E3 ligase activity[18], through breakdown of Fur4. A strain expressing a Fur4-GFP fusion protein was constructed to examine the first possibility. The Fur4 protein was tagged with GFP at the C-terminus and chromosomally integrated into the native *fur4* locus to express under the native promoter. The tagged strain was checked for its sensitivity to 5-FU in EMM medium. No significant difference was observed by fusing GFP with *fur4* (Fig. 2-3A). Δ*ura4* cells expressing Fur4-GFP carrying pAU-gms1-RFP (*ura4*$^{+}$) were grown on YES or EMM medium and the localization of Fur4-GFP was observed. Fur4-GFP localized to subcellular compartments (Fig. 2-4A). Fur4-GFP co-localized with RFP-tagged Gms1 (Arrow), which was known to localize to the Golgi[47] (Fig. 2-4A, top panel). Fur4-GFP also localized to other structures (Arrow head). To examine the localization of Fur4-GFP in more detail, vacuolar membranes were stained with FM4-64. The Fur4-GFP exhibited a vacuolar pattern of fluorescence corresponding to the staining pattern of FM4-64 (Fig 2-4A, bottom panel).

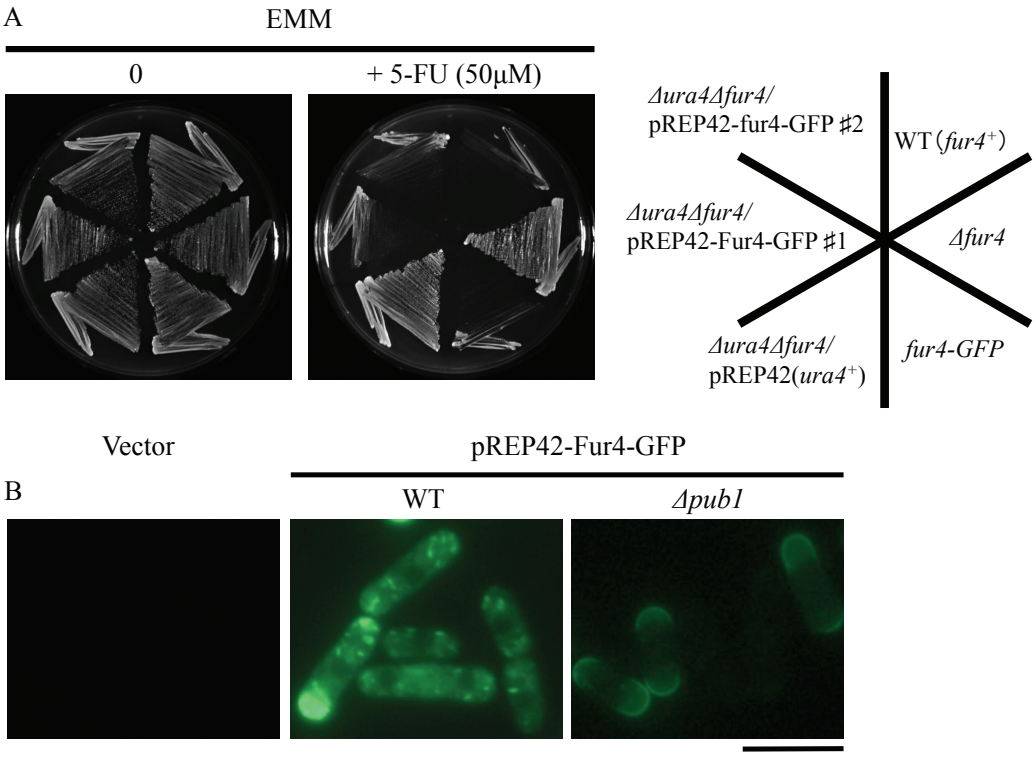


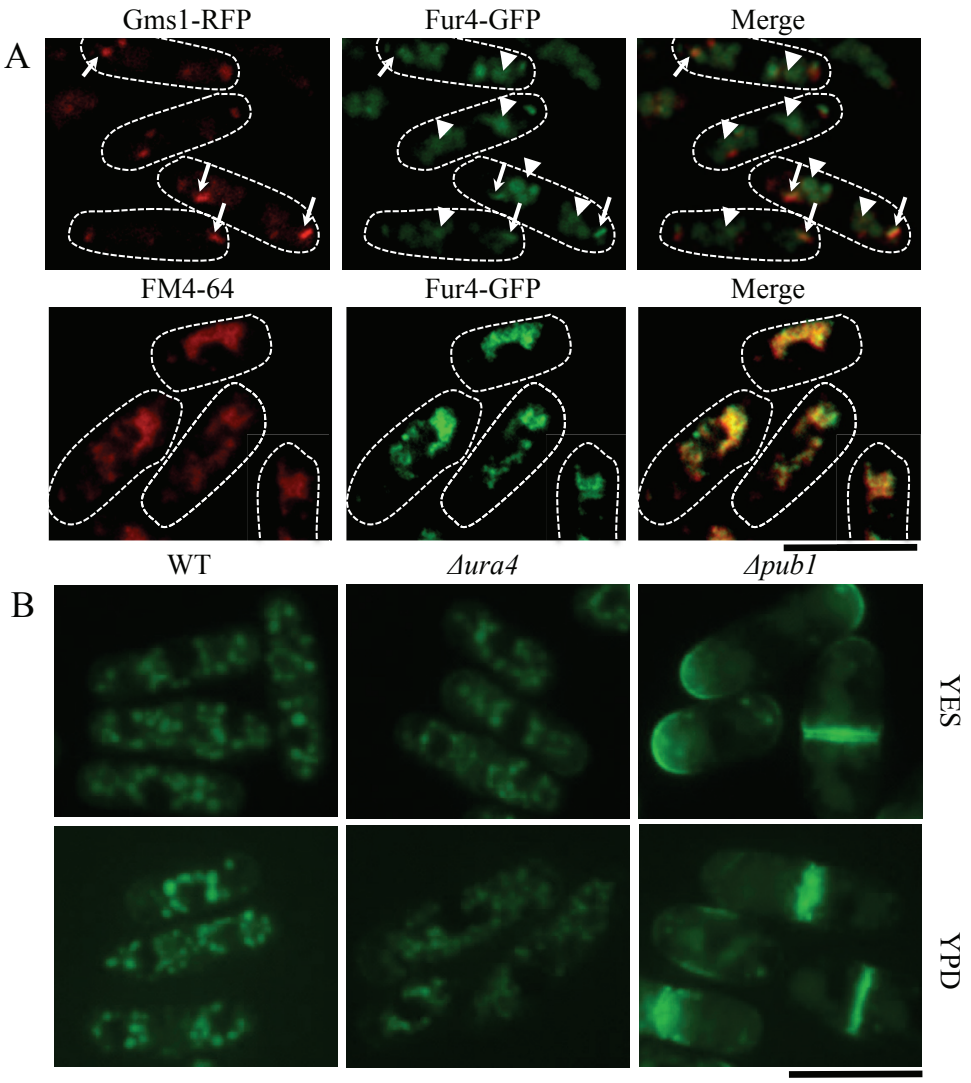
Figure 2-3. Fur4-GFP retains the function indistinguishable with Fur4.

(A) L972 (WT), KNP16 (Δ*fur4*), KNP76 (Fur4-GFP) and KNP27 (Δ*ura4* Δ*fur4*) carrying pREP42 or pREP42-Fur4-GFP (#1 and #2 only differ in an isolated transformant) were grown on YES or EMM plate for 24 h. The indicated cells were streaked on EMM in the absence or the presence of 5-FU (50 μM) and incubated at 30°C for 3 days. (B) PR109 (WT) and KNP63 (Δ*pub1*) carrying pREP42 or pREP42-Fur4-GFP cells were grown in EMM+thiamine liquid medium for 12 h. Cells were washed twice with sterile water and suspended in EMM and incubated at 30°C for 18 h. Cells were observed by fluorescence microscopy. Bar: 10μm.

Localization of Fur4-GFP was next examined in wild-type, Δ*ura4* or Δ*pub1* cells. When grown in YES or YPD media, Fur4-GFP localized primarily to the plasma membrane in Δ*pub1* cells, indicating that Pub1 was important for localization of Fur4 at the Golgi apparatus (Fig. 2-4B).

In *S. cerevisiae*, Fur4p localizes to the membrane upon uracil starvation. To determine whether such regulation occurred in *S. pombe*, Fur4 was examined in wild-type and Δ*ura4* cells, but no apparent difference in localization was observed (Fig. 2-4C). Localization of Fur4-GFP was no significant different when cells were cultured in long time (0, 6 or 12h) (Fig 2-6). The author next examined the localization of Fur4-GFP cells cultured in EMM, EMM(-N), EMMU, and EMMU(-N) medium. In contrast to a slight membrane localization of Fur4-GFP in cells grown in EMM and YES, clear membrane

localization of it was observed in both wild-type and Δ*ura4* cells grown in EMMU(-N) medium (Fig. 2-4). Some membrane localization was also seen in EMM(-N) medium. This demonstrated that nitrogen starvation, but not uracil starvation, induced membrane localization of Fur4 in *S. pombe*, indicating that the regulation of Fur4 in *S. pombe* differed from that of Fur4p in *S. cerevisiae*.



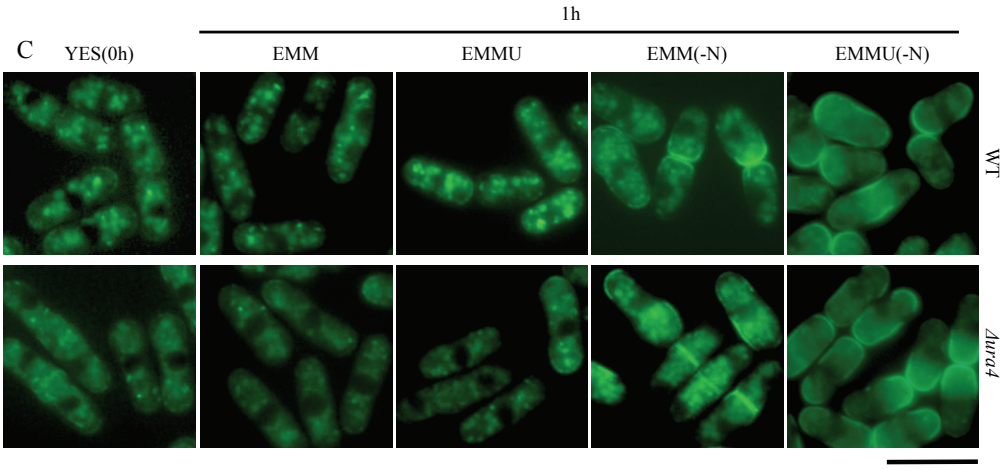


Figure 2-4. Localization of Fur4-GFP.

(A) KNP95 (Δ*ura4 fur4-GFP*) cells carrying pAU-gms1-RFP (*ura4*$^{+}$) were cultured in EMM medium for 12 h at 30°C and observed using fluorescence microscopy. Arrows show co-localization of Fur4-GFP with Golgi marker and arrow heads show only localization of Fur4-GFP (top panel). KNP76 (*fur4-GFP*) cells were cultured in EMM medium for 12h at 30℃ and shifted to EMM containing 8μM FM4-64. These cells were cultured for 1h and washed twice with EMM medium to visualize vacuoles and Fur4-GFP (bottom panel). (B) KNP76 (WT *fur4-GFP*), KNP95 (Δ*ura4 fur4-GFP*), and KNP83 (Δ*pub1 fur4-GFP*) cells were grown in YES medium for 12 h. These cells were washed twice with sterile water and suspended in YES or YPD medium and incubated at 30°C for 1 h. These cells were observed using fluorescence microscopy. Bars: 10 μm.
(C) KNP76(*ura4*$^{+}$), and KNP95 (Δ*ura4*) cells expressing Fur4-GFP were grown in YES liquid medium for 12 h. Cells were washed twice with sterile water and suspended in EMM, EMMU, EMM(-N), or EMMU(-N) media and incubated at 30°C for 1 h. Cells were observed using fluorescence microscopy. Bar: 10 μm.

Fur4 acts as a uracil transporter with uracil uptake activity in *S. pombe*[48]. However, it was unclear whether, as with *S. cerevisiae* Fur4p, *S. pombe* Fur4 was a uracil-specific transporter. To examine this, the author tested the substrate specificity of the Fur4 transporter. Uracil-auxotrophic strains that lacked *ura4* were unable to grow on EMM minimum medium, but could grow on minimum medium containing 75 mg/l of uracil (Fig. 2-5). Double Δ*ura4* Δ*fur4* deletion mutants could not grow on EMMU medium containing 75 mg/l of uracil, but could grow when the amount of uracil was raised to 225 mg/l. This showed that Fur4 was necessary for the efficient utilization of extracellular uracil, but also that Fur4 was not the sole transporter of uracil in *S. pombe*. Cytosine, uridine, and UMP were also tested for their

impact on growth (Fig. 2-5). The addition of these components supported growth of the Δ*ura4* strain. This was as expected because the salvage metabolic pathway from cytosine to uracil should be functional in this strain. However, while the Δ*ura4* Δ*fur4* double mutant was able to grow with uridine supplementation, no growth was seen on medium containing cytosine, and only minimal growth was observed with UMP. These results showed that Fur4 enhanced the utilization of extracellular uracil and UMP, but not uridine, and was absolutely required for the utilization of extracellular cytosine.

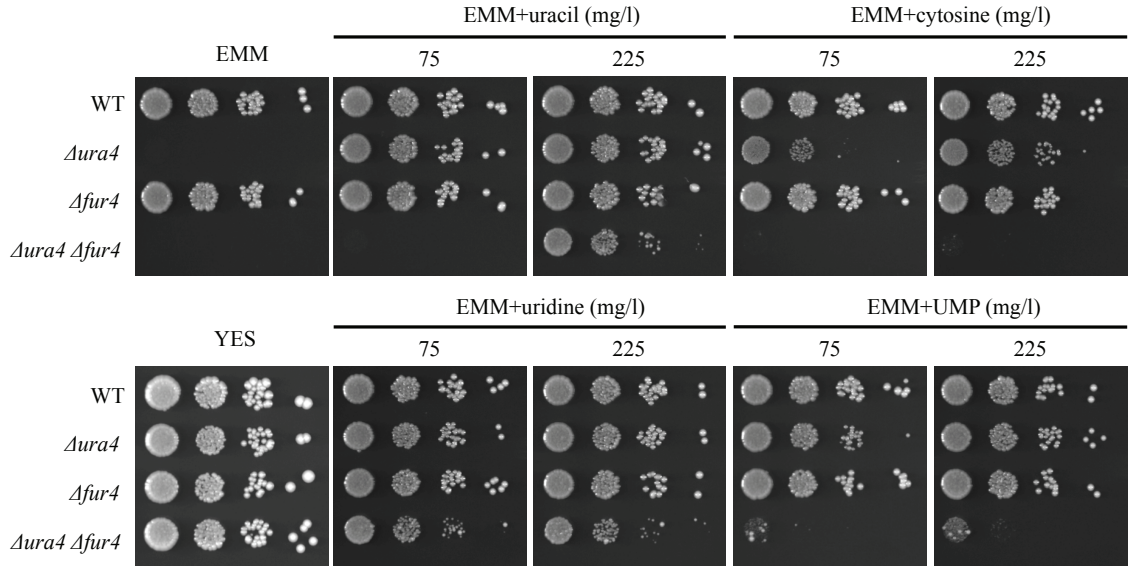


Figure 2-5. Growth recovery by supplementation of various nucleotide derivatives.

L972 (*ura4*$^{+}$), KNP16 (Δ*fur4*), UMP31 (Δ*ura4*), and KNP27 (Δ*ura4* Δ*fur4*) cells were grown in YES medium for 12 h. Cells were spotted on EMM containing uracil, cytosine, uridine, or UMP (75 or 225 mg/l) and incubated at 30°C for 3 days.

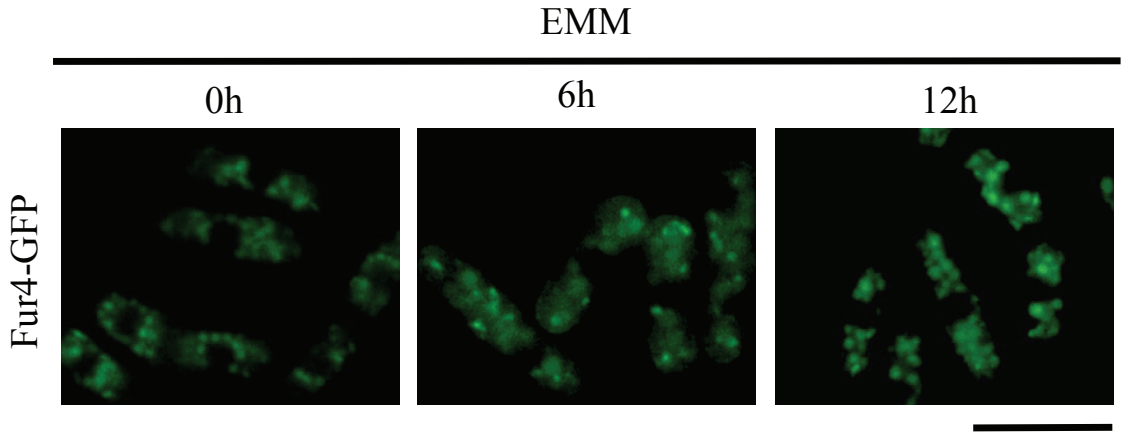


Figure 2-6. Localization of Fur4-GFP does not differ after long time culture. KNP76 (Fur4-GFP) was grown in YES liquid medium for 12 h. Cells were washed twice with sterile water and suspended in EMM media and incubated at 30°C for 0, 6, and 12 h. Cells were observed by fluorescence microscopy. Bar: 10μm.

Uracil concentration impacts cell lysis

Previous research suggested that cell lysis in Δ*ura4* cells was induced during the stationary phase[13]. The suppression of lysis, even at the stationary phase, by uracil supplementation suggested that uracil starvation was the cause of cell lysis in Δ*ura4* cells. To test this, the author examined induction of cell lysis in three Δ*ura4* cultures initiated with differing cell numbers (5×10^5, 5×10^6, or 1×10^7 cells/ml). Cell lysis and uracil concentration in the medium were determined. The proportion of lysed cells increased with increasing initial cell concentration, and uracil concentration in the media decreased more rapidly with higher initial cell concentrations (Fig. 2-7A and B). Cell lysis was strongly induced when Δ*ura4* cells were grown in EMM medium, which did not contain uracil (Fig. 2-7C), supporting the supposition that uracil starvation was responsible for the induction of cell lysis. Cell lysis in EMM was also suppressed in the presence of cycloheximde or 1 M sorbitol (Fig. 2-7C). Sorbitol prevents bursting by maintenance of high osmolarity. Cycloheximide inhibits protein synthesis, preventing the cellular processes that lead to lysis. Lysis of Δ*ura4* cells was suppressed by the addition of uracil, but not by supplementation with uridine, UMP, or cytosine (Fig. 2-7D).

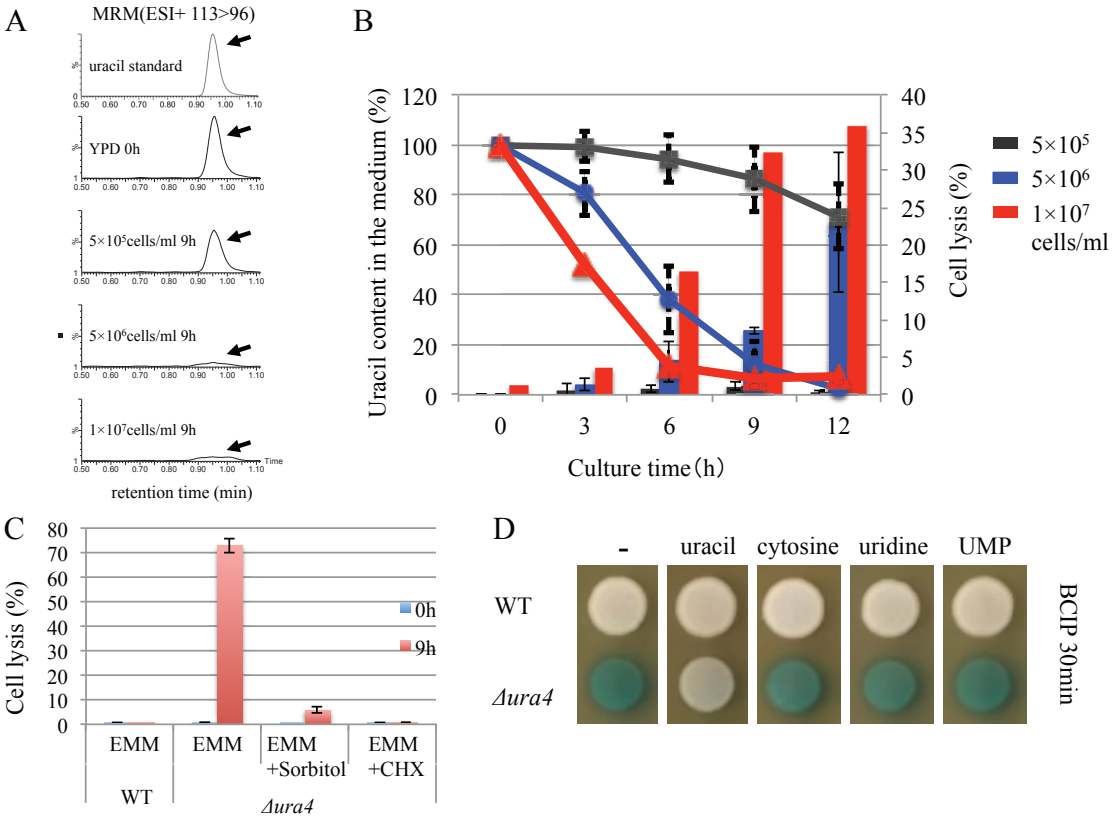


Figure 2-7. Induction of cell lysis by uracil starvation.

(A) UMP31 (Δ*ura4*) cells were grown in YES medium for 12 h. Cells were washed twice with sterile water and then grown in YPD medium at initial cell counts of 5×10^5, 5×10^6, or 1×10^7 cells/ml. The chromatography of LC-MS data (MRM (ESI+ 113>96)) obtained at 0 and 9h are shown as representatives. Arrows show uracil peaks. (B) All data of uracil concentration measured by LC-MS are plotted in the graph. The uracil concentration in the medium (lines) and the extent of cell lysis (bars) were measured after 0, 3, 6, 9, and 12 h of incubation (right). Error bars indicate standard deviation. (C) L972 (*ura4*$^+$) and UMP31 (Δ*ura4*) cells were grown in YES medium for 12 h, washed twice with sterile water, and suspended in EMM medium with or without CHX (100 μg/ml) or 1 M sorbitol and incubated at 30°C. Cell lysis was measured after 0 and 9 h of incubation. Error bars indicate standard deviation. (D) Wild-type and Δ*ura4* cells were grown in YES medium for 12 h. Cells were then spotted on YPD containing uracil, cytosine, uridine, or UMP (each 300 mg/l) and incubated at 30°C for 3 days. For the alkaline phosphatase assay, BCIP was used as described in Figure 2-1.

Uracil transport activity is down-regulated in Δ*fur4* strains

The uracil transporter Fur4 localized predominantly to the membrane in the Δ*pub1* strain, which suggested that uracil uptake might be up-regulated in Δ*pub1* cells. To test this, the author used a [U-^{14}C]

uracil assay to measure uracil uptake in mutant and wild-type strains (Fig. 2-8). Consistent with previous results[46], only minimal uracil uptake activity was observed in the Δ*fur4* strain. Uracil uptake was up-regulated in Δ*ura4* Δ*pub1* cells and, to a lesser extent, in Δ*ura4* cells. Uptake was down-regulated in Δ*ura4* Δ*pub1* Δ*fur4* cells (Fig. 2-8). These results were consistent with the resistance to 5-FU exhibited by strains containing the *fur4* deletion (Fig. 2-2B). Taken together, these data suggest that up-regulation of uracil uptake is dependent on Fur4 and that Pub1 regulates uracil uptake via Fur4.

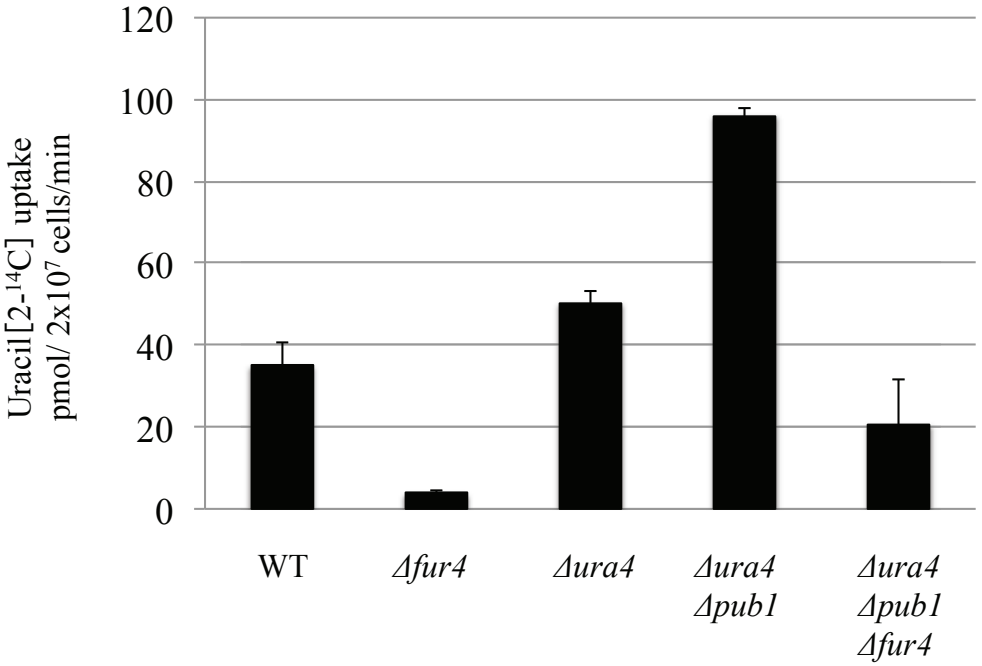


Figure .2-8 Uracil transport assay.

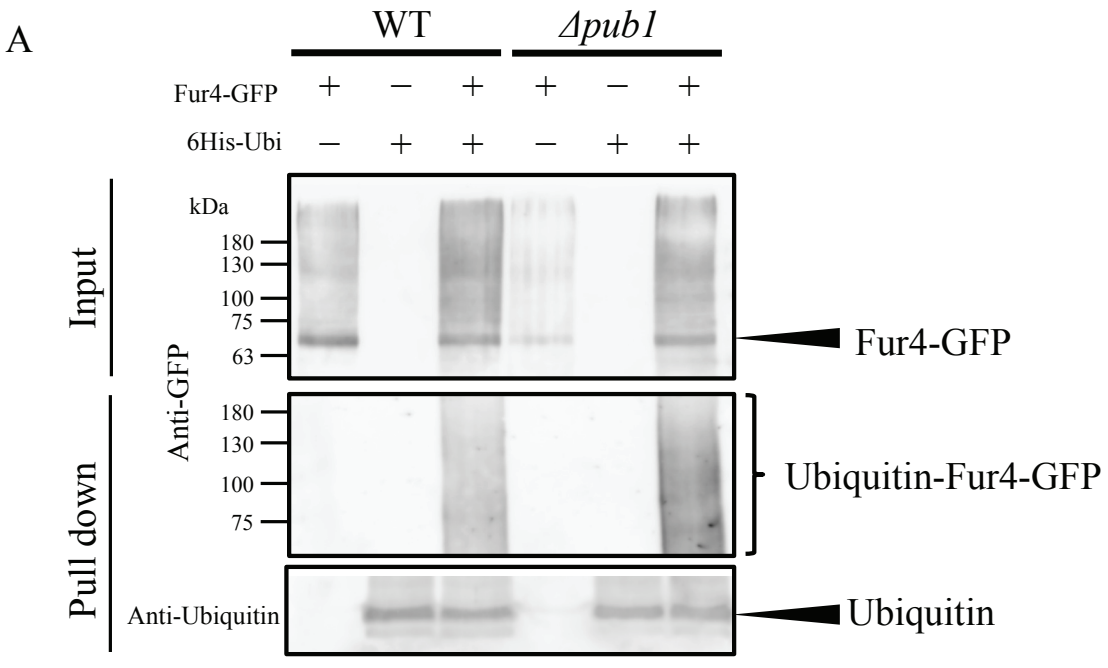
Exponentially growing L972 (WT), KNP16 (Δ*fur4*), UMP31 (Δ*ura4*), KNP32 (Δ*ura4* Δ*pub1*), and KNP38 (Δ*ura4* Δ*pub1* Δ*fur4*) cells were grown in YES medium for 12 h. Uracil uptake was monitored using [U-^{14}C]uracil. After 1 min, cells were filtered on cellulose acetate membrane filter and washed three times. Radioactivity was measured using a liquid scintillation counter with xylene scintillator. Experiments were performed in triplicate, and error bars indicate standard deviation.

Fur4 is ubiquitinated in wild-type and Δ*pub1* cells

Pub1, which is an E3 ubiquitin ligase, regulates localization of target proteins in some cases[18]. The author reasoned that Pub1 might ubiquitinate Fur4 and thereby regulate its localization. To test this, Fur4-GFP was ectopically expressed in wild-type and Δ*pub1* strains and its ubiquitination was examined. Prior to this experiment, the author verified the function of Fur4-GFP does not differ with Fur4 by checking 5-FU sensitivity and localization in Δ*ura4*Δ*fur4* and Δ*pub1* cells (Fig. 2-3A and B). A set of

plasmids pREP42 (or pREP42-Fur4-GFP) and pREP1 (or pREP1-6His-Ubiquitin) were introduced into wild-type and Δ*pub1* strains. After these transformants were growth in EMM medium, cellular proteins were extracted and detected on western blots using an anti-GFP antibody. Fur4-GFP was observed as smears in wild-type and Δ*pub1* cells. Extracts were then incubated with Ni^2-NTA agarose beads to purify 6His tagged ubiquitin conjugated proteins. Purified extracts were detected on western blots with anti-GFP or anti-ubiquitin (control) antibodies (Fig. 2-9A). Ni^2-NTA-purified Fur4 was ubiquitinated in both wild-type and Δ*pub1* cells, which suggested that Pub1 was not directly involved in ubiquitination of Fur4.

The author next examined ubiquitination of Fur4-GFP after cells were incubated under nitrogen starved condition, since the author observed membrane localization of Fur4-GFP increased after nitrogen starvation (Fig. 2-4). The author cultured cells harboring pREP42-Fur4-GFP and pREP1-6His-ubiquitin in EMM medium and further incubated in EMM (-N) medium for 2 h. After confirming intensive membrane localization of Fur4-GFP as in Fig. 2-4, proteins were extracted. The proteins were detected on western blot using anti-GFP and anti-ubiquitin antibodies (Fig. 2-9B). Extracts were then incubated with Ni^2-NTA agarose beads to purify 6His tagged ubiquitin conjugated proteins. In this pull down experiment, ubiquitination of Fur4-GFP was detected before cells were incubated without nitrogen, but it gradually disappeared after nitrogen starvation. After 2 h nitrogen starvation, ubiquitinated Fur4-GFP was not detected in the pulled down samples both in wild type and Δ*pub1* deletion background.



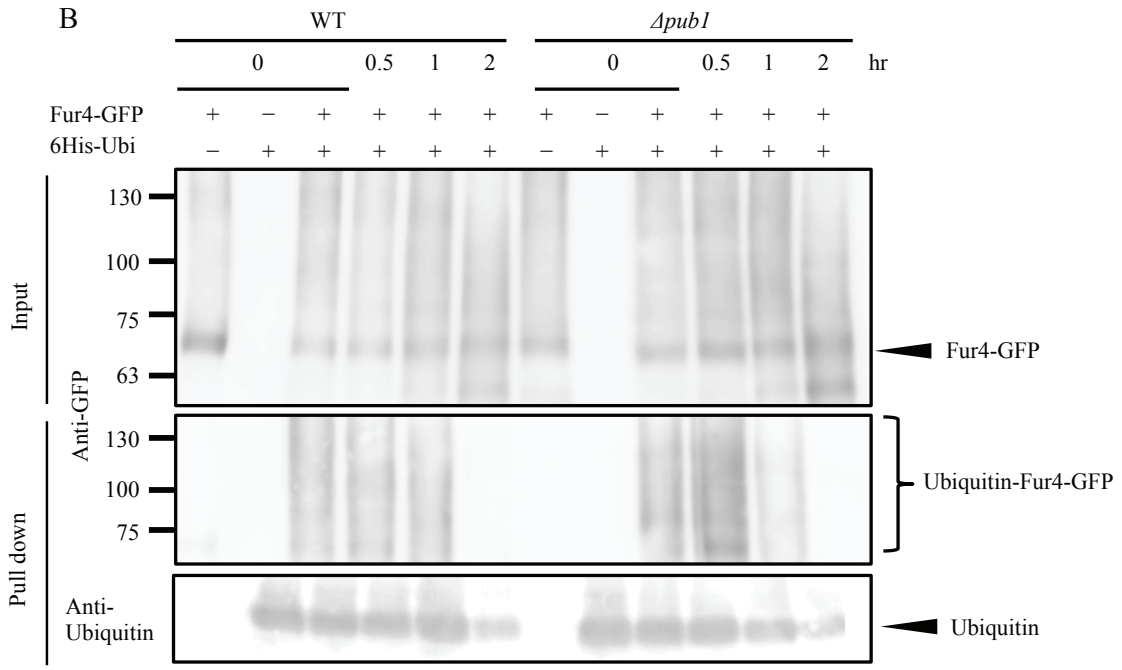


Figure 2-9. Ubiquitination of Fur4.

(A) PR109 (WT) and KNP63 (Δ*pub1*) carrying pREP42(-) or pREP42-Fur4-GFP and carrying pREP1 (-) or pREP1-6His-Ub (+) were grown in EMM. Proteins tagged with 6His-Ubiquitin were purified from protein extracts using Ni^2-NTA. Immunoblotting was performed with an anti-GFP antibody (upper and middle) and with an anti-ubiquitin antibody as a loading control (bottom). (B) The same transformants were grown in EMM for 16h. Cells were harvested by centrifugation, washed twice with dH_2O and further grown in EMM(-N) medium for 2h. After proteins were extracted and pulled down by Ni^2-NTA, immunoblotting was performed as in (A).

Discussion

Cell lysis in *ura4* mutants is suppressed in the absence of *pub1*

In this study, the author explored the mechanisms by which cell lysis was induced in *S. pombe ura4* mutants through the analysis of suppressors obtained from screening a *S. pombe* 3,400 non-essential gene deletion library. Several suppressors were identified. Deletion of the *pub1* gene, which encoded E3 ubiquitin ligase, produced a particularly strong suppression phenotype (Fig. 2-1A). Previously identified suppressors were either directly involved in *de novo* UMP synthesis, such as mutations in *ura1*, *ura2*, *ura3*, or *ura5*, or were indirectly involved in UMP synthesis, such as mutations affecting synthesis of

CoQ (a cofactor of the Ura3 reaction) [13,49]. However, *pub1* deletion did not render uracil auxotrophy, and the Δ*pub1* strain had high 5-FU sensitivity, indicating that the mechanism of suppression was not related to a *de novo* UMP pathway. Cell lysis was suppressed by supplementation of uracil in YPD, suggesting that Pub1 might be involved in uracil uptake. The involvement of Pub1 in uracil uptake was confirmed by its alteration of the localization of the uracil transporter Fur4, which was identified in a previous study[46,48].

Genetic experiments consistently supported the relationship between Pub1 and Fur4. High sensitivity to 5-FU in the Δ*pub1* strain was suppressed by deletion of *fur4*. Furthermore, the Δ*ura4* Δ*pub1* Δ*fur4* strain, but not the Δ*ura4* Δ*pub1* strain, underwent lysis when grown on YPD medium (Fig. 2-2). Fur4 predominantly localized to the Golgi apparatus and vacuoles under normal growth conditions but accumulated at the cell membrane in the *pub1* mutant, underlining the importance of Pub1 for Fur4 localization (Fig. 2-4). The high sensitivity to 5-FU and the suppression of cell lysis observed in the Δ*pub1* strain could be explained by the accumulation of Fur4 at the membrane. Consistent with Fur4 membrane localization in Δ*pub1*, uracil uptake was higher in the *pub1* mutant than in the wild type (Fig. 2-8). Thus, the identification of *pub1* in the initial suppressor screen could be explained by the increased uptake of uracil in Δ*pub1* mutants as a result of Fur4 localizatin at the membrane.

A diagram outlying the proposed interactions of Fur4 and Pub1 is given in Figure 2-10. This cartoon is supported by the auhor's experimental observations, but at least two major questions relating to this mechanism remain. First, why does supplementation with uracil suppress cell lysis, and second, how does Pub1 affect Fur4 localization?

The author used a supplementation approach to address the first question. Uracil, but not cytosine, uridine, or UMP, was able to suppress cell lysis (Fig. 2-5); however, all four supplements rescued the inability of *ura4* mutants to grow on minimal medium (Fig. 2-5). These results suggested that uracil had a specific role in the suppression of cell lysis and that the suppression did not involve enforcement of the UMP synthesis salvage pathway. Previous experiments showed that OMP, a precursor of the Ura4 reaction, accumulated when Δ*ura4* cells were grown in YPD medium and that addition of uracil decreased OMP accumulation[13]. It was therefore possible that OMP acted as a key component in the cell lysis pathway. Uracil is converted to UMP by condensation with 5-phosphoribosyl 1-pyrophosphate (PRPP). This suggests that supplementation with uracil may provide a competitor for the Ura5 reaction (condensation of PRPP with orotate), thereby, as the author observed, decreasing levels of OMP. Cytosine, uridine, and UMP are unlikely to compete with the Ura5 reaction. However, this competition hypothesis remains to be proved.

To address the second question, the author asked whether, given that *pub1* encodes E3 ubiquitin ligase, variable ubiquitination might explain the effect of Pub1 on Fur4 localization. However, Fur4 ubiquitination did not appear to differ between wild-type and Δ*pub1* strains (Fig. 2-9A). This suggested that the E3 ubiquitin ligase activity of Pub1 did not act directly on Fur4. Similarly, a GPI anchored protein, Ecm33, whose localization is also regulated by Pub1, exhibits no clear change in ubiquitination pattern in the absence of Pub1[21]. Conversely, the amino acid transporter Aat1 is ubiquitinated by Pub1, and this ubiquitination regulates Aat1 localization[18]. The author's observations led to the hypothesis that Fur4 might have multiple mono-ubiquitination sites that play distinct roles in its regulation. Such subtle modification differences would not have been discernible in the western analysis. Alternatively, Pub1 may act on a separate target that then regulates Fur4. Multiple ubiquitinations were proposed to play a role in the activity of the amino acid transporter Cat1[20], and the ubiquitination of adaptor protein Any1 was involved in the regulation of amino acid transporter Aat1 and Cat1[18,20]. However, the question of how Fur4 is regulated by Pub1 remains to be answered.

Mechanisms of uracil uptake differ between *S. cerevisiae* and *S. pombe*

The regulation of the *S. cerevisiae* uracil transporter Fur4p has been elucidated[23,25,50]. Degradation of Fur4p is induced by ubiquitination of two lysine residues (Lys38 and 41) by Rsp5p, a homolog of *S. pombe* Pub1[51,52]. The ubiquitinated Fur4p is rapidly internalized and degraded in a multivesicular body (MVB) pathway. When cells are grown on medium containing uracil, Fur4p is internalized and degraded. This regulatory pathway can also be induced by stressors such as heat or H_2O_2[52].

In *S. pombe*, down-regulation of Fur4 was not induced by uracil (Fig. 2-4C). The author also observed that, when wild-type and Δ*ura4* strains were grown under nitrogen starvation conditions, Fur4 localized to the cell surface and septum (Fig. 2-4). No similar observations were seen with *S. cerevisiae* Fur4p[53]. Intriguingly, ubiquitined Fur4 was not detected in the pull down experiment of the samples taken from the cells grown under nitrogen starved condition for 2 h (Fig. 2-9B). But ubiquitination of Fur4 did not disappear in *pub1* gene deletion mutants without starvation. The author's result indicates that nitrogen starvation is critical for de-ubiquitination of Fur4-GFP. The author thinks that the balancing of ubiquitination (possibly mediated by Pub1) and de-ubiquitination of Fur4-GFP through nitrogen starvation is operating in this regulation. Further analysis is necessary to understand this regulatory mechanism of Fur4.

In addition, Fur4p is a high-affinity uracil transporter that does not transport cytosine[53]. Genetic data indicated that *S. pombe* Fur4 was involved in transport of uracil, cytosine, and UMP (Fig. 5). Collectively,

these data indicate that the regulation of uracil uptake by Fur4 and Fur4p differs substantially between *S. pombe* and *S. cerevisiae*.

Uracil concentration is critical for cell lysis in Δ*ura4* cells

The author's experiments demonstrated that the cellular uracil concentration was critical for cell lysis in the Δ*ura4* strain (Fig. 2-7A and C). The initial finding that cell lysis was induced when *ura4* mutants were grown in YPD medium was complemented by the observation that cell lysis was induced immediately upon cultivation of Δ*ura4* cells in EMM medium (Fig. 2-7A). The time of uracil consumption in YPD coincided with the time of cell lysis (Fig. 2-7B). Lysis of Δ*ura4* cells is strongly related to the level of uracil concentration. The current hypothesis is that polypeptone within the media contains some factor(s) that facilitates uracil consumption, but this remains to be proved.

In conclusion, cell lysis in *S. pombe* Δ*ura4* cells is triggered by cellular uracil depletion and consequent OMP accumulation. OMP then likely weakens the cell wall structure by an unknown mechanism, allowing cells to be easily broken.

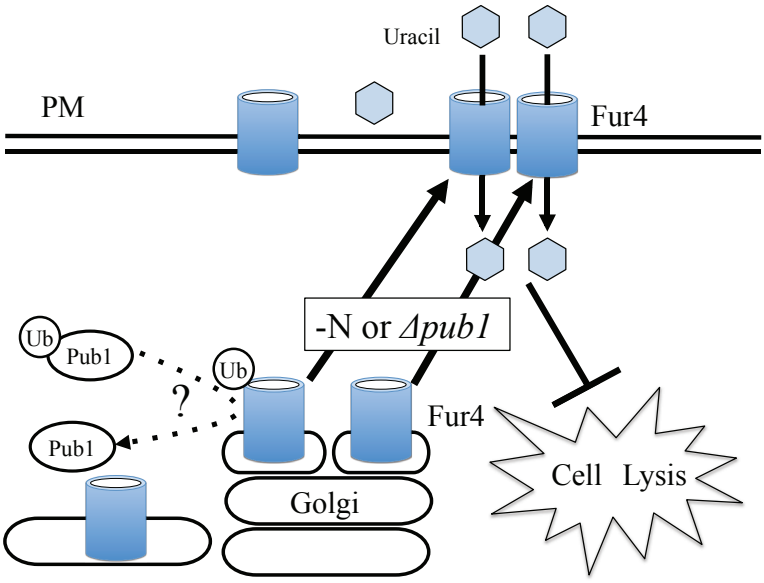


Figure 9. Proposed model for interaction of Pub1 and Fur4.

The uracil transporter Fur4 localizes to Golgi or vacuoles under normal growth conditions and shifts to the plasma membrane when nitrogen is consumed. Fur4 is also localized to the plasma membrane when the *pub1* gene is deleted. Increased uracil uptake in *pub1* mutants by the accumulated Fur4 localization in the membrane rescues the cell lysis phenotype. Ubiquitination of Fur4 is observed, but the mechanisms underlying Pub1 regulation of Fur4 localization remains to be determined. PM, Plasma Membrane

Chapter 3

Urea enhances cell lysis of *Schizosaccharomyces pombe ura4* mutants

Introduction

Media composition is an important factor in phenotypic analyses of yeasts. *Saccharomyces cerevisiae* and closely related species are generally grown in YPD medium, which contains yeast extract, polypeptone, and dextrose (glucose). Meanwhile, *Schizosaccharomyces pombe* is usually grown on YE, which contains yeast extract, and glucose, but not polypeptone[30,40]. Polypeptone, a protease degradation product of casein protein, is used as an amino acid and nitrogen source in YPD media, but has a deleterious effect on growth of *S. pombe*[1]. Yeast extract is sufficient to support growth when a carbon source is supplied. However, the composition of polypeptone and yeast extracts varies by producer, or even by lot from the same supplier. Growth of auxotrophic strains requires supplementation of media with nucleosides and amino acids. Auxotrophic markers are used for plasmid transformation, gene deletion, or diploidization, but auxotrophy can also affect phenotype: in *S. cerevisiae*, amino acid, inositol, and uracil concentrations influence growth of BY family strains[28,29], whereas in *S. pombe*, G418 can be used to select on YE or EMM containing glutamate, but not on EMM medium containing ammonium chloride as nitrogen source[30]. Thus, the relationship between auxotrophic strains and compounds in the medium must be considered carefully.

The fission yeast *S. pombe* is a eukaryotic model organism used to study a wide range of molecular and cellular biological processes, including cell cycle regulation, signal transduction, transcription, chromatin structure, cell wall synthesis, and biotechnological applications[33,49,54-57]. It was found that cell lysis was induced in *S. pombe* Δ*ura4* cells by addition of polypeptone or depletion of uracil in the media. Deletion of either one of *ura* genes (*ura1*, *ura2*, *ura3* and *ura5*) suppressed cell lysis of *Δ ura4* cells. Orotidine-5-monophosphate (OMP), a key compound in cell lysis, did not accumulate in these cells[13]. Furthermore, it was found previously that cell lysis of Δ*ura4* cells was suppressed by addition of uracil or deletion of the *pub1* gene, which encodes an E3 ubiquitin ligase involved in the regulation of the *S. pombe* uracil transporter Fur4[58]. Deletion of the *pub1* gene increased membrane localization of Fur4 protein, thereby increasing uracil uptake ability[58]. Thus, intracellular uracil concentration is critical for cell lysis of Δ*ura4* cells. Uracil is used for synthesis of uridine-5-monophosphate (UMP) by the salvage pathway. Alternatively, UMP is also synthesized by the *de novo* pathway comprising Ura1, Ura2, Ura3, Ura4, and Ura5 (Fig. 1-1).

In this study, the author investigated the relationship between cell lysis of *S. pombe* Δ*ura4* cells and components in yeast extract or polypeptone. Uracil concentration differed among five different yeast extracts and correlated with the cell lysis phenotype. GC-MS analysis identified 172 peaks in yeast extracts and polypeptone; among them, the author determined that urea is an inducer of cell lysis in Δ*ura4* cells.

Materials and methods

Strains and media

The *S. pombe* strains used in this study are listed in Table 3-1. Standard yeast culture media and genetic manipulations were used. *S. pombe* strains were grown in complete YES medium (0.5% yeast extract, 3% glucose, and 225 mg/L each of adenine, leucine, uracil, histidine, and lysine hydrochloride)[40], YE (1% yeast extract and 2% glucose), or YPD [1% yeast extract, 2% glucose, and 2% polypeptone (Nihon Pharmaceuticals Co. Ltd.)]. The author used several different yeast extracts (Kyokuto Pharma. Ind., Co., Ltd; OXOID Ltd., BD & Co.; and Oriental Yeast Co., Ltd.) for comparison. Yeast extract from Kyokuto was used unless otherwise indicated. *S. pombe* strains were also grown in EMM medium (0.3% potassium hydrogen phthalate, 0.56% sodium phosphate, 0.5% ammonium chloride, 2% glucose, vitamins, minerals, and salts)[40]. The appropriate auxotrophic supplements were added as necessary (10 or 225mg/l of uracil) to EMM. Polypeptone was added to a final concentration of 1 or 2%. Urea was added to a final concentration of 0.05, 0.1 or 0.4%.

Table 3-1. Strains used in this chapter

Strain	Genotype	Reference
L972	*h⁻*	Lab stock
PR109	*h⁻ ura4-D18 leu1-32*	Lab stock
UMP31	*h⁻ ura4::kanMX6*	[13]
UMP34	*h⁻ ura1::kanMX6*	[13]
UMP35	*h⁻ ura2::kanMX6*	[13]
UMP36	*h⁻ ura3::kanMX6*	[13]
UMP37	*h⁻ ura5::kanMX6*	[13]
UMP42	*h⁻ ura4::hphMX6 ura5::kanMX6*	[13]
KNP16	*h⁻ fur4::hphMX6*	This study
KNP27	*h⁻ ura4::kanMX6 fur4::hphMX6*	This study
KNP32	*h⁻ura4::kanMX6 pub1::natMX6*	This study
KNP38	*h⁻ura4::kanMX6 pub1::natMX6 fur4::hphMX6*	This study
KNP174	*h⁻ ure2::natMX6*	This study
KNP176	*h⁻ ura4::kanMX6 ure2::natMX6*	This study
KNP178	*h⁻ ura1::kanMX6 ure2::natMX6*	This study
KNP180	*h⁻ ura2::kanMX6 ure2::natMX6*	This study
KNP182	*h⁻ ura3::kanMX6 ure2::natMX6*	This study
KNP184	*h⁻ ura5::kanMX6 ure2::natMX6*	This study

Gene disruption

The chromosomal *ure2* gene was disrupted by homologous recombination using PCR-generated fragments[59]. The 1.2 kb *natMX6* modules were amplified with flanking homology sequences of the *ure2* gene[42] using primers listed in Table 3-2. Correct disruption of the gene of interest was verified by colony PCR using the appropriate primers[60].

Table 3-2: Primer used in this chapter

Primer	Sequence
ure2-d-W	5'-TTGCTGCGTGTAAACACAAC-3'
ure2-d -X	5'-GGGGATCCGTCGACCTGCAGCGTACGAGTTGGAACAACGCTAGAGTG-3'
ure2-d -Y	5'-GTTTAAACGAGCTCGAATTCATCGATGTTTGAGGCCGAGAAATTCC-3'
ure2-d -chk1	5'-TGGAATGTGAGCCTGTAGAC-3'
ure2-d -chk2	5'-ACAGTGCAAATGTAGACGGC-3'
nb2	5'-GTTAACGAGCTCGAATTC-3'

Spotted and BCIP assay

Cells were pre-cultured in YES liquid medium for 12 h at 30°C, and then re-suspended in water at a density of 2×10^6 cells/mL. Cell suspensions were serially diluted (1:10) and plated on YES, YPD, YE, or YE+urea plates and incubated for 3 days at 30°C. For the alkaline phosphatase assay, each plate was overlaid for 10, 30, or 60 min with a phosphatase assay solution containing 0.05 M glycine-NaOH (pH9.8), 1% agar, and 2.5 mg/mL 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Medium sample preparation for GC-MS

Samples from media were prepared by adding 900 µL of extract buffer (methanol:chloroform:water = 5:2:2) to 100 µL of medium, and proteins were removed by centrifugation (14,000 rpm, 4°C, 10 min). Then, 600 µL of supernatant was transferred to a new tube, dried in a centrifugal evaporator, and freeze-dried. For oximation, 80 µL of methoxyamine hydrochloride in pyridine (20 mg/ml) was added, and the sample was incubated at 30°C for 90 min. For trimethylsilylation, 40 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroaceramide (MSTFA) was added, and the sample was incubated at 37°C for 30 min.

Uracil concentration using stable isotopic uracil

Samples from media were prepared by adding 900 µL of extract buffer (methanol:chloroform:water = 5:2:2) to 100 µL of medium, and proteins were removed by centrifugation (14,000 rpm, 4°C, 10min).

Supernatant (600 µL) was transferred to a new tube and dried on a centrifugal evaporator. Next, 150 µL of 50% acetonitrile was added to the dry sample and diluted 10-fold. Then, an equal amount of 0.5 ppm ^{13}C uracil was added to the diluted sample. LC-MS data were obtained using a MassLynx system (Waters) coupled to a Xevo-TQ mass spectrometer (Waters). LC separation was performed on an ACQUITY UPLC BEH Amide column (Merck SeQuant; 2.1×100 mm, 1.7 µm particle size). Buffer A (acetonitrile + 0.1% formic acid) and buffer B (H_2O + 0.1% formic acid) were used as the mobile phase, with gradient elution from 99% A (1% B) to 30% A (80% B) in 7 min at a flow rate of 0.4 mL/min. The initial conditions were restored after 10 min and maintained for 5 min at a flow rate of 0.4 mL/min. ^{12}C Uracil was detected using MRM mode [ESI(+) 113>96], and ^{13}C uracil was detected using MRM mode [ESI(+) 114>97].

Urea measurement

Urea assay Kit (BioAssay Systems) was used. 50 µL samples, water (blank), and standard urea (5 mg/dL) in duplicate were transferred into wells of a clear bottom 96-well plate. 200 µL working reagent was added and tapped lightly to mix. Samples were incubated 50 min at room temperature and optical density at 430nm was measured.

Results

Polypeptone supported growth of uracil auxotrophic strains

To better understand the role of polypeptone on the inducing effect of cell lysis of *S. pombe ura4* gene mutants[13], the author first tested the effect of polypeptone on the growth of uracil auxotrophic strains. The author inoculated five uracil auxotrophic strains (*Δura1*, *Δura2*, *Δura3*, *Δura4*, and *Δura5* strains), all of which are defective in *de novo* UMP synthesis, onto various media and incubated for 3 days at 30°C (Fig.3-1A). None of the strains grew without uracil (Fig. 3-1A; comparison of second and third panels), but polypeptone restored growth without additional uracil (Fig. 3-1A, fourth panel; grown on PG media (2% polypeptone and 2% glucose)), indicating that polypeptone contains uracil (or compounds related to uracil) for the synthesis of UMP. Polypeptone is generally used as a source for amino acids or nitrogen for culture medium, but our result indicates that it apparently contains other ingredients that support UMP synthesis. The author next tested the growth of uracil auxotrophic strains on EMM+polypeptone medium (fifth panel). None of the uracil auxotrophic strains grew well on this medium, but they grew much better on EMM+polypeptone in the absence of ammonium salt as a nitrogen source (sixth panel), indicating that excess nitrogen inhibits their growth under this condition. Although uracil auxotrophic strains grew on EMM medium containing a low amount of uracil (10 mg/mL), the growth of uracil auxotrophic strains was retarded by the addition of 2% polypeptone (Fig. 3-1B). These results indicate that excess nitrogen source, such as polypeptone, in the medium inhibits the growth of uracil auxotrophic strains. Thus, while polypeptone supported the growth of uracil auxotrophic strains, it also inhibited growth under different

conditions. These results are consistent with a previous study showing that growth of *ura4* deletion mutants is inhibited by 5 g/L NH_4Cl in YE medium[60].

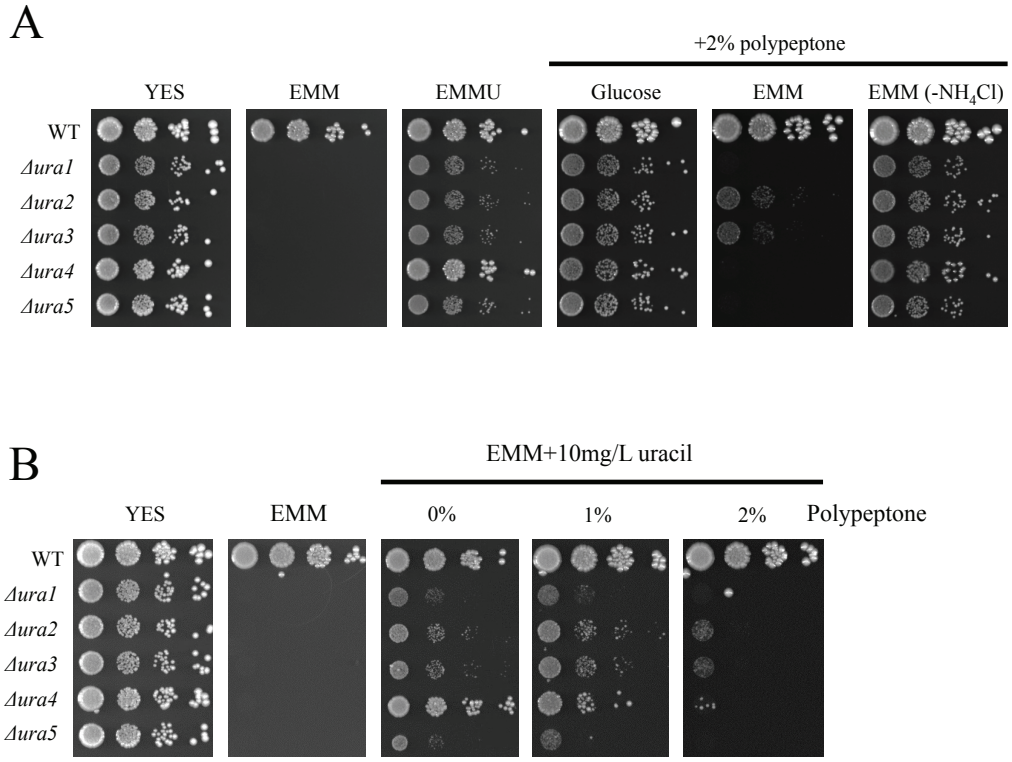


Fig. 3-1. Excess nitrogen source inhibits growth of uracil auxotrophic strains. (A) L972 (WT; *ura4*$^+$), UMP34 (Δ*ura1*), UMP35 (Δ*ura2*), UMP36 (Δ*ura3*), UMP31 (Δ*ura4*), and UMP37 (Δ*ura5*) were pre-cultured for 12 h, and then spotted onto YES, EMM, EMMU (225 mg/L uracil), PG (Polypeptone 2% and Glucose 2%), EMM+Polypeptone, or EMM (-NH_4Cl)+Polypeptone media, and incubated for 3 days at 30°C. (B) The same strains were pre-cultured for 12 h, and then spotted onto YES, EMM, and EMMU (10 mg/L uracil) in the presence or absence of polypeptone (0%, 1%, or 2%) and incubated for 3 days at 30°C.

Uracil concentration in yeast extracts are different.

The author previously showed that Δ*ura4* cells underwent cell lysis in YE medium, which does not contain polypeptone[13], but this result was not always reproducible. The author suspected that this difference was caused by differences in the yeast extract used in the author's previous two experiments.

The author then tested various kinds of yeast extracts made by different suppliers. Cell lysis of Δ*ura4* cells on YE medium was observed when the author used yeast extracts from BD, OXOID, Oriental, and Difco, but not when the author used Kyokuto yeast extract (Fig. 3-2A). Cell lysis was observed when polypeptone was included in any media. Deletion of the *fur4* gene, which encodes a uracil transporter, in Δ*ura4* cells induced cell lysis even in medium containing Kyokuto yeast extract. These results indicate that components of yeast extract differ among suppliers. Because the author knew that uracil suppressed cell lysis of Δ*ura4* cells when it was grown on YE medium[13], the author hypothesized that these differences were related to a difference in the uracil concentration among yeast extracts. The author then measured the uracil concentration of five yeast extracts by LC-MS/MS, using the stable isotope ^{13}C-uracil as an internal standard. Indeed, the uracil concentration of Kyokuto yeast extract was two to four times higher than those of the four other yeast extracts (Fig. 3-2B). By contrast, Difco yeast extract contained the lowest uracil concentration. *Δura4* cells did not lyse in YE (Difco) media when more than 20 mg/L uracil was added (Fig. 3-2 C). The author also tested the effect of high concentrations of yeast extract in YE media (4% yeast extract and 2% glucose), expecting that increasing the uracil concentration would suppress cell lysis. However, Δ*ura4* cells lysed in media containing 4% yeast extract (Kyokuto, OXOID, BD, oriental, and Difco) (Fig. 3-2 D). These results indicate that uracil is a factor that can reverse cell lysis of Δ*ura4* cells, but is not the sole determinant of lysis, suggesting that there are other factors in yeast extract that affect cell lysis.

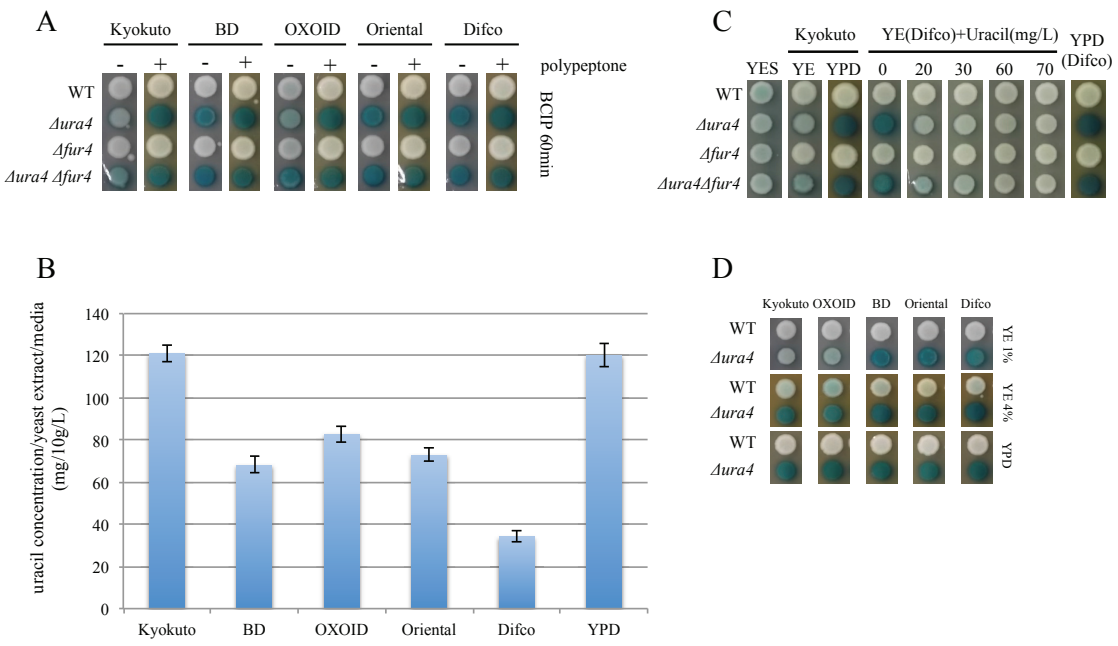


Fig. 3-2. Uracil is an important determinant of cell lysis of Δ*ura4* strains in YE medium. (A) L972 (WT; *ura4*$^{+}$), UMP31 (Δ*ura4*), KNP16 (Δ*fur4*), and KNP27 (Δ*ura4* Δ*fur4*) were grown for 12 h, spotted onto YE medium containing yeast extract from the indicated supplier (Kyokuto, BD, OXOID, Oriental Yeast, or Difco) in the presence or absence of polypeptone (2%), and incubated for 3 days at 30°C. For the alkaline phosphatase assay, the plates were overlaid for 60 min with a phosphatase assay solution containing 50 mM glycine-NaOH (pH 9.8), 1% agar, and 2.5 mg/mL of BCIP. (B) Uracil concentration of yeast extract from each supplier, measured by LC-MS/MS. (C) L972 (WT; *ura4*$^{+}$), UMP31 (Δ*ura4*), KNP16 (Δ*fur4*), and KNP27 (Δ*ura4* Δ*fur4*) were grown for 12 h, spotted onto YE in the presence or absence of uracil (20, 30, 60, or 70 mg/l), and incubated for 3 days. (D) L972 (WT; *ura4*$^{+}$) and UMP31 (Δ*ura4*) cells were grown for 12 h, spotted onto YE medium (1% or 4% OXOID, BD, Oriental, or Difco yeast extract), and incubated for 3 days. For the alkaline phosphatase assay, BCIP was used as described in (A).

GC-MS analysis of yeast extract

The results described above suggested that yeast extract and polypeptone contain unidentified compounds that induce cell lysis of Δ*ura4* cells. To identify these compounds, the author subjected yeast extract and polypeptone to GC-MS (Shimadzu GC-MS 2010 ultra) analysis after treatment with TMS (trimethylsilylation). Raw data were analyzed using the Smart Metabolites Database system. In the author's analysis, the author detected about 172 peaks from yeast extract and polypeptone (all compounds are indicated in Table 3-3). Uracil concentration in Kyokuto yeast extract was 2.2–2.9 times higher than those of the OXOID and BD yeast extracts, respectively (Table 3-4), consistent with the LC-MS/MS data (Fig. 3-2B). Based on this result, the author believe that the GC-MS data are reliable. Nine kinds of compounds detected in GC-MS analysis had higher peak areas in Kyokuto yeast extract than in OXOID and BD yeast extracts (Table 3-4). On the other hand, ten kinds of compounds were detected in OXOID and BD yeast extracts, but not in Kyokuto yeast extract (Table 3-5). Furthermore, 27 compounds were specifically detected in polypeptone (Table 3-6). Among these 27 compounds, 14 were sugars, but the isomeric structures of these compounds could not be determined. The author speculates that these compounds are potentially related to cell lysis of Δ*ura4* cells, either as suppressors or inducers.

Table 3-3 all compounds

compound number	peak number		Kyokuto	OXOID	BD	Polypeptone
1	73	1,6-Anhydroglucose-3TMS	N.D	N.D	1358	N.D
2	17	2-Aminoethanol-3TMS	224825	97896	65752	23443

3	5	2-Hydroxybutyric acid-2TMS	N.D	1685	3105	N.D
4	49	2-Isopropylmalic acid-3TMS	N.D	N.D	5466	N.D
5	139	2'-Deoxyuridine-3TMS	5047	N.D	N.D	N.D
6	50	3-Aminoglutaric acid-3TMS	N.D	1592	N.D	N.D
7	34	3-Aminopropanoic acid-3TMS	5245	N.D	N.D	1827
8	8	3-Hydroxybutyric acid-2TMS	N.D	5070	2948	N.D
9	9	3-Hydroxyisobutyric acid-2TMS	N.D	5070	2948	N.D
10	7	3-Hydroxypropionic acid-2TMS	32829	35113	12726	4814
11	81	3-Methoxy-4-hydroxybenzoic acid-2TMS	N.D	N.D	N.D	2712
12	68	3-Sulfinoalanine-3TMS	N.D	2829	N.D	N.D
13	22	4-Aminobutyric acid-2TMS	281651	258099	239030	N.D
13	45	4-Aminobutyric acid-3TMS	880718	N.D	255409	3502
14	15	4-Hydroxybutyric acid-2TMS	N.D	2890	N.D	N.D
15	103	4-Hydroxyphenyllactic acid-3TMS	N.D	N.D	N.D	1011216
16	33	5-Aminovaleric acid-2TMS	9657	9225	9135	3064
17	109	5-Dehydroquinic acid-5TMS	N.D	N.D	1870	N.D
18	46	5-Hydroxy-2-furoic acid-2TMS	2367	N.D	N.D	N.D
19	138	5-Methoxytryptamine-2TMS	3658	N.D	N.D	24431
20	43	5-Oxoproline-2TMS	712989	589970	531308	310031
21	142	Adenosine-4TMS	877094	985869	580830	48411
22	2	Alanine-2TMS	411861	342341	357115	60154
23	*21	allo-Isoleucine-2TMS	281651	258099	239030	118631
24	*95	Allose-5TMS	N.D	N.D	N.D	449801
24	101	Allose-meto-5TMS(1)	N.D	N.D	N.D	35481
24	*106	Allose-meto-5TMS(2)	155839	824235	87633	54552
25	54	Arabinose-4TMS(1)	15573	12091	6935	16475
25	62	Arabinose-4TMS(2)	N.D	N.D	6141	N.D
26	78	Arabitol-5TMS	38299	N.D	N.D	N.D
27	87	Arginine-3TMS	114026	60672	51512	68782

28	115	Ascorbic acid-4TMS	N.D	N.D	11428	N.D
29	66	Asparagine-3TMS	111592	86790	75683	40567
29	56	Asparagine-4TMS	53897	49669	37723	25046
30	40	Aspartic acid-3TMS	109539	86705	75331	10705
31	83	Azelaic acid-2TMS	N.D	9951	N.D	N.D
32	16	Benzoic acid-TMS	N.D	N.D	719	1873
33	52	Cadaverine-3TMS	1019	N.D	N.D	N.D
34	89	Citric acid-4TMS	4017	113592	60429	856710
35	*114	Coniferyl aldehyde-meto-TMS(1)	N.D	14295	N.D	35139
35	*114	Coniferyl aldehyde-meto-TMS(2)	N.D	14295	N.D	35139
36	133	Cystamine-nTMS	2898	7802	6122	12524
37	47	Cysteine-3TMS	43035	58625	40624	2295
38	42	Cytosine-2TMS	N.D	7130	12345	N.D
39	129	Dopamine-4TMS	7600	6641	N.D	34322
41	*137	Fructose 1-phosphate-meto-6TMS(1)	1157	N.D	N.D	5540
41	*137	Fructose 1-phosphate-meto-6TMS(2)	1157	N.D	N.D	5540
40	*92	Fructose-5TMS(3)	N.D	N.D	N.D	15660
40	*102	Fructose-meto-5TMS(1)	58707	111939	34376	3571980
40	*102	Fructose-meto-5TMS(2)	58707	111939	34376	3571980
42	69	Fucose-4TMS(1)	5779	7216	N.D	N.D
42	76	Fucose-4TMS(2)	166691	N.D	5207	N.D
43	28	Fumaric acid-2TMS	40609	88593	50469	17133
44	*116	Galactitol-6TMS	48052	48152	217417	8769
45	100	Galactosamine-5TMS(1)	7425	7885	5608	N.D
46	85	Galactose-5TMS(1)	9283	5491	12575	3819
46	*95	Galactose-5TMS(2)	N.D	N.D	N.D	449801
46	*106	Galactose-meto-5TMS(1)	155839	824235	87633	54552
46	*112	Galactose-meto-5TMS(2)	N.D	11876	N.D	80307

47	128	Galacturonic acid-5TMS(2)	N.D	N.D	N.D	85723
47	120	Galacturonic acid-meto-5TMS(2)	4388	N.D	9575	1789
48	127	Glucaric acid-6TMS	N.D	N.D	N.D	447120
49	124	Gluconic acid-6TMS	N.D	N.D	N.D	8277
50	111	Glucono-1,4-lactone-4TMS	N.D	7227	N.D	28388
51	110	Glucosamine-5TMS(1)	N.D	N.D	14254	N.D
52	104	Glucose-5TMS(1)	N.D	N.D	N.D	3914471
52	121	Glucose-5TMS(2)	N.D	19463	14140	40093
52	*106	Glucose-meto-5TMS(1)	155839	824235	87633	54552
52	*112	Glucose-meto-5TMS(2)	N.D	11876	N.D	80307
54	90	Glucuronic acid lactone-3TMS(1)	15488	N.D	3058	N.D
54	93	Glucuronic acid lactone-3TMS(2)	N.D	N.D	N.D	22360
53	118	Glucuronic acid-5TMS(1)	4022	4318	16580	45304
53	125	Glucuronic acid-5TMS(2)	N.D	N.D	2427	8967
55	53	Glutamic acid-3TMS	265246	355779	336582	30427
56	13	Glyceraldehyde-meto-2TMS(2)	10082	9777	8184	4360
57	27	Glyceric acid-3TMS	24236	15172	27856	4528
58	19	Glycerol-3TMS	224880	84526	154046	100127
59	3	Glycine-2TMS	6906	4672	8273	N.D
60	26	Glycine-3TMS	982932	917193	714496	104874
61	1	Glycolic acid-2TMS	8619	7696	8150	2240
62	86	Glycyl-Glycine-4TMS	1608	3177	9103	9683
63	132	Guanine-3TMS	54397	19676	11863	N.D
64	108	Histidine-3TMS	44601	29193	19399	21713
65	65	Homocysteine-3TMS	24509	15635	39392	2919
66	35	Homoserine-3TMS	23004	N.D	N.D	N.D
67	94	Hydroxylysine (2 isomers)	N.D	N.D	N.D	32495
68	84	Hypoxanthine-2TMS	18115	22451	13362	N.D
69	117	Indol-3-acetic acid-2TMS	N.D	21624	N.D	N.D

71	152	Inosine monophosphate-5TMS	32076	N.D	N.D	N.D
70	141	Inosine-4TMS	15002	N.D	N.D	12216
72	131	Inositol-6TMS(2)	347049	137177	110955	73395
73	*21	Isoleucine-2TMS	281651	258099	239030	118631
74	150	Isomaltose-meto-8TMS(1)	7251	25503	N.D	888157
74	151	Isomaltose-meto-8TMS(2)	N.D	3594	N.D	115175
75	*148	Lactitol-9TMS	5768	5035	7352	159466
76	144	Lactose-meto-8TMS(1)	N.D	6974	N.D	3305
76	145	Lactose-meto-8TMS(2)	N.D	10085	2555	6980
77	58	Lauric acid-TMS	N.D	N.D	N.D	17670
78	18	Leucine-2TMS	487404	433561	414345	264530
79	107	Lysine-4TMS	2267420	1891073	1482713	1631091
80	64	Lyxose-4TMS(2)	4348	N.D	N.D	N.D
81	24	Maleic acid-2TMS	4700	4196	4033	N.D
82	37	Malic acid-3TMS	32725	111850	52784	54503
83	10	Malonic acid-2TMS	N.D	N.D	N.D	17976
84	149	Maltitol-9TMS	N.D	12577	5268	19223
85	*147	Maltose-meto-8TMS(1)	4699445	13991600	11352001	472774
85	*148	Maltose-meto-8TMS(2)	5768	5035	7352	159466
86	*116	Mannitol-6TMS	48052	48152	217417	8769
87	*95	Mannose-5TMS(2)	N.D	N.D	N.D	449801
87	*105	Mannose-meto-5TMS(1)	N.D	N.D	N.D	35481
87	*106	Mannose-meto-5TMS(2)	155839	824235	87633	54552
88	41	Methionine-2TMS	139873	122181	96350	44595
89	11	Methylmalonic acid-2TMS	18720	17949	17583	5205
90	146	Monostearin-2TMS	1649165	1614359	1404164	2996677
91	91	Myristic acid-TMS	13661	N.D	N.D	129341
92	119	N-Acetyl-Ornithine-4TMS	130644	N.D	N.D	N.D
93	130	N-Acetylmannosamine-meto-4TMS	N.D	2973	3469	N.D
94	39	N-Acetylserine-2TMS	N.D	N.D	3257	N.D
95	36	Niacinamide-TMS	11757	40001	35144	N.D

96	30	Nonanoic acid-TMS	N.D	N.D	984	N.D
97	51	Ornithine-3TMS	73927	46557	34346	70973
97	88	Ornithine-4TMS	1097934	184625	391498	30076
98	4	Oxalic acid-2TMS	N.D	1685	3105	N.D
99	126	Palmitic acid-TMS	509296	475264	445836	1112065
100	122	Pantothenic acid-3TMS	1999	18243	11595	N.D
101	55	Phenylalanine-2TMS	3677121	3462839	3213885	2715916
102	20	Phosphoric acid-3TMS	2456325	1387338	1325872	208571
103	23	Proline-2TMS	175172	135182	128880	9862
104	*105	Psicose-5TMS(4)	N.D	N.D	N.D	35481
104	96	Psicose-meto-5TMS(1)	N.D	N.D	N.D	5041
104	*99	Psicose-meto-5TMS(2)	N.D	N.D	N.D	46282
105	80	Putrescine-4TMS	361486	N.D	67535	7893
106	57	Rhamnose-4TMS(1)	44777	403055	379336	42291
106	77	Rhamnose-meto-4TMS(1)	10683	N.D	N.D	N.D
107	79	Ribitol-5TMS	38299	N.D	N.D	N.D
108	72	Ribonolactone-3TMS	9475	4477	6116	N.D
109	60	Ribose-4TMS(1)	18723	19160	13356	7571
109	61	Ribose-4TMS(2)	N.D	N.D	6141	N.D
109	63	Ribose-4TMS(3)	4348	N.D	N.D	N.D
109	67	Ribose-4TMS(4)	27843	N.D	18137	8906
109	71	Ribose-meto-4TMS	1623	3814	N.D	N.D
110	70	Ribulose-meto-4TMS	2646	2342	N.D	N.D
111	6	Sarcosine-2TMS	N.D	N.D	N.D	1636
112	31	Serine-3TMS	224821	202134	179003	60815
113	*116	Sorbitol-6TMS	48052	48152	217417	8769
114	*99	Sorbose-meto-5TMS(1)	N.D	N.D	N.D	46282
114	*102	Sorbose-meto-5TMS(2)	58707	111939	34376	3571980
115	136	Spermidine-5TMS	51661	41864	43739	5046
116	134	Stearic acid-TMS	316434	322200	289537	757994
117	25	Succinic acid-2TMS	201012	175077	285520	1763
118	143	Sucrose-8TMS	1508	N.D	N.D	48744

119	*92	Tagatose-5TMS(1)	N.D	N.D	N.D	15660
119	*92	Tagatose-5TMS(2)	N.D	N.D	N.D	15660
119	*105	Tagatose-5TMS(5)	N.D	N.D	N.D	35481
119	98	Tagatose-meto-5TMS(2)	N.D	N.D	N.D	6356
120	59	Tartaric acid-4TMS	2475	1502	N.D	N.D
121	44	Thiodiglycolic acid-2TMS	15360	12142	10514	N.D
122	38	Threitol-4TMS	6710	24998	11097	13065
123	48	Threonic acid-4TMS	7049	10545	35850	3723
124	32	Threonine-3TMS	776404	736850	621762	220432
125	*147	Trehalose-8TMS	4699445	13991600	11352001	472774
126	135	Tryptamine-3TMS	12297	17554	17321	N.D
127	113	Tyrosine-3TMS	66092	48292	64064	109553
128	29	Uracil-2TMS	218414	97050	75445	1697
129	14	Urea-2TMS	N.D	23325	34099	26788
130	140	Uridine-4TMS	21635	4229	6856	N.D
131	12	Valine-2TMS	1259763	1218080	1084922	449982
132	97	Vanilmandelic acid-3TMS	35739	43840	33972	41132
133	123	Xanthine-3TMS	73320	107646	31470	N.D
134	75	Xylitol-5TMS	N.D	N.D	N.D	2494
135	74	Xylose-4TMS(1)	N.D	N.D	7092	1495
135	82	Xylose-4TMS(2)	N.D	N.D	N.D	1528

Table 3-4. Compounds abundant in Kyokuto yeast extract

	Fold difference	
Compound	Kyokuto/OXOID	Kyokuto/BD
Ornithine-4TMS	5.95	2.80
Inositol-6TMS	2.53	3.13
2-Aminoethanol-3TMS	2.30	3.42
Uracil-2TMS	2.25	2.90
Arginine-3TMS	1.88	2.21
Ornithine-3TMS	1.59	2.15

Guanine-3TMS	2.76	4.59
Uridine-4TMS	5.12	3.16
Ribonolactone-3TMS	2.12	1.55

Table 3-5. Compounds not detected in Kyokuto yeast extract

	Peak area	
Compound	OXOID	BD
Glucose-5TMS(2)	19463	14140
Urea-2TMS	23325	34099
Maltitol-9TMS	12577	5268
Lactose-meto-8TMS(2)	10085	2555
Cytosine-2TMS	7130	12345
3-Hydroxybutyric acid-2TMS	5070	2948
3-Hydroxyisobutyric acid-2TMS	5070	2948
N-Acetylmannosamine-meto-4TMS	2973	3469
Oxalic acid-2TMS	1685	3105
2-Hydroxybutyric acid-2TMS	1685	3105

Table 3-6. Compounds detected only in polypeptone

	Compound	Area
	Glucose-5TMS(1)	3914471
	4-Hydroxyphenyllactic acid-3TMS	1011216
One peak	Galactose-5TMS(2)	449801
	Mannose-5TMS(2)	449801
	Allose-5TMS	449801
	Glucaric acid-6TMS	447120
	Galacturonic acid-5TMS(2)	85723
One peak	Psicose-meto-5TMS(2)	46282
	Sorbose-meto-5TMS(1)	46282
One peak	Allose-meto-5TMS(1)	35481

	Mannose-meto-5TMS(1)	35481
	Tagatose-5TMS(5)	35481
	Psicose-5TMS(4)	35481
	Hydroxylysine (2 isomers)	32495
	Glucuronic acid lactone-3TMS(2)	22360
	Malonic acid-2TMS	17976
	Lauric acid-TMS	17670
One peak	Tagatose-5TMS(1)	15660
	Fructose-5TMS(3)	15660
	Tagatose-5TMS(2)	15660
	Gluconic acid-6TMS	8277
	Tagatose-meto-5TMS(2)	6356
	Psicose-meto-5TMS(1)	5041
	3-Methoxy-4-hydroxybenzoic acid-2TMS	2712
	Xylitol-5TMS	2494
	Sarcosine-2TMS	1636
	Xylose-4TMS(2)	1528

Urea induces cell lysis of Δ*ura4* cells

Based on the results described above, the author next tested the compounds detected in polypeptone for their ability to induce cell lysis. For this purpose, the author made YE medium containing seven different sugars or urea, and spotted Δ*ura4* cells on the various media. None of the sugars the author tested (sucrose, maltose, lactose, galactose, mannose, mannitol, and xylose) significantly affected the growth and lysis of Δ*ura4* or Δ*ura4* Δ*fur4* cells. However, cell lysis in Δ*ura4* and Δ*ura4* Δ*fur4* cells was induced when they were grown on YE containing 0.1% urea (Fig. 3-3A). Because 1% inhibited the growth of all tested strains, the author used 0.1% urea for subsequent experiments. The author also noticed that deletion of *fur4* increased sensitivity to urea (Fig. 3-3A and B). The author then spotted Δ*ura4* cells onto YE containing different amount of urea (0%, 0.1%, 0.15%, and 0.2%). Cell lysis of Δ*ura4* cells was induced on YE containing urea (0.1%, 0.15%, and 0.2%) but suppressed by supplementation with uracil (300 mg/L) (Fig. 3-3B). Moreover, deletion of the *pub1* gene, which encodes E3 ubiquitin ligase, or the *ura5* gene, which encodes orotate phosphoribosyltransferase, strongly suppressed cell lysis of Δ*ura4* cells on YE containing urea. Because deletion of Pub1 enhances uracil incorporation, it antagonizes the role of

urea. Deletion of *ura5* in Δ*ura4* cells suppressed cell lysis by urea, as observed in YPD, because orotidine-5-monophasphate (OMP), a key compound in cell lysis, does not accumulate in Δ*ura5* Δ*ura4* cells. Because polypeptone inhibited growth of uracil auxotrophic strains in uracil-containing medium, as shown in Fig. 3-1, the author asked whether a similar effect could be seen in medium containing urea. The author spotted uracil auxotrophic (Δ*ura1*, Δ*ura2*, Δ*ura3*, Δ*ura4*, and Δ*ura5*) strains on low uracil-containing medium (10 mg/L) with or without urea. Growth of uracil auxotrophic strains was inhibited by urea (Fig. 3-3C). This growth inhibition of uracil auxotrophic strains by urea was suppressed by addition of a high concentration of uracil (Fig 3-3D). Cell lysis was clearly observed under the microscope by the addition of urea (Fig. 3-3E). The author further examined the effect of uracil transporter Fur4 on the growth of EMM in the presence of polypeptone or urea. Δ*ura4* Δ*fur4* cells exhibited stronger sensitivity to polypeptone or urea than Δ*ura4* and Δ*fur4* cells (Fig. 3-3F). Thus, urea has an effect similar to that of polypeptone on cell lysis of Δ*ura4* cells and inhibition of growth on EMMU. However, when the author measured concentration of urea in yeast extracts and polypeptone by urea assay kit, urea concentrations were about 0.0017-0.0022% in BD yeast extract, OXOID yeast extract or polypeptone (Fig. 3-4). These concentrations of urea are too low to induce cell lysis.

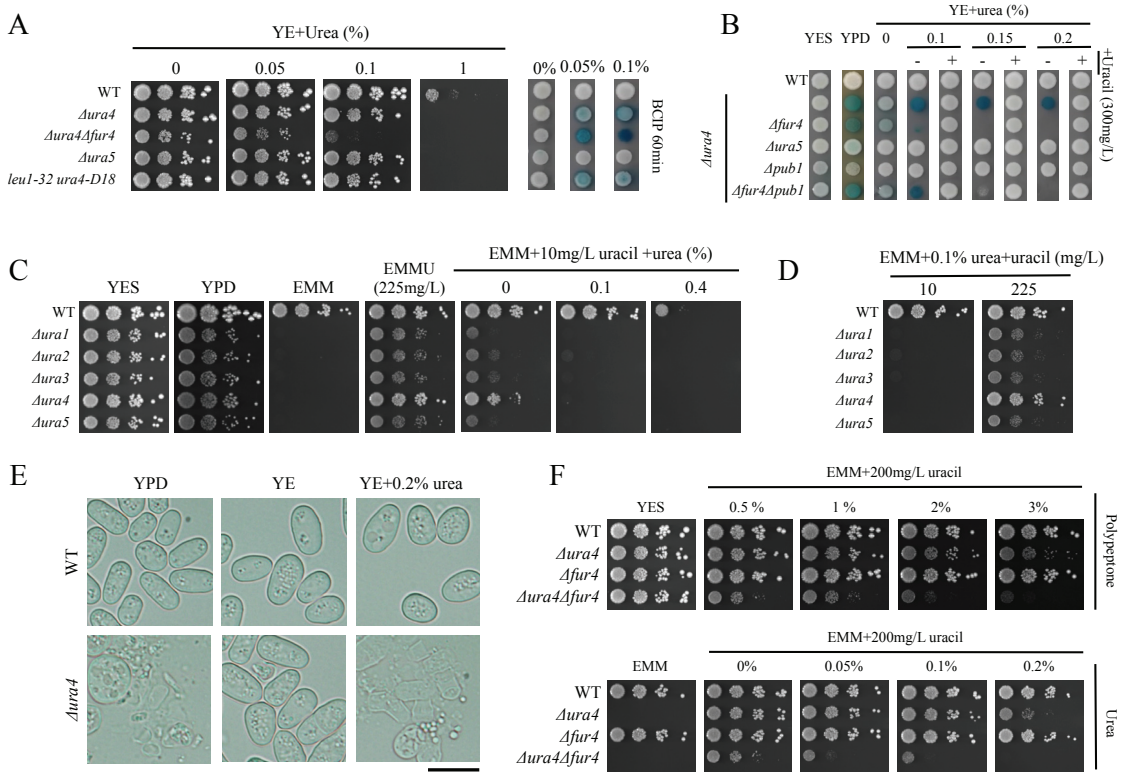


Fig. 3-3. Urea induces cell lysis of Δ*ura4* strains in YE medium. (A) L972 (WT; *ura4*$^{+}$), UMP31 (Δ*ura4*), KNP27 (Δ*ura4* Δ*fur4*), UMP37 (Δ*ura5*), and PR109 (*ura4-D18*) were grown for 12 h, spotted onto YE in the presence or absence of urea (0.05%, 0.1%, or 1%), and incubated for 3 days. For the alkaline phosphatase assay, BCIP was used as described in Fig. 3-2. (B) L972 (WT; *ura4*$^{+}$), UMP31 (Δ*ura4*), KNP27 (Δ*ura4* Δ*fur4*), UMP42 (Δ*ura4* Δ*ura5*), KNP32 (Δ*ura4* Δ*pub1*), and KNP38 (Δ*ura4* Δ*pub1* Δ*fur4*) were grown for 12 h, spotted onto YE in the presence or absence of urea (0.1%, 0.15%, or 0.2%)

or uracil (300 mg/L), and incubated for 3 days. For the alkaline phosphatase assay, BCIP was used as described in Fig. 3-1. Cells were also observed by microscopy after 3 days of growth on YE+urea. (C) L972 (WT; *ura4*$^{+}$), UMP34 (Δ*ura1*), UMP35 (Δ*ura2*), UMP36 (Δ*ura3*), UMP31 (Δ*ura4*), and UMP37 (Δ*ura5*) were grown for 12 h, and then spotted onto EMMU (10mg/L uracil) in the presence or absence of urea (0%, 0.1%, or 0.4%) and incubated for 3 days. (D) L972 (WT; *ura4*$^{+}$), UMP34 (Δ*ura1*), UMP35 (Δ*ura2*), UMP36 (Δ*ura3*), UMP31 (Δ*ura4*), and UMP37 (Δ*ura5*) were grown for 12 h, and then spotted onto EMMU (10 or 225mg/L uracil) in the presence of urea (0.1%) and incubated for 3 days. (E) L972 (WT; *ura4*$^{+}$) and UMP31 (Δ*ura4*) were also observed by microscopy after 3 days on YE, YE+urea, or YPD. (F) L972 (WT; *ura4*$^{+}$), UMP31 (Δ*ura4*), KNP16 (Δ*fur4*), and KNP27 (Δ*ura4* Δ*fur4*) were grown for 12 h, spotted onto EMMU (200 mg/L uracil) in the presence or absence of polypeptone (0%, 0.5%, 1%, 2%, or 3%) or urea (0.05%, 1%, or 2%), and incubated for 4 days at 30°C.

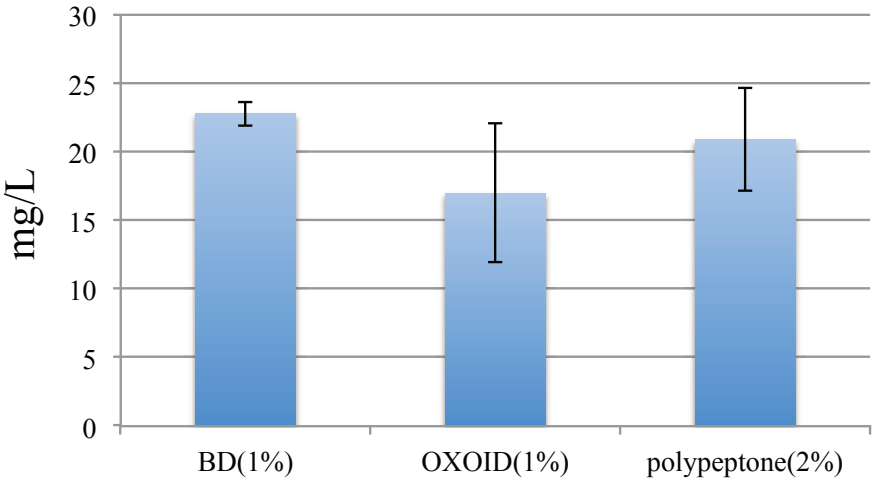


Fig. 3-4. Urea measurement in yeast extract and polypeptone

Urea concentration of yeast extract(BD and OXOID) and polypeptone, measured by urea assay kit.

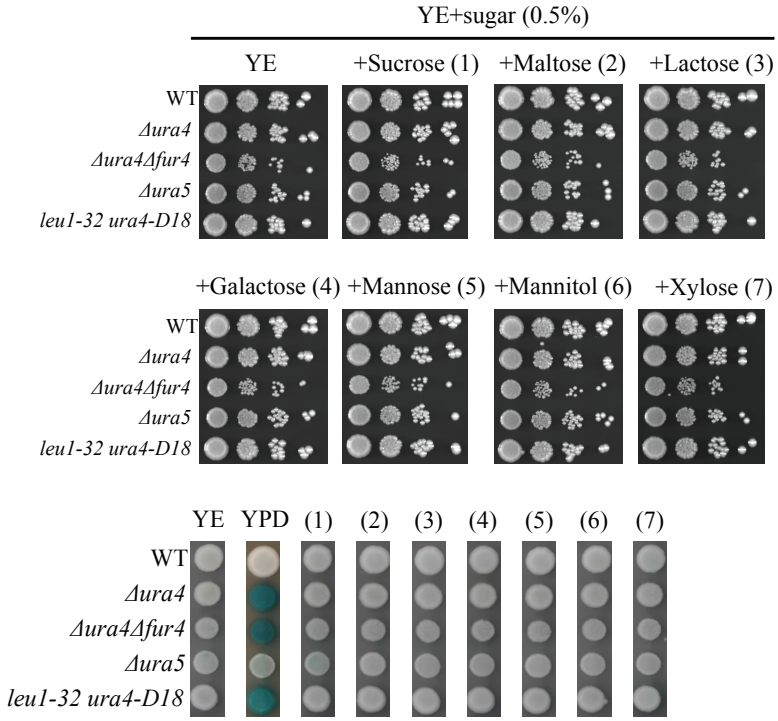


Fig. 3-5. Effect of sugars on cell lysis

L972 (WT; *ura4*$^{+}$), UMP31 (Δ*ura4*), KNP27 (Δ*ura4*Δ*fur4*), UMP37 (Δ*ura5*), and PR109 (*ura4-D18*) were grown for 12 h, and then spotted onto YE in the presence of indicated sugars. For the alkaline phosphatase assay, BCIP was used as described in Fig. 3-1.

Deletion of *ure2* suppressed cell lysis of Δ*ura4* cells grown on YE containing urea

The author next tested the role of urea in cell lysis by deleting the *ure2* gene, which encodes an ATP-independent urease that catalyzes the formation of $2NH_3$ from urea[61]. (The *ure2* gene has been mapped[62] and shown to be identical to the *ure1* gene[63]). Urea can be used as a nitrogen source in *S. pombe* expressing the Ure2 protein[63]; consequently, Δ*ure2* cells cannot grow on medium containing urea (0.01 M) as the sole nitrogen source. The author constructed Δ*ura4*Δ*ure2* double mutants to further examine the effect of *ure2* on the cell growth and cell lysis of Δ*ura4* cells. Deletion of the *ure2* gene suppressed the growth inhibitory effect of urea (Fig. 3-7A) and significantly suppressed cell lysis of Δ*ura4* cells on YE containing urea (Fig. 3-7B). However, Δ*ura4* Δ*ure2* cells still lysed on YPD medium, suggesting that polypeptone contains another inducible factor in addition to urea. Next, the author investigated whether Δ*ura4* Δ*ure2* cells would lyse on YE (OXOID, BD, Oriental, or Difco) medium. Deletion of the *ure2* gene significantly suppressed cell lysis of Δ*ura4* cells on YE (OXOID) medium and weakly suppressed cell lysis of Δ*ura4* cells on YE (BD or Oriental) medium (Fig. 3-5C). However, deletion of the *ure2* gene in Δ*ura1*, Δ*ura2,* Δ*ura3,* Δ*ura4* or Δ*ura5* cells did not suppress growth inhibition by urea in low uracil-containing medium (10mg/mL) (Fig. 3-7A).

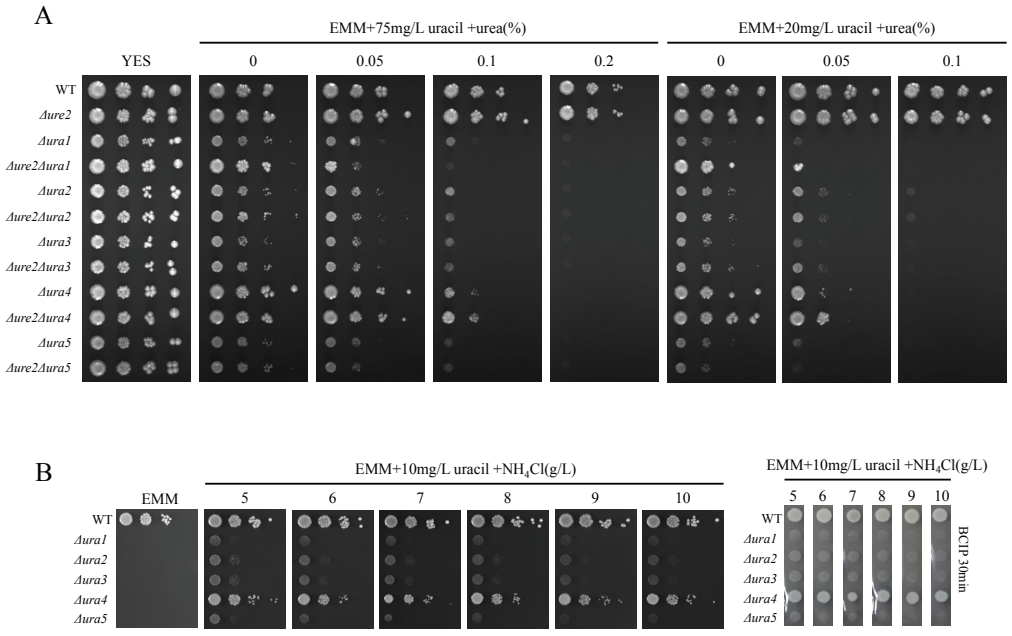


Fig. 3-6. Deletion of the *ure2* gene does not suppress growth of uracil auxotrophic strains on EMMU medium. L972 (WT; *ura4*$^{+}$), UMP34 (Δ*ura1*), UMP35 (Δ*ura2*), UMP36 (Δ*ura3*), UMP31 (Δ*ura4*), UMP37 (Δ*ura5*), KNP174 (*Δure2*), KNP178 (Δ*ura1 Δure2*), KNP180 (Δ*ura2 Δure2*), KNP182 (Δ*ura3 Δure2*), KNP176 (Δ*ura4 Δure2*), and KNP184 (Δ*ura5 Δure2*)were grown for 12 h, and then spotted onto EMMU (75, 20mg/L uracil) in the presence or absence of urea (0%, 0.05%, or 0.1%) and incubated for 5 days.

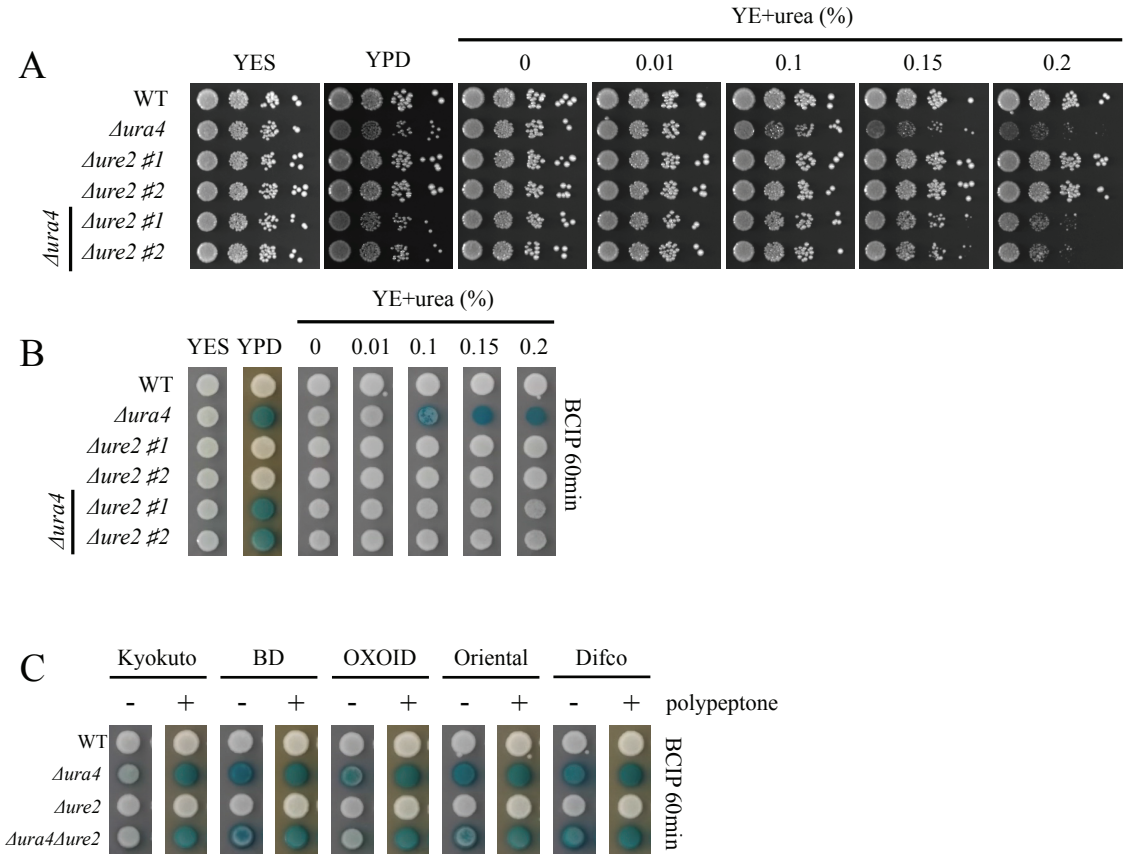


Fig. 3-7. Deletion of the *ure2* gene suppresses cell lysis of Δ*ura4* on YE+urea medium. (A) L972 (WT; *ura4*$^{+}$), UMP31 (Δ*ura4*), KNP174♯1 (Δ*ure2*), KNP174＃2 (Δ*ure2*), KNP176＃1 (Δ*ura4* Δ*ure2*), and KNP176＃2 (Δ*ura4* Δ*ure2*) were grown for 12 h, spotted onto YE in the presence or absence of urea (0.01%, 0.1%, 0.15%, or 0.2%), and incubated for 3 days. Strains #1 and #2 are different isolates and are otherwise of the same genetic background. (B) For the alkaline phosphatase assay, BCIP was used as described in Fig. 3-1. (C) L972 (WT; *ura4*$^{+}$), UMP31 (Δ*ura4*), KNP174 (Δ*ure2*), and KNP176 (Δ*ura4* Δ*ure2*) were grown for 12 h, spotted onto YE medium (Kyokuto, BD, OXOID, Oriental Yeast, and Difco) in the presence or absence of polypeptone (2%), and incubated for 3 days at 30°C. For the alkaline phosphatase assay, BCIP was used as described in Fig. 3-1.

Discussion

The components of yeast extract affect the cell lysis phenotype of *S. pombe* Δ*ura4*.

In this study, the author sought to identify the medium components that induce cell lysis of Δ*ura4* cells in YPD media. The author reported previously that polypeptone induces cell lysis of Δ*ura4* cells in YPD medium[13], but as shown in this study, yeast extract also affected cell lysis (Fig. 3-2A). The main factor explaining why yeast extract affects cell lysis is the difference in the concentration of uracil among yeast extract from different suppliers (Fig. 3-2B). In light of the author's previous observation that depletion of

uracil induced cell lysis, whereas supplementation of uracil suppressed cell lysis in YPD media, it is reasonable to postulate that the amount of uracil in yeast extract affects the cell lysis phenotype. However, the uracil in yeast extract is not the sole determinant of the cell lysis-inducing effect. Increasing the amount of yeast extract to 4% did not reverse the effect in Δ*ura4* cells (Fig. 3-2C and D), indicating that some factors (such as urea) antagonize the increased uracil amount by increasing yeast extract.

The author measured compounds in yeast extract and polypeptone using GC-MS, and identified 172 peaks in total. Nine kinds of compounds detected by GC-MS analysis were more abundant in Kyokuto yeast extract than in OXOID and BD yeast extracts (Table 3-4), whereas ten peaks were detected in BD and OXOID but not in Kyokuto yeast extract (Table 3-5). The author added some of the products identified by GC-MS to the media, and tested cell lysis by BCIP assay (Fig. 3-5). The seven sugars tested did not affect cell lysis. However, urea, which was not detected in the Kyokuto yeast extract, clearly induced cell lysis in Δ*ura4* cells (Fig. 3-3A). Urea was also detected in polypeptone (Table 3-3)(Fig. 3-4) and had an effect similar to that of polypeptone, as addition of uracil suppressed cell lysis in YE containing urea. In addition, the cell lysis of Δ*ura4* cells in YE containing urea was suppressed by deletion of *pub1*, which encodes an E3 ligase that regulates uracil transporter, or *ura5*, which encodes orotate phosphoribosyltransferase (Fig. 1-1). The author concluded that urea is one of the compounds that induces cell lysis of Δ*ura4* cells, and that the balance between suppression of cell lysis by uracil and induction of lysis by urea determines the overall extent of cell lysis. Even though the author observed 0.1 % urea induced cell lysis, the concentrations of urea included in yeast extracts and polypeptone were very low (about 0.002 %). This indicates urea is not a sole determinant in inducing cell lysis and suggests that other nitrogen sources such as peptides or amino acids together can induce cell lysis in Δ*ura4* cells.

Urea induces cell lysis of Δ*ura4* cells by increasing the intracellular NH_3 concentration

Deletion of the *ure2* gene, which encodes an ATP-independent urease, suppressed cell lysis of Δ*ura4* cells in YE+urea media. However, deletion of the *ure2* gene did not clearly suppress cell lysis of Δ*ura4* cells in YPD media (Fig. 3-7A and B). Therefore, the author believe that urea does not directly affect cell lysis of *Δura4* cells, but instead induces lysis by increasing the intracellular concentration of ammonia (NH_3), which is generated through hydrolysis of urea by urease. Because polypeptone contains low molecular weight peptides and various amino acids, these are converted to NH_3. Thus, intracellular NH_3 may be the inducer of cell lysis resulting from treatment with urea or various amino acids (Fig. 3-8).

Excess nitrogen source may inhibit transport of uracil or the UMP salvage pathway

Based on the author's results, the author hypothesizes that excess nitrogen inhibits uracil transport and/or the UMP salvage pathway, for the following reasons. 1) Uracil auxotrophic strains, all defective in the *de novo* UMP synthetic pathway, exhibited growth retardation by addition of polypeptone in EMM (Fig. 3-1A). 2) Growth of uracil auxotrophic strains was inhibited by polypeptone or urea on EMM medium containing low concentrations of uracil (Fig. 3-1B and 3-3C). 3) Growth of Δ*ura4* mutants on YE media was sensitive to addition of NH_4Cl or urea. 4) Δ*ura4* Δ*fur4* cells were more sensitive to

polypeptone or urea on EMMU media than Δ*ura4* cells. 5) Cell lysis was induced in Δ*ura4* Δ*fur4* cells on YE containing urea (0.1%) (Fig. 3-3B and E). All of these results can be explained if nitrogen, probably NH_3, inhibits the transport of uracil and/or the salvage pathway of UMP synthesis. Uracil in the media is necessary for transport, mainly (but not solely) through Fur4, into the cell, where it is converted to UMP by the salvage pathway. Thus, transport of uracil and the UMP salvage pathway are necessary for growth of uracil auxotrophic strains. The author previously found that localization of Fur4 was regulated by a nitrogen source in media. However, because Δ*ura4* Δ*fur4* cells were more sensitive to polypeptone (or urea) than Δ*ura4* cells when grown on EMMU medium (Fig. 3-3F), the author's results suggest that excess nitrogen source is also inhibitory to UMP salvage pathway. Moreover, Δ*ura4* Δ*fur4* cells were grown on EMMU(200mg/l uracil) media without urea or polypeptone. If the transporter (Fur4) is the target of polypeptone or urea, no such phenotype should be observed. At present, the author does not know whether regulation of the transporter or the UMP salvage pathway is more relevant to the mechanism by which excess nitrogen source affects uracil auxotrophic strains. However, the phenotypes the author observed are closely related to the availability of uracil inside the cell.

Excess NH_4Cl inhibits growth of the Δ*leu1* or Δ*eca39* mutants in YE media[60,64]. This phenotype can be suppressed by nitrogen signaling factors (NSFs) or supernatant from a prototrophic strain. However, the growth of *ade6* and *ura4* single mutants was not promoted by these conditions. Thus, the mechanism of sensitivity to excess nitrogen differs between *leu1* and *ura4* mutants, but apparently excess nitrogen affects cell growth. Excess NH_4Cl did not inhibit growth of uracil auxotrophic strains and not induce cell lysis of Δ*ura4* cells in low uracil-containing EMM medium (Fig. 3-6B). The author thinks that the effect of NH_4Cl in EMM media is different from YE media and that other compounds in yeast extract affect the phenotype.

In conclusion, the author found in this study that among the components included in polypeptone and yeast extracts, uracil is a suppressor and urea is an inducer of cell lysis in *S. pombe* Δ*ura4* cells. To be effective, urea must be converted into ammonia, and excess intracellular nitrogen somehow inhibits the availability of uracil inside cells. Thus, uracil depletion induces cell lysis of *S. pombe* Δ*ura4*.

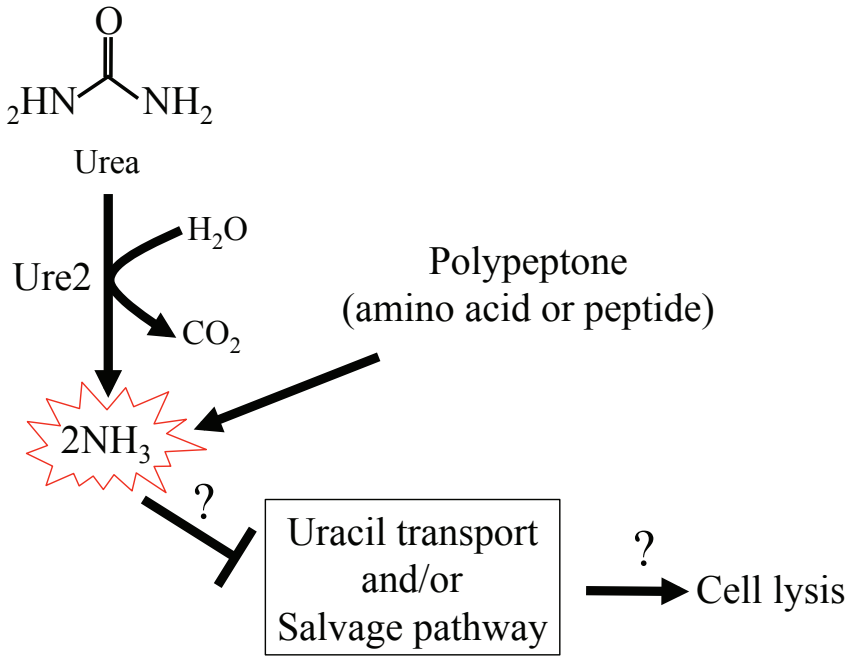


Fig. 3-8. A model for induction of cell lysis

In fission yeast, urease (Ure2) catalyzes the hydrolysis of urea to yield $2NH_3$ and CO_2. Polypeptone supplies amino acid or low molecular weight peptide, and therefore increases the intracellular concentration of NH_3. The increase in intracellular concentration of NH_3 is likely to inhibit uracil transport and/or the salvage pathway of UMP synthesis. Depletion of intracellular uracil then causes cell lysis.

References

1. Hoffman CS, Wood V, Fantes PA. An Ancient Yeast for Young Geneticists: A Primer on the *Schizosaccharomyces pombe* Model System. Genetics 2015; 201:403–23.

2. Rhind N, Chen Z, Yassour M, Thompson DA, Haas BJ, Habib N, Wapinski I, Roy S, Lin MF, Heiman DI, et al. Comparative functional genomics of the fission yeasts. Science 2011; 332:930–6.

3. Miyata M, Kitamura J, Miyata H. Lysis of growing fissin yeast cells induced by aculeacin A, a new antifungal antibiotic. Arch Microbiol 1980; 127:11–6.

4. Johnson BF. Lysis of yeast cell walls induced by 2-deoxyglucose at their sites of glucan synthesis. Journal of Bacteriology 1968; 95:1169–72.

5. Cortés JCG, Sato M, Muñoz J, Moreno MB, Clemente-Ramos JA, Ramos M, Okada H, Osumi M, Durán A, Ribas JC. Fission yeast Ags1 confers the essential septum strength needed for safe gradual cell abscission. J Cell Biol 2012; 198:637–56.

6. Cortes JCG. The novel fission yeast (1,3) -D-glucan synthase catalytic subunit Bgs4p is essential during both cytokinesis and polarized growth. Journal of Cell Science 2005; 118:157–74.

7. Cortés JCG, Konomi M, Martins IM, Muñoz J, Moreno MB, Osumi M, Durán A, Ribas JC. The (1,3)beta-D-glucan synthase subunit Bgs1p is responsible for the fission yeast primary septum formation. Mol Microbiol 2007; 65:201–17.

8. Gupta S, Govindaraghavan M, McCollum D. Cross talk between NDR kinase pathways coordinates cytokinesis with cell separation in *Schizosaccharomyces pombe*. Eukaryotic Cell 2014; 13:1104–12.

9. Balazs A, Batta G, Miklos I, Acs-Szabo L, Vázquez de Aldana CR, Sipiczki M. Conserved regulators of the cell separation process in *Schizosaccharomyces*. Fungal Genet Biol 2012; 49:235–49.

10. Perez P. Cell wall analysis. Methods 2004; 33:245–51.

11. de Medina-Redondo M, Arnáiz-Pita Y, Clavaud C, Fontaine T, del Rey F, Latgé J-P, de Aldana CRV. β(1,3)-Glucanosyl-Transferase Activity Is Essential for Cell Wall Integrity and Viability of Schizosaccharomyces pombe. PLoS ONE 2010; 5:e14046.

12. Osumi M. Visualization of yeast cells by electron microscopy. J Electron Microsc (Tokyo) 2012; 61:343–65.

13. Matsuo Y, Nishino K, Mizuno K, Akihiro T, Toda T, Matsuo Y, Kaino T, Kawamukai M. Polypeptone induces dramatic cell lysis in *ura4* deletion mutants of fission yeast. PLoS ONE 2013; 8:e59887.

14. Hochstrasser M. Ubiquitin-dependent protein degradation. Annu Rev Genet 1996; 30:405–39.

15. Ciechanover A. The ubiquitin-proteasome pathway: on protein death and cell life. The EMBO Journal 1998; 17:7151–60.

16. Husnjak K, Dikic I. Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. Annu Rev Biochem 2012; 81:291–322.

17. Zhao Y, Macgurn JA, Liu M, Emr S. The ART-Rsp5 ubiquitin ligase network comprises a plasma membrane quality control system that protects yeast cells from proteotoxic stress. Elife 2013; 2:e00459.

18. Nakase M, Nakase Y, Chardwiriyapreecha S, Kakinuma Y, Matsumoto T, Takegawa K. Intracellular trafficking and ubiquitination of the *Schizosaccharomyces pombe* amino acid permease Aat1p. Microbiology 2012; 158:659–73.

19. Nakase Y, Nakase M, Kashiwazaki J, Murai T, Otsubo Y, Mabuchi I, Yamamoto M, Takegawa K, Matsumoto T. The fission yeast β-arrestin-like protein Any1 is involved in TSC-Rheb signaling and the regulation of amino acid transporters. Journal of Cell Science 2013; 126:3972–81.

20. Nakashima A, Kamada S, Tamanoi F, Kikkawa U. Fission yeast arrestin-related trafficking adaptor, Arn1/Any1, is ubiquitinated by Pub1 E3 ligase and regulates endocytosis of Cat1 amino acid transporter. Biol Open 2014; 3:542–52.

21. Fang Y, Jaiseng W, Ma Y, Hu L, Yamazaki S, Zhang X, Hayafuji T, Shi L, Kuno T. E3 ubiquitin ligase Pub1 is implicated in endocytosis of a GPI-anchored protein Ecm33 in fission yeast. 2014; 9:e85238.

22. Tamai KK, Shimoda C. The novel HECT-type ubiquitin-protein ligase Pub2p shares partially overlapping function with Pub1p in *Schizosaccharomyces pombe*. Journal of Cell Science 2002; 115:1847–57.

23. Blondel M-O, Morvan J, Dupré S, Urban-Grimal D, Haguenauer-Tsapis R, Volland C. Direct sorting of the yeast uracil permease to the endosomal system is controlled by uracil binding and Rsp5p-dependent ubiquitylation. Mol Biol Cell 2004; 15:883–95.

24. Lin CH, Macgurn JA, Chu T, Stefan CJ, Emr SD. Arrestin-related ubiquitin-ligase adaptors regulate endocytosis and protein turnover at the cell surface. Cell 2008; 135:714–25.

25. Keener JM, Babst M. Quality control and substrate-dependent downregulation of the nutrient transporter Fur4. Traffic 2013; 14:412–27.

26. Babst M. Quality control: quality control at the plasma membrane: one mechanism does not fit all. J Cell Biol 2014; 205:11–20.

27. Boeke JD, Croute F, Fink GR. A positive selection for mutants lacking orotidine-5-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol Genet Genomics 1984; 197:345–6.

28. Corbacho I, Teixidó F, Velázquez R, Hernández LM, Olivero I. Standard YPD, even supplemented with extra nutrients, does not always compensate growth defects of *Saccharomyces cerevisiae* auxotrophic strains. 2010; 99:591–600.

29. Hanscho M, Ruckerbauer DE, Chauhan N, Hofbauer HF, Krahulec S, Nidetzky B, Kohlwein SD, Zanghellini J, Natter K. Nutritional requirements of the BY series of *Saccharomyces cerevisiae* strains for optimum growth. FEMS Yeast Res 2012; 12:796–808.

30. Petersen J, Russell P. Growth and the Environment of *Schizosaccharomyces pombe*. Cold Spring Harb Protoc 2016; 2016:pdb.top079764.

31. R E. Fission Yeast in General Genetics. Springer-Verlag 2003; :1–12p.

32. Tanae K, Horiuchi T, Matsuo Y, Katayama S, Kawamukai M. Histone chaperone Asf1 plays an essential role in maintaining genomic stability in fission yeast. PLoS ONE 2012; 7:e30472.

33. Kato H, Kira S, Kawamukai M. The transcription factors Atf1 and Pcr1 are essential for transcriptional induction of the extracellular maltase Agl1 in fission yeast. PLoS ONE 2013; 8:e80572.

34. Hayashi K, Ogiyama Y, Yokomi K, Nakagawa T, Kaino T, Kawamukai M. Functional conservation of coenzyme Q biosynthetic genes among yeasts, plants, and humans. PLoS ONE 2014; 9:e99038.

35. Losson R, Lacroute F. Plasmids carrying the yeast OMP decarboxylase structural and regulatory genes: transcription regulation in a foreign environment. Cell 1983; 32:371–7.

36. Bach ML. Cloning and expression of the OMP decarboxylase gene URA4 from *Schizosaccharomyces pombe*. Current Genetics 1987; 12:527–34.

37. Kim D-U, Hayles J, Kim D, Wood V, Park H-O, Won M, Yoo H-S, Duhig T, Nam M, Palmer G, et al. Analysis of a genome-wide set of gene deletions in the fission yeast *Schizosaccharomyces pombe*. Nat Biotechnol 2010; 28:617–23.

38. Nefsky B, Beach D. Pub1 acts as an E6-AP-like protein ubiquitiin ligase in the degradation of cdc25. The EMBO Journal 1996; 15:1301–12.

39. Kitamura K, Nakase M, Tohda H, Takegawa K. The Ubiquitin ligase Ubr11 is essential for oligopeptide utilization in the fission yeast *Schizosaccharomyces pombe*. Eukaryotic Cell 2012; 11:302–10.

40. Moreno S, Klar A, Nurse P. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. In: Guide to Yeast Genetics and Molecular Biology. Elsevier; 1991. pages 795–823.

41. Bähler J, Wu JQ, Longtine MS, Shah NG, McKenzie A, Steever AB, Wach A, Philippsen P, Pringle JR. Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. Yeast 1998; 14:943–51.

42. Sato M, Dhut S, Toda T. New drug-resistant cassettes for gene disruption and epitope tagging in *Schizosaccharomyces pombe*. Yeast 2005; 22:583–91.

43. Matsuo Y, Fisher E, Patton-Vogt J, Marcus S. Functional characterization of the fission yeast phosphatidylserine synthase gene, *pps1*, reveals novel cellular functions for phosphatidylserine. Eukaryotic Cell 2007; 6:2092–101.

44. Shiozaki K, Russell P. Stress-activated protein kinase pathway in cell cycle control of fission yeast. Meth Enzymol 1997; 283:506–20.

45. Silve S, Volland C, Garnier C, Jund R, Chevallier MR, Haguenauer-Tsapis R. Membrane insertion of uracil permease, a polytopic yeast plasma membrane protein. Molecular and Cellular Biology 1991; 11:1114–24.

46. Chevallier MR, Lacroute F. Expression of the cloned uracil permease gene of *Saccharomyces cerevisiae* in a heterologous membrane. The EMBO Journal 1982; 1:375–7.

47. Tanaka N, Takegawa K. Functional characterization of Gms1p/UDP-galactose transporter in *Schizosaccharomyces pomb*. Yeast 2001;

48. de Montigny J, Straub ML, Wagner R, Bach ML, Chevallier MR. The uracil permease of *Schizosaccharomyces pombe*: a representative of a family of 10 transmembrane helix transporter proteins of yeasts. Yeast 1998; 14:1051–9.

49. Kawamukai M. Biosynthesis of coenzyme Q in eukaryotes. Biosci Biotechnol Biochem 2015; 80:23–33.

50. Séron K, Blondel M-O, Haguenauer-Tsapis R, Volland C. Uracil-induced down-regulation of the yeast uracil permease. J. Bacteriol. 1999; 181:1793-1800.

51. Galan JM, Moreau V, Andre B, Volland C, Haguenauer-Tsapis R. Ubiquitination mediated by the Npi1p/Rsp5p ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease. Journal of Biological Chemistry 1996; 271:10946–52.

52. Decottignies A, Owsianik G, Ghislain M. Casein kinase I-dependent phosphorylation and stability of the yeast multidrug transporter Pdr5p. Journal of Biological Chemistry 1999; 274:37139–46.

53. Chevallier MR. Cloning and transcriptional control of a eucaryotic permease gene. Molecular and Cellular Biology 1982; 2:977–84.

54. Egel R. Fission Yeast in General Genetics. In: The Molecular Biology of *Schizosaccharomyces pombe*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2004. pages 1–12.

55. Nurse P. A long twentieth century of the cell cycle and beyond. Cell 2000; 100:71–8.

56. Kawamukai M, Gerst J, Field J, Riggs M, Rodgers L, Wigler M, Young D. Genetic and biochemical analysis of the adenylyl cyclase-associated protein, cap, in *Schizosaccharomyces pombe*. Mol Biol Cell 1992; 3:167–80.

57. Matsuo Y, Kawamukai M. cAMP-dependent protein kinase involves calcium tolerance through the regulation of Prz1 in *Schizosaccharomyces pombe*. Biosci Biotechnol Biochem 2017; 81:231–41.

58. Nishino K, Kushima M, Matsuo Y, Matsuo Y, Kawamukai M. Cell lysis in *S. pombe ura4* mutants is suppressed by loss of functional Pub1, which regulates the uracil transporter Fur4. PLoS ONE 2015; 10:e0141796.

59. Krawchuk MD, Wahls WP. High-efficiency gene targeting in *Schizosaccharomyces pombe* using a modular, PCR-based approach with long tracts of flanking homology. Yeast 1999; 15:1419–27.

60. Takahashi H, Sun X, Hamamoto M, Yashiroda Y, Yoshida M. The SAGA histone acetyltransferase complex regulates leucine uptake through the Agp3 permease in fission yeast. J Biol Chem 2012; 287:38158–67.

61. Lubbers MW, Rodriguez SB, Honey NK, Thornton RJ. Purification and characterization of urease from *Schizosaccharomyces pombe*. Can J Microbiol 1996; 42:132–40.

62. Lubbers MW, Thornton RJ, Honey NK. Mapping of *ure1*, *ure2* and *ure3* markers in fission yeast. Yeast 1997; 13:1195–7.

63. Tange Y, Niwa O. Identification of the *ure1+* gene encoding urease in fission yeast. Current Genetics 1997; 32:244–6.

64. Sun X, Hirai G, Ueki M, Hirota H, Wang Q, Hongo Y, Nakamura T, Hitora Y, Takahashi H, Sodeoka M, et al. Identification of novel secreted fatty acids that regulate nitrogen catabolite repression in fission yeast. Sci Rep 2016; 6:20856.

List of Publications

Chapter 2

(1) Kohei Nishino, Misaki Kushima, Yuzy Matsuo, Yasuhiro Matsuo, Makoto Kawamukai :
「Cell Lysis in S. pombe ura4 Mutants Is Suppressed by Loss of Functional Pub1, Which Regulates the Uracil Transporter Fur4」
PLoS ONE 10(11): e0141796. doi:10.1371/journal. pone.0141796

Chapter 3

(2) Kohei Nishino, Misaki Kushima, Tomohiro Kaino, Yasuhiro Matsuo, Makoto Kawamukai :
「Urea enhances cell lysis of Schizosaccharomyces pombe ura4 mutants」
Bioscience,Biotechology,and Biochemistry DOI:10.1080/09168451.2017.1303360 (2017)

Proposed Conclusion

In this thesis, the author studied the following two aspects regarding cell lysis of *S. pombe ura4* deletion strains.

(1) Cell lysis of *S. pombe ura4* deletion strains was suppressed by deletion of *pub1* gene, which encodes E3 ligase. Uracil uptake via Fur4, a uracil transporter, was increased in *pub1* deletion strains. And cell lysis of *ura4* deletion strains was suppressed by excess uracil in media. Concentration of intracellular uracil is critical for cell lysis. Cell lysis in *S. pombe ura4* deletion strains is triggered by cellular uracil depletion and subsequent OMP accumulation. OMP then likely weakens the cell wall structure by an unknown mechanism.

(2) Cell lysis of *S. pombe ura4* deletion strains was induced by polypeptone and high-concentration of yeast extract. I succeeded to identify about 172 peaks from yeast extract and polypeptone and focused on urea in one of these compounds. Cell lysis of *ura4* deletion strains was induced by 0.15% urea, and suppressed by *ura5* or *pub1* deletion and excess uracil. Cell lysis was suppressed by deletion of *ure2* gene, which encodes urease, in YE medium containing urea but not YPD medium. This indicates that cell lysis of *S. pombe ura4* deletion strains is induced by increasing the intracellular NH_3 concentration.

Acknowledgements

The author would like to express his grateful acknowledgement to his supervisor Professor Makoto Kawamukai, Department of Applied Bioscience and Biotechnology, Shimane University, for his appropriate advices, English proofreading, valuable suggestions, and warm encouragement during the course of this study.

The author would like to express his sincere gratitude to Professor Dr. Tsuyoshi Nakagawa, Department of Molecular and Functional Genomics, Center for Integrated Research in Science, Shimane University, for his valuable suggestions and warm encouragement during the course of this study.

The author would like to express his sincere acknowledgement to Associate Professor Dr. Tomohiro Kaino, Department of Applied Bioscience and Biotechnology, Shimane University, for his advices, fixing typos of paper and warm encouragement during the course of this study.

The author would like to express his sincere gratitude to Assistant Professor Dr. Yasuhiro Matsuo, Department of Applied Bioscience and Biotechnology, Shimane University, for his appropriate advices, technical instruction of several experiments and warm encouragement during the course of this study.

The author would like to express his acknowledgement to Assistant Professor Dr. Takashi Akihiro, Faculty of Life and Environmental Science, Shimane University, for his experimental advices on LC-MS.

The author would like to express his sincere gratitude to Assistant Professor Dr. Kenjiro Furuta, Department of Applied Bioscience and Biotechnology, Shimane University, for his experimental advices on LC-MS.

The author would like to express his thanks to Dr. Yuzy Matsuo and Dr. Katsuhiro Tanae for technical instruction, valuable suggestions, and warm encouragement during the course of this study.

The author would like to express his thanks to Ms. Misaki Kushima for their experimental supports, and encouragement during the course of this study.

The author would like to express his tanks to Dr. Daisuke Moriyama, Dr. Shinya Nakamura, Dr. Yuji Tanaka, Ms. Chikako Yokogi, Mr. Kazuhiro Hayashi, Ms. Yuriko Takashima and all other members of Laboratory of Applied Microbiology, Shimane University for their kind supports, technical advices, and warm encouragement during the course of this study.

Summary

Fission yeast *S. pombe* has been studied as a model organism of eukaryotic organisms. *S. pombe* encodes 5,000 genes in 12.57Mbp genome on three chromosomes. *S. pombe* is stable in haploid, and it is easy to construct a gene deletion strains or tagging strains. Because splicing, post-transcriptional regulation and cell division are remarkably similar to those of animal cells than *S. cerevisiae*, *S. pombe* is beneficial to study cell cycle, morphology and cellular responses. This study is based on the finding that dramatic cell lysis is induced in *ura4* deletion strains of *S. pombe,* Ura4 protein catalyze decarboxylation of OMP to synthesis UMP in *de novo* UMP pathway. OMP is significantly accumulated by deletion of the *ura4* gene. But, how OMP is involved in cell lysis is not elucidated.

To find out detailed mechanism of cell lysis in *S. pombe ura4* mutants. I performed the screening using the *S. pombe* 3,400 non-essential gene deletion strains. I spot the 3,400 cells on YPD medium, to select strains that did not undergo cell lysis in *ura4* mutants background. As a result, I found that cell lysis of *ura4* deletion strains was dramatically suppressesed by deletion of *pub1* gene, which encodes E3 ubiquitin ligase. I then tested the sensitivity of 5-FU, a toxic analog of uracil, in *pub1* deletion strains, and found that the *pub1* gene deletion strains showed high sensitivity to 5-FU. High-sensitivity of 5-FU to the *pub1* gene deletion strains was suppressed by concomitant deletion of *fur4* gene, encoding uracil transporter. Because Pub1 controls localization of membrane proteins, I tested the Fur4 affects cell lysis in using *ura4pub1fur4* triple gene deletion strains. Deletion of *fur4* gene reversed the suppressive effect by deletion of *pub1*. I thought that suppression of cell lysis by *pub1* gene disruption is via localization control of membrane transporter Fur4. In fact, the membrane localization of Fur4 increases in *pub1* deletion strain. Accordingly, increased uracil uptake via Fur4 suppressed cell lysis of *ura4* deletion strains in the *pub1* deletion strains. Uracil of uptake was significantly enhanced in *pub1* deletion strain. In addition, cell lysis of *ura4* deletion strain was suppressed by adding excess uracil. Additionaly, I analyzed membrane localization of Fur4 protein and found that Fur4 protein in ubiquitinated in WT and *pub1* deletion strains. Membrane localization of Fur4 increased under nitrogen source depletion condition and ubiquitination of Fur4 disappeared. This result indicates that ubiquitination of Fur4 controls its membrane localization.

I concluded that cell lysis of *ura4* gene deletion strains is suppressed by increase of uracil uptake in *pub1* deletion strains.

Next, I examined the relationship between media components and cell lysis. Cell lysis of *ura4* deletion strain was induced in YPD medium. However, what components in the medium induce cell lysis is not known. So, I decided to study the effect and components of the yeast extract which contains a variety of nutrients. Previously cell lysis was observed in YE medium without polypeptone, but in the other cases, the cell lysis was not observed. I thought this difference is due to the difference of the manufacturer of yeast extract. Therefore, I investigated whether cell lysis occurs in YE medium using yest extract from the five manufacturers. While cell lysis was not observed when yeast extract of Kyokuto was used, it was observed when when OXOID, BD, Oriental or Difco were used. Next, I made high-concentration yeast extract (Kyokuto, OXOID, BD, Oriental, Difco), and found cell lysis of *ura4* gene deletion strains was induced in all media. This result suggests that the yeast extract contains the inducer of cell lysis.

Then I decided to analyze the medium components using GC-MS. In yeast extract and polypeptone, I identified the peaks of 172 compounds by using GC-MS. Among them, ten kinds of compounds were detected in OXOID and BD yeast extracts, but not in Kyokuto yeast extract. There was urea among ten compounds. In fact, urea induced cell lysis of *ura4* deletion strains in YE medium of Kyokuto. Cell lysis by addition of urea was suppressed by deletion of *ura5* or *pub1*. In addition, suppression was observed by the addition of uracil. The effect of urea is very similar to the effect of polypeptone.

I examined the cell lysis in a *ura4* with deletion of *ure2* which encodes urease an enzyme converting urea to ammonium. As a result, deletion of *ure2* gene suppressed cell lysis of *ura4* deletion strains. These results suggest increasing the concentration of ammonium concentration may cause the cell lysis but not urea itself.

In this these, I concluded that the suppression of cell lysis by *pub1* gene deletion is caused by increase uracil uptake via Fur4. I revealed that cell lysis of *ura4* deletion strains was induced by urea and urea increase the nitrogen source concentration insides cells.

These findings elucidated that the specific component in media and uracil uptake mechanism via Fur4 in *S. pombe*.

要約

分裂酵母は真核生物のモデル生物として研究されている。分裂酵母は染色体が 3 本であり、ゲノム配列も決定しており、遺伝子破壊や遺伝学的な解析が容易であることから分裂酵母は優れた真核生物のモデル生物である。また、出芽酵母と比較するとイントロンの割合、翻訳後修飾、分裂様式、細胞極性の観察の容易さ等の点から細胞周期や形態に関する研究で使用されている。

我々の研究室において分裂酵母の *ura4* 遺伝子破壊株が劇的な細胞溶解を誘導することを見出した。そこで、私は *ura4* 遺伝子破壊株の細胞溶解の詳細な仕組みを明らかにするために研究を行った。Ura4 タンパク質は *de novo* UMP 合成経路の下流に位置しており、OMP を脱炭酸し UMP を合成することが知られている。その為、*ura4* 遺伝子の破壊により反応の前駆体である OMP が蓄積することを明らかにした。しかし、OMP が細胞溶解にどのように関与しているかは不明である。

その為に私は分裂酵母非必須遺伝子破壊株ライブラリーを用いたスクリーニングを行なうことにした。ライブラリーの 3400 株を YPD 培地にスポットし細胞溶解抑圧株を単離した。結果として、*ura4* 遺伝子破壊株における細胞溶解は *pub1* 遺伝子の破壊により顕著に抑圧されることを見出した。

Pub1 は E3 ユビキチンリガーゼであり、膜タンパク質の局在制御に関与していることが分かっている。そこで、*pub1* 遺伝子破壊株の表現型を調べるとウラシルのアナログである 5-FU に対して強い感受性を示すことを明らかにした。また、この感受性はウラシルトランスポーターである *fur4* 遺伝子の破壊により抑圧した。これは *pub1* 遺伝子破壊株の 5-FU 感受性は Fur4 を介していることを示唆している。そこで、*pub1* 遺伝子破壊株における Fur4 の局在を調べると Fur4 の膜局在が増加していることを明らかになった。次に Fur4 の膜局在の増加による影響を調べた。結果として、*pub1* 遺伝子破壊株では Fur4 依存的なウラシルの取り込みが亢進していることが明らかになった。そこで、過剰なウラシルが *ura4* 遺伝子破壊株の細胞溶解を抑圧するかを調べるために YPD 培地にウラシルを添加した。すると、過剰なウラシルの添加により *ura4* 遺伝子破壊株の細胞溶解が抑圧された。

また、Fur4 の局在制御機構に関して解析を進めると、Fur4 はユビキチン化していることを明らかにした。このユビキチン化は *pub1* 遺伝子の破壊では消失しなかった。一方、Fur4 は窒素源枯渇条件下において膜局在が増加しユビキチン化も消失する。このことから Fur4 の膜局在の制御にはユビキチン化が関与する可能性が示唆された。

最終的に*pub1*遺伝子破壊株においてFur4を介した細胞溶解抑圧因子であるウラシルの取り込みが亢進することで*ura4*遺伝子破壊株の細胞溶解が抑圧されたと考えている。

次に私は培地成分と細胞溶解との関係性を調べることにした。*ura4*遺伝子破壊株の細胞溶解はYPD培地において誘導されるがどの成分が細胞溶解に関与しているかは不明である。そこで、私は培地中の様々な栄養成分を含んでいる酵母エキスに着目した。過去の結果から細胞溶解はポリペプトンを含まないYE培地において観察される。一方で、YE培地において細胞溶解が起こらないこともある。この原因は使用した酵母エキスの製造メーカーが異なるからと考えている。そこで、私は5つのメーカー(極東製薬、OXOID、BD、オリエンタル酵母、Difco)の異なる酵母エキスを使い細胞溶解が起こるかを調べることにした。その結果、極東製薬の酵母エキスを使用した場合のみYE培地での細胞溶解が観察されなかった。また、5つのメーカーの酵母エキスの濃度を上げた場合いずれの条件においても細胞溶解が観察された。このことから、私は酵母エキスには細胞溶解誘導因子が含まれていると考えました。

次に、私はGC-MSを用いた培地成分の解析を行った。酵母エキスとポリペプトンの成分をGC-MSを用いて同定した結果172のピークを検出した。その中で10個の化合物はOXOIDとBDで検出され、極東製薬の酵母エキスでは検出されなかった。10種類の中に含まれていた尿素を極東製薬のYE培地に添加することで*ura4*遺伝子破壊株の細胞溶解が誘導されることを見出した。尿素添加による細胞溶解は*ura5*や*pub1*遺伝子の破壊やウラシルの添加により抑圧した。この結果からもYPD培地による細胞溶解と尿素添加による細胞溶解は同一であると考えられる。

さらに、尿素の影響はその分解物に依存するかを知るためにウレアーゼをコードする*ure2*遺伝子破壊株を作製しその影響を調べた。*ura4ure2*二重遺伝子破壊株において尿素の添加による細胞溶解は抑圧された。しかし、*ure2*遺伝子の破壊ではYPD培地における細胞溶解の抑圧は観察されなかった。これらの結果から、私は尿素自身が細胞溶解を誘導するわけではなく細胞内における窒素源濃度の上昇が細胞溶解を誘導するのではないかと考えている。

このように本論文では*pub1*遺伝子破壊株における細胞溶解の抑圧はFur4を介したウラシルの取り込み量の増加によると結論づけた。また、*ura4*遺伝子破壊株による細胞溶解は尿素により誘導されることを明らかにした。しかし、尿素そのものが細胞溶解の原因ではなく尿素添加による細胞内の窒素源濃度の上昇が原因であると考えている。

本論文では*ura4*遺伝子破壊株の細胞溶解現象の解析を通じて、分裂酵母のウラシル取り込みメカニズムと複雑な培地成分が酵母に与える影響に関して新たな知見を提供したと考えている。