Title

Yeast PRPP amidotransferase assembles into fine granules to promote *de novo* purine

synthesis through intermolecular substrate channeling

Masak Takaine[1,2], Rikuri Morita[3]

[1]GIAR and [2]IMCR, Gunma University, Maebashi, Japan; [3]Center for Computational

Sciences, University of Tsukuba, Ibaraki, Japan

Keywords: purine biosynthesis, *de novo* purine synthesis, PRPP amidotransferase

(PPAT), Ade4, yeast

*Correspondence should be addressed to: masaktakaine@gunma-u.ac.jp (M.T.)

Highlights

- Budding yeast PRPP amidotransferase (PPAT) forms intracellular dynamic granules in response to the deprivation of environmental purine bases.
- The assembly of PPAT granules is driven by ribosome crowding in the cytoplasm induced by TORC1.
- Pure PPAT proteins condensate into granule-sized particles *in vitro* under molecular crowding conditions.
- The interaction of PPAT with purine nucleotides prevents granule assembly.
- The condensation of PPAT in granules permits the intermolecular channeling of intermediates, thereby facilitating *de novo* purine synthesis.

Summary

De novo purine synthesis (DPS) is up-regulated under conditions of high purine demands to ensure the mass production of genetic materials and chemical energy, thereby supporting cell proliferation. However, the mechanisms by which DPS is activated remain unclear. We herein demonstrated that PRPP amidotransferase (PPAT), the rate-limiting enzyme in DPS, condensated into granules in *Saccharomyces cerevisiae* cells under purine-depleted conditions. The assembly of granules required phase separation driven by target of rapamycin complex 1 (TORC1)-induced ribosome biosynthesis. A biochemical analysis revealed that the self-assembly of PPAT molecules accounted for granule formation and submillimolar levels of purine nucleotides inhibit self-assembly. Cells unable to form PPAT granules showed growth defects, indicating the physiological importance of granules for the promotion of DPS. These results suggest that the condensation of PPAT by phase transition is a mechanism that regulates DPS sensitively responding to both TORC1 activity and cellular purine demands.

Introduction

Purine nucleotides and their derivatives are involved in many cellular processes and, thus, are essential metabolites for every living organism. The purine nucleotide triphosphates ATP and GTP serve as the energy currencies of cells. Purine metabolites are also incorporated into genetic material, namely, DNA and RNA, as well as coenzymes, such as NAD and coenzyme A. Purine nucleotides are synthesized via both salvage and *de novo* pathways. In mammalian cells, as long as extracellular purine bases are available, purine nucleotides are preferentially synthesized from the recycling of bases through the salvage pathway[1]. Under the condition of a high purine demand (*e.g.*, in actively proliferating cells), the *de novo* pathway is activated to supply higher levels of purine nucleotides[1]. Disorders in the regulation of purine metabolism are associated with various diseases, such as cancer, hyperuricemia (gout), and immunological defects [2, 3]. A more detailed understanding of the regulatory mechanisms underlying purine metabolism will provide insights into the molecular basis of these diseases and facilitate the identification of their therapeutic targets.

The *de novo* purine biosynthetic pathway comprises ten highly conserved sequential chemical reactions that are performed by six enzymes and transforms phosphoribosyl pyrophosphate (PRPP) into inosine 5′-monophosphate (IMP). IMP is subsequently converted into adenosine 5′-monophosphate (AMP) or guanine 5′-monophosphate (GMP) via two branched paths (Fig. 1H). Among these enzymes, only the activity of PRPP amidotransferase (PPAT) (EC 2.4.2.14) is regulated by purine nucleotides [4]. Therefore, PPAT is presumed to be the rate-limiting enzyme in the pathway and plays an important role in the regulation of *de novo* purine synthesis. PPAT catalyzes the first step of the *de novo* pathway, namely, the transfer of amino groups from glutamine onto PRPP to produce 5-phosphoribosylamine (PRA) (eq. 1). This reaction is further divided into two half-reactions: the deprivation of ammonia (NH_3) from glutamine (eq. 2) and the ligation of NH_3 with PRPP (eq. 3). PPAT is a bifunctional enzyme with two catalytic domains: an N-terminal glutamine amidotransferase (GATase) domain that catalyzes the former reaction and a C-terminal phosphoribosyltransferase (PRTase) domain that catalyzes the latter reaction.

PRPP + glutamine + H$_2$O → PRA + glutamate + PP$_i$ (eq. 1)

glutamine + H$_2$O → NH$_3$ + glutamate (eq. 2)

PRPP + NH$_3$ → PRA + PP$_i$ (eq. 3)

The enzymatic properties and catalytic mechanisms of PPAT have been extensively investigated for decades mainly using enzymes purified from *Escherichia coli* [5] and *Bacillus subtilis* [6]. One of the important features of this enzyme is feedback inhibition by the end-products of the *de novo* pathway, such as AMP and GMP [5-7]. The feedback inhibition of PPAT has been implicated in the suppression of *de novo* purine synthesis in the presence of extracellular salvageable purine bases (and its activation in purine starvation) in mammalian culture cells [7]. Moreover, in the budding yeast *Saccharomyces cerevisiae* and filamentous fungus *Ashbya gossypii*, the expression of PPAT is repressed by the presence of extracellular purine bases [8, 9].

Although the feedback inhibition of PPAT by purine nucleotides appears to play a pivotal role in the regulation of purine synthesis, millimolar levels of AMP or GMP are required for the inhibition of PPAT activity[5, 7, 9]. Intracellular concentrations of AMP and GMP are estimated to be 40–80 and 10–20 µM, respectively, in yeast or mammalian cells under nutrient-rich conditions[10]. The 10- to 100-fold difference between physiological and inhibitory concentrations of AMP and GMP raises the question whether the feedback inhibition of PPAT is directly relevant to the regulation of purine synthesis *in vivo*. Other layers of regulation of PPAT activity, such as post-translational modifications and intracellular signaling, may be involved; however, this has not yet been investigated.

A growing body of evidence reveals that many metabolic enzymes form condensates in cells in response to external stimuli in order to regulate cellular metabolism [11]. For example, the pyruvate kinase Cdc19 in budding yeast reversibly aggregates into large foci upon glucose starvation and heat shock [12, 13]. The assembly of foci protects Cdc19 from stress-induced degradation and inactivates the enzyme, both of which are important for the efficient formation of stress granules and the regrowth of cells after stress relief. Cytidine triphosphate synthase (CTPS) forms filamentous structures in bacteria, yeast, human, and fly germline cells [14-17]. Bacterial CTPS polymerizes into filaments corresponding to the inactive state of the enzyme, while the polymerization of human CTPS into filaments activates the enzyme [18, 19]. PPAT also reversibly localizes to cytoplasmic condensates upon the depletion of extracellular purine bases in budding yeast and HeLa cells [20, 21]. In the case of HeLa cells, PPAT colocalizes with five other enzymes involved in *de novo* purine synthesis in condensates. This multi-enzyme cellular complex is called a purinosome. The molecular basis that regulates the assembly of these PPAT condensates and the physiological importance of the condensates remain unclear.

We herein demonstrated that PPAT formed dynamic intracellular granules through phase separation in budding yeast cells in the absence of external purine bases. Active ribosome synthesis promoted by target of rapamycin complex 1 (TORC1) is essential for the molecular crowding of the cytoplasm harnessed to drive phase transition. A biochemical analysis demonstrated that PPAT by itself was sufficient for the assembly of granules, and purine nucleotides at submillimolar concentrations may suppress their assembly. Furthermore, we found that the formation of PPAT granules was important for *de novo* purine synthesis to promote cell proliferation under purine-depleted conditions. Consistent with this result, a computer simulation supported the possible condensation-dependent activation of the enzyme. Collectively, the present results suggest that the condensation of PPAT by phase transition is a mechanism that quickly regulates purine synthesis in response to both TORC1 activity and the concentrations of purine nucleotides.

Results

The yeast phosphoribosylpyrophosphate amidotransferase Ade4 concentrates in fine granules

We visualized the localization of the Ade4 protein, the yeast PPAT, by C-terminally tagging the chromosomal *ADE4* gene with a single yeast codon-optimized GFP (hereafter referred to as GFP). As demonstrated in previous studies [20, 22], Ade4-GFP showed a large focus in cells transferred into medium lacking purine bases, but not in the presence of purine bases (Figs. 1A and 1S1A). However, Ade4-mGFP, Ade4 tagged with a GFP bearing the monomeric mutation A206K[23], appeared as numerous fine dim dots under the same conditions. Ade4-monomeric NeonGreen (mNG) also formed fine granules, but with markedly higher fluorescence intensities (Fig. 1A-B). Moreover, Ade4 tagged with the monomeric red fluorescent protein mKate2 (Ade4-mKate2) showed similar granule localization (Fig. 1S1B). Therefore, we concluded that Ade4-GFP concentrated into 1-2 foci through the dimerization of the GFP moiety. In subsequent experiments, we mainly examined the granules of Ade4-mNG to elucidate the molecular mechanisms regulating the condensation of Ade4 molecules. In some cases, we examined the assembly of Ade4-GFP foci because they basically have the same properties as Ade4-mNG granules and are easily detectable under a microscope because they are brighter than granules and, thus, are suitable for high-content imaging. A close examination of Ade4-mNG granules revealed that they were highly motile and localized throughout the cytoplasm (Fig. 1C). Moreover, time-lapse imaging revealed that the granules were a dynamic structure: they assembled within 40 min after the depletion of an extracellular purine base (adenine) (Figs. 1D, F and 1S1C, and Movies S1-S3) and disassembled within 10 min after supplementation with adenine (Figs. 1E, G and 1S1D, and Movies S4-S6).

Ade4 granules have liquid-like properties

The reversible assembly and dynamic nature of Ade4-mNG granules prompted us to speculate that they are liquid-like assemblies rather than static, solid-like aggregates. Therefore, we treated cells having Ade4-mNG with the aliphatic alcohol 1,6-hexanediol, which is known to dissolve a range of intracellular membrane-less structures [24]. The permeabilization of yeast cells by digitonin did not affect the assembly of Ade4-mNG granules, whereas the granules significantly disassembled within 10 min when exposed to 10% 1,6-hexanediol plus digitonin, revealing their liquid-like properties (Fig. 2A-B).

Previous findings suggested the importance of an intrinsically disordered region (IDR) in

the formation of intracellular membrane-less structures[25]. Therefore, we examined the
amino acid sequences of Ade4 and other PPATs using the DISOPRED algorithm[26], which
predicts IDR using a neural network-based method. We identified an IDR at the C
terminus of Ade4 (Fig. 2C). The C-terminal IDR was highly conserved in Ade4
homologues of Ascomycota and whole fungi (Fig. 2S1). It was also conserved in the
PPATs of species in other kingdoms, particularly those in bacteria and mammals (Fig.
2S2). On the other hand, the amino acid sequences of IDRs vary across species, even in
yeasts (Fig. 2S3). A mNG-tagged Ade4 mutant lacking the IDR, Ade4ΔIDR-mNG, was
incapable of forming granule-like structures, suggesting that the IDR is required for the
assembly of Ade4 granules (Fig. 2D). Taken together, these results suggest that Ade4
molecules condensate into fine motile granules through IDR-dependent phase separation.
170
Formation of Ade4 granules requires active TORC1 and ribosome biogenesis
We examined the factors required for phase separation, which drives the assembly of
Ade4 granules. During the course of this study, TORC1 has been suggested to control
phase separation in the cytoplasm of yeast and mammalian cells by tuning ribosome
crowding[27]. Therefore, we investigated whether TORC1 plays a role in the formation of
Ade4 granules. In cells, rapamycin has been shown to bind to FKBP12, and this complex
potently inhibited TORC1[28]. The addition of rapamycin suppressed the assembly of
Ade4-GFP foci in wild-type cells, while rapamycin did not affect the foci in cells lacking
Fpr1 (budding yeast FKBP1) (Fig. 3A). Rapamycin also significantly suppressed the
assembly of Ade4-mNG granules in wild-type cells (Fig. 3B-C). These results suggested
that the activity of TORC1 was necessary for the assembly of Ade4 granules. To further
identify the TORC1-related factors responsible for Ade4 granule formation, we observed
Ade4-GFP foci in 37 single-gene deletion mutants, each of which lacked one of the 17
upstream regulators, the 17 downstream effectors of TORC1 signaling, or 3 genes of
interest (Fig. 3S1A-B). Consistent with the inhibitory effects of rapamycin, the assembly
of Ade4-GFP foci was liable to be attenuated in mutants of the upstream activators of
TORC1 signaling (such as the Pib2 or EGO complex). Most of the downstream effectors
of TORC1 signaling appeared to have no or only a negligible effect on Ade4-GFP foci.
However, the assembly of the foci and granules of Ade4-mNG was significantly
suppressed in *sfp1Δ* and *sit4Δ* cells (Figs. 3D-E and 3S1A-B).
191
Sfp1 and Sit4 have both been implicated in cytoplasmic crowding through ribosome
biogenesis [27, 29, 30]. Therefore, we examined the importance of ribosome synthesis on Ade4
granule formation. Diazaborine has been shown to specifically inhibit the maturation of

the 60S ribosomal subunit and reduce the level of the 60S subunit in yeast cells [31]. The

treatment of cells with diazaborine strongly suppressed the formation of Ade4-GFP foci

and Ade4-mNG granules, suggesting that the active synthesis of the ribosomal 60S

subunit is required for the assembly of these condensates (Figs. 3F and 3S1C-D). Taken

together, macromolecular crowding of the cytoplasm by TORC1-mediated ribosome

synthesis may be the main driving force of phase separation required for the assembly of

Ade4 granules.

Intracellular derivatives of purine nucleotides prevent the formation of Ade4

condensates

Although phase separation driven by macromolecular crowding in the cytoplasm was

necessary for the assembly of Ade4 granules, it was only one of the prerequisites. Another

layer of regulation is required to enable cells to suppress the assembly of granules in the

presence of extracellular purine bases. Purine bases are actively transported into cells by

the purine-cytosine permease Fcy2[32] and converted to various metabolites (Fig. 4A).

Therefore, we investigated the metabolites responsible for the suppression of Ade4

granule formation. Foci were dissolved in wild-type cells with Ade4-GFP foci treated

with medium containing adenine or hypoxanthine (Fig. 4B-E, wt). In *aah1Δapt1Δ* cells,

in which incorporated adenine was not further metabolized, the addition of hypoxanthine,

but not adenine, dissolved Ade4 foci (Fig. 4B-C). These results suggested that

intracellular adenine itself did not affect Ade4 granules. In *hpt1Δ* cells in which

incorporated hypoxanthine was not further metabolized, the addition of hypoxanthine did

not disassemble Ade4 foci, whereas the addition of adenine slightly dissolved the foci

(Fig. 4B, D). This result indicated that intracellular hypoxanthine itself did not affect

Ade4 granules, and also that intracellular adenine was efficiently converted to

hypoxanthine. In *ade12Δ* cells in which IMP was not converted to AMP via

adenylosuccinate (S-AMP), the addition of hypoxanthine did not disassemble Ade4 foci,

whereas that of adenine moderately dissolved the foci (Fig. 4B, E). The inhibition of

Ade12 by alanosine, a specific inhibitor of adenylosuccinate synthetase [33], gave similar

results: in alanosine-treated cells, the addition of hypoxanthine partially dissolved Ade4-

GFP foci (Fig. 4S1A-C). Based on these results, we concluded that adenine nucleotides,

including S-AMP derived from external purine bases, were responsible for inhibiting the

assembly of Ade4 condensates in the presence of extracellular purine bases.

Interaction of purine nucleotides with Ade4 is implicated in granule assembly

We initially suspected that Ade4 itself was the site of action of the inhibitory adenine

nucleotide suppressing its granule assembly because PPATs have been shown to directly
bind to purine nucleotides, particularly AMP and GMP [1, 6, 34]. Interactions with these
purine nucleotides down-regulate PPATs (see the Introduction). A structural analysis of *B.*
subtilis PPAT proposed the involvement of K316 and R318 in interactions with adenine
nucleotides (Fig. 5A), and the replacement of these residues with glutamine reduced
affinity for AMP or GMP[35] [34]. Furthermore, D293 has been suggested to interact with
purine nucleotides, and the replacement of the residue with valine reduced affinity for
purine nucleotides[9]. Structural data showed that these three amino acids formed hydrogen
bonds with ADP or GMP (Fig. 5A). These three amino acid residues, and their adjacent
amino acid sequences, were well conserved among the PPATs of various species,
including budding yeast (Fig. 5B). Therefore, we introduced these corresponding
mutations into Ade4 (D310V, K333Q, and R335Q, hereafter Ade4-VQQ) and assessed
whether the interaction with purine nucleotides affected the assembly of Ade4 granules.
In comparisons with the granules of Ade4-mNG, those of Ade4-VQQ-mNG were
detected in a higher percentage of cells (Fig. 5C, D) and had a higher fluorescence
intensity (Fig. 5E). These results suggested that the mutant Ade4 was more liable to
assemble and condense into granules. In other words, the binding of purine nucleotides
to Ade4 may play an inhibitory role in granule assembly.
249
Ade4 protein condensates into particles under molecular crowding conditions *in*
vitro
Regarding the molecular mechanisms regulating the assembly of Ade4 granules, two
mutually exclusive possibilities appeared. The first possibility was that Ade4 itself was
sufficient for granule assembly, and the second was that other cellular components
(including proteins, lipids, and organelles) were required to assemble Ade4 into granules.
To distinguish the two possibilities, we investigated the properties of the pure Ade4
protein *in vitro*. A previous study reported that the expression of Ade4 in the active form
was negligible in bacteria [36]. Therefore, we purified Ade4-mNG from budding yeast cells
(Fig. 6S1A). Using data derived from twenty quantitative proteomic studies, intracellular
concentrations of Ade4 were estimated to be approximately 0.5 µM (Fig. 6S2). In
subsequent *in vitro* assays, we selected the concentrations of Ade4-mNG that were close
to or slightly lower than the estimated concentration. Under physiological ionic
conditions (0.15 M NaCl, 4 mM $MgCl_2$, pH 7.5), purified Ade4-mNG homogenously
dispersed, showing no aggregates ("0%" in Fig. 6A). We then tested the effects of
macromolecular crowding using polyethylene glycol (PEG) as a crowding agent. The
addition of PEG8000 induced the formation of sub-micron-sized particles of Ade4-mNG

in a concentration-dependent manner; a large amount of particles formed in >10% PEG
(Fig. 6A-C). The aggregation of the pure mNG protein was not induced by 10% PEG (Fig.
6S1B). We also investigated the effects of PEG sizes and found that Ade4-mNG
assembled into particles in the presence of >4 kDa PEG (Fig. 6S1C). Consistent with this
size dependency, other crowding agents, 2% lysozyme (MW: 14 kDa) and Ficoll-400
(MW: 400 kDa) induced the assembly of Ade4-mNG particles (Fig. 6S1F). These results
clearly suggested that Ade4 by itself was capable of assembling into sub-micron particles
under macromolecular crowding conditions.
275
We then examined the role of phase separation in the self-assembly of Ade4-mNG *in vitro*.
In the presence of 1, 6-hexanediol, the mean fluorescence intensity and total mass of
Ade4-mNG particles were both significantly reduced (Fig. 6D-F). Similarly, Ade4ΔIDR-
mNG self-assembled into particles less effectively than Ade4-mNG (Fig. 6G-I).
Therefore, we concluded that Ade4 molecules partially aggregated independently of the
IDR moiety, and phase separation was important for the full assembly of Ade4 particles
in vitro.
283

Purine nucleotides attenuate the assembly of Ade4 particles *in vitro*

As demonstrated above, purine nucleotides were expected to suppress the aggregation of
Ade4 in yeast cells (Figs. 4 and 5). Therefore, we investigated whether purine nucleotides
exerted some effects on the *in vitro* assembly of Ade4-mNG particles. In the presence of
GMP, AMP, ADP, and ATP, the condensation of Ade4-mNG was significantly reduced
(Fig. 7A and Fig. 7S1C). AMP, ADP, and ATP were more effective than GMP, while S-
AMP unexpectedly had no significant effect. Adenine and inosine, a degradation product
of IMP, did not affect the *in vitro* condensation of Ade4 (Fig. 7S1A-B), which is consistent
with *in vivo* results (Fig. 4). More than 250 μM AMP, ADP, or ATP was required to
achieve maximal inhibitory effects, whereas the inhibitory effects of GMP plateaued at
100 μM (Fig. 7B-D and Fig. 7S1D). Importantly, AMP also inhibited the condensation of
Ade4ΔIDR-mNG as well as that of Ade4-mNG (Fig. 7E). These results suggested that
purine nucleotides, including AMP, ADP, ATP, and GMP, repressed the condensation of
Ade4 molecules by inhibiting IDR-independent aggregation.
298

Formation of Ade4 granules is important for efficient *de novo* purine synthesis during cell growth

We examined the physiological significance of Ade4 granules. On solid medium in the
absence of adenine, the growth of cells expressing Ade4ΔIDR-3HA, which failed to

assemble into granules, was markedly slowly than those expressing Ade4-3HA (Fig. 8A).

The protein level of Ade4ΔIDR-3HA in cells grown in adenine-free medium was similar

to that of Ade4-3HA (Fig. 8B). Supplementation with adenine restored the growth of the

ADE4ΔIDR mutant to the wild-type rate, indicating that the slow growth of the mutant

was due to the limited activity of the enzyme and not to dominant-negative inhibition

caused by the mutant protein. We also noted that the phenotype of the *ADE4ΔIDR* mutant

depended on the nitrogen source of the medium: the mutant grew more slowly as

concentrations of ammonium ions in the medium decreased (Fig. 8A). Therefore, we

quantitatively examined cell growth by measuring growth curves in liquid medium. The

ADE4ΔIDR mutant showed significantly slower growth, particularly in the absence of

ammonium ions, whereas the growth rate of the wild-type strain was almost independent

of ammonium ion concentrations in the medium (Fig. 8C-D and Fig. 8S1A-B). These

results suggested that the formation of Ade4 granules was important for enhancing

enzymatic activities in cells to support efficient cell growth, particularly in a low-

ammonium environment.

Biochemical analyses previously revealed that PPAT directly utilized NH_3 as a nitrogen

donor in place of glutamine to produce PRA[5]. Glutamine-dependent PRA synthesis (Gln-

PRA activity) (Fig. 8S1C) and NH_3-dependent PRA synthesis (NH_3-PRA activity) (Fig.

8S1C) have both been implicated in the *de novo* biosynthesis of purine nucleotides *in vivo*

[37] [38]. Moreover, biochemical assays revealed that NH_3-PRA activity depended on the

concentrations of NH_4Cl, a fraction of which was in the form of NH_3, whereas Gln-PRA

activity was inhibited by NH_3 [5]. Therefore, Gln-PRA activity and NH_3-PRA activity may

be activated in cells under low and high NH_3 (ammonium) conditions, respectively (Fig.

8S1C-D). We speculated that the slow ammonium concentration-dependent growth of the

granule-hypoplasia *ADE4ΔIDR* mutant was due to the inefficient enhancement of the

Gln-PRA activity of Ade4.

MD calculations predict the possible leakage of glutamine-derived NH_3 from PPAT molecules

In the process of PRA synthesis from glutamine, NH_3 is drawn from glutamine at the first

glutamine amidotransferase (GATase) site and then needs to be efficiently transferred to

PRPP loaded on the second phosphoribosyltransferase (PRTase) site to prevent the release

of intermediate NH_3 into bulk solution. A structural analysis of *E. coli* PPAT suggested

that the binding of PRPP activated PPAT and resulted in the formation of a hydrophobic

channel connecting the GATase and PRTase sites [39]. Therefore, the channel was expected

to permit the efficient intramolecular transfer of the intermediate NH_3 between the two

catalytic sites (Figs. 9F, H and 9S1A). However, we hypothesized that the extreme

condensation of granules enabled the intermolecular transfer of the intermediate NH_3

between Ade4 molecules and, hence, promoted Gln-PRA activity. Therefore, we

investigated whether glutamine-derived NH_3 escaped from the initial site through paths

other than the intramolecular channel by examining the behavior of NH_3 on the PPAT

molecule using a molecular dynamics (MD) simulation. The calculation of MD was based

on the crystal structure of *E. coli* PPAT in the active form (PDB: 1ECC) [39].

We performed MD simulations for the holo condition of PPAT that contained glutamic

acid, NH_3, PRPP, and Mg^{2+}. During ten individual trials, NH_3 was not released from PPAT

and remained in the initial or adjacent positions (Fig. 9A). NH_3 did not reach the catalytic

site. We then performed MD simulations for the semi-holo condition that contained only

glutamic acid and NH_3. NH_3 was released through the hydrophobic channel once in the

ten trials. NH_3 was released through a transient channel that was directed to Arg333,

which was conserved in Ade4, hereinafter referred to as the front channel, four times in

ten trials (Fig. 9B, D). Prior to the release of NH_3, a hydrophobic side chain of Phe260

was flipped from the initial structure. Furthermore, Arg333 functioned as a gate for NH_3

and water by forming hydrogen bonds with the backbone oxygen of Tyr259, Phe260,

His26, and the phosphate group of PRPP (Fig. 9D, E). In the semi-holo condition, the

lack of PRPP opened the Arg gate. To confirm the existence of the Arg gate, additional

MD simulations were performed on the Arg333Ala mutant under the holo condition. As

expected, the flipping of the Phe260 side chain and NH_3 release were both observed six

times in ten trials even though PRPP was in the catalytic site (Fig. 9C).

These results suggested that after the production of NH_3 from glutamine, the attack of

external NH_3 released PRA, which opened the Arg gate, making the front channel, and

internal NH_3 then escaped from the channel (Fig. 9G and 9S1C).

Discussion

We herein demonstrated that the budding yeast PPAT Ade4 formed intracellular fine granules in purine-depleted media. These granules were highly dynamic and possessed liquid-like properties, and phase separation was involved in granule assembly. We also showed that phase separation was driven by the molecular crowding of the cytoplasm induced by active TORC1 and its downstream ribosome biogenesis. Purified Ade4 self-assembled into sub-micron-sized particles, and sub-millimolar purine nucleotides inhibited the formation of particles, as indicated by the genetic analysis. Cells unable to form Ade4 granules showed slower growth, suggesting the importance of the granules for the promotion of *de novo* purine synthesis. The MD simulation implied the condensation-dependent activation of PPAT by enabling the intermolecular channeling of the intermediate NH_3. These results indicate that the formation of Ade4 granules is a biologically relevant mechanism that regulates purine synthesis in response to both TORC1 activity and purine nucleotide levels.

We visualized intracellular fine granules of the budding yeast Ade4 using the bright, monomeric fluorescent protein mNG. These structures were overlooked in previous studies in which Ade4 was tagged with non-monomeric GFP[20, 22]. The large foci of Ade4 reported in previous studies and granules appeared to share basic properties; however, since the pure Ade4 protein assembles into granule-sized particles, the latter is likely to be the actual structure of Ade4. A close examination of granules revealed their inherently dynamic nature in that they quickly assembled or disassembled in response to environmental purine bases.

A previous study demonstrated that Ade4 played an indirect, but pivotal role in regulating the expression of AMP biosynthesis genes in response to extracellular purine bases [36]. The present results suggest that Ade4 is also crucial for the more rapid, short-term regulation of purine biosynthesis, in addition to its long-term regulation at the transcriptional level.

Assembly mechanism of Ade4 granules

We found that phase separation dependent on both TORC1 activity and the C-terminal IDR of Ade4 was essential for the assembly of Ade4 granules. In addition, we identified Sfp1 and Sit4 as genes downstream of TORC1 that are critically involved in granule assembly. Since both genes are important for ribosome synthesis, the requirement of ribosome synthesis for granule assembly was tested and confirmed. These results are

consistent with the theory that TORC1 regulates the macromolecular crowding of the
cytoplasm through ribosome synthesis and degradation, which, in turn, controls
biomolecular condensation by phase separation [27]. This study is the first experimental
demonstration of a metabolic enzyme assembling into condensates by the TORC1-
regulated phase transition of the cytoplasm in yeast cells (Figure 7F, left panel). Since
TORC1 and PPAT IDRs are both conserved in many species, the condensation of PPAT
by TORC1 may be a common feature in eukaryotic cells.
411
Purified Ade4 was capable of self-assembling into fine particles under molecular
crowding conditions. Therefore, we speculated that Ade4 by itself also assembled into
granules in cells; however, unidentified auxiliary factors may participate in their
assembly. *In vitro* assays also revealed that the condensation of Ade4 comprised IDR-
independent aggregation and IDR-dependent phase separation. The former may be a
prerequisite for the latter (Fig. 7F, right panel). IDR-independent aggregates were small
and appeared to be unstable in living cells and, hence, were undetectable (Fig. 2D).
Phase separation may be required for the stable condensation of aggregates into
granules. There was a threshold size and concentration of PEG required to induce the
formation of Ade4 particles *in vitro*, which suggested that the assembly of Ade4
granules is only allowed within a narrow range of ribosome concentrations *in vivo*.
423
The genetic analysis indicated an inhibitory role for purine nucleotides in the assembly
of Ade4 granules, and purine nucleotides suppressed the IDR-independent aggregation
of Ade4 *in vitro*. Therefore, we speculated that the intracellular purine nucleotide
concentration, which fluctuates in response to extracellular purine bases, controlled the
assembly of Ade4 granules as another regulatory layer of PPAT activity (Fig. 7F, right
panel). Human PPAT has been shown to form an inactive tetramer in complex with
AMP, but an active dimer in the apo form. [7, 40]. Moreover, human PPAT bearing a
mutation that corresponds to K333Q, one of the VQQ mutations, predominantly formed
active dimers, even in the presence of AMP[7]. Therefore, interactions with purine
nucleotides are closely associated with the transition between the dimer and tetramer in
eukaryotic PPAT. The present results further suggest that dimers (depicted by hexagons
on the right in Fig. 7F), but not tetramers (depicted by circles), are prone to aggregate
under molecular crowding conditions. This model indicates that the affinity of each
purine nucleotide for Ade4 affects the strength of inhibition. Satyanarayana and Kaplan
reported that AMP, ADP, and ATP inhibited the enzymatic activity of partially purified
Ade4 more strongly than GMP [41], which is consistent with the present results.

Biological significance of Ade4 granules

Biological significance of Ade4 granules

The condensation of Ade4 into granules is of physiological significance because cells

devoid of granules show slower growth as environmental NH_3 levels decrease. This

phenotype is suppressed in the presence of purine bases, in which purine synthesis

through the salvage pathway is available. This suggests that condensation activates the

rate-limiting enzyme Ade4 and, thus, is important for the activation of *de novo* purine

synthesis. Under low ammonium conditions, it is more important to use glutamine as a

substrate. We hypothesized that condensation promotes the Gln-PRA activity of Ade4.

The MD simulation revealed a previously unidentified path through which glutamine-

derived intermediate NH_3 leaked out of the PPAT molecule (Figs. 9G and 9S1C). By its

nature, the NH_3 molecule rapidly diffuses throughout the cytoplasm. However, in Ade4

granules, in which the local concentration of the enzyme is very high, another enzyme

was more likely to trap NH_3 and process it by the PRTase domain (Figs. 9H and 9S1D).

In other words, since the transfer efficiency of the intermediate NH_3 is not necessarily

perfect, the condensation of Ade4 with two catalytic domains is equivalent to the co-

clustering of two enzymes having GATase and PRTase, respectively, in the microspace.

This type of activation by intermolecular NH_3 channeling corresponds to the clustering-

mediated metabolic channeling (of intermediates) demonstrated theoretically and

experimentally by Castellana et al [42].

The activity of PPAT is known to be regulated in a feedback manner by millimolar

levels of AMP and GMP *in vivo* [43]. However, since intracellular purine mononucleotide

concentrations are generally in the micromolar range of tens to hundreds, this regulation

does not appear to be physiologically relevant. The present study elucidated a

mechanism that regulates intracellular PPAT activity in a more acute, sensitive, and

physiologically valid manner.

TORC1 is activated by sensing nutrients, such as amino acids, glucose, and ATP [44].

Therefore, the requirement of TORC1 activity for PPAT condensation ensures that the

de novo synthesis of purines is only up-regulated under conditions that are favorable for

cell growth. A previous study also reported that mammalian TORC1 transcriptionally

up-regulated enzymes involved in the mitochondrial tetrahydrofolate cycle, thereby

increasing *de novo* purine synthesis [45]. Correlating the metabolic flux of purine

synthesis with the nutritional status via TORC1 activity appears to be a conserved and

elaborate strategy for cell proliferation.

Recent studies showed that many metabolic enzymes formed intracellular condensates by phase separation, allowing cell metabolism to adapt to changing growth conditions. [11]. These condensates were mostly a temporary store of inactive enzymes to survive adverse environmental conditions and, in a few cases, an activated form of enzymes. As already described in the Introduction, human CTPS polymerizes into filaments with an active conformation[18]. Mammalian acetyl-CoA carboxylate 1 (ACC1) has also been shown to form filamentous structures *in vivo* and *in vitro* for decades. A recent study revealed that filament formation in the presence of citrate locked human ACC1 into an active conformation[46]. The present results provide another example where the condensation of a metabolic enzyme leads to its activation. Ade4 condensation is unique in that it forms highly dynamic structures and activates its enzymatic activity through the cluster-mediated channeling of intermediates. Although Ade4 does not appear to polymerize into filaments, future research is awaited to elucidate the molecular organization within granules. A more detailed understanding of the architecture of PPAT condensates will provide insights into the mechanism of condensation. Based on that information, PPAT condensation has potential as a therapeutic target for diseases associated with abnormal purine metabolism, such as cancer and gout.

Materials and Methods

Yeast strains and plasmids

The yeast strains, plasmids, and primers used in the present study are listed in Supplementary Tables S1, S2, and S3 respectively. These strains were constructed by a PCR-based method [47] and genetic crosses. The yeast knockout strain collection was originally purchased from GE Healthcare (cat. # YSC1053).

Regarding the construction of plasmids used for the C-terminal tagging of monomeric GFP or mKate2, DNA fragments encoding the corresponding fluorescent proteins were artificially synthesized (Eurofin Genomics, Ebersberg, Germany). These fragments were replaced with GFP of pYM25[47] using *Sal*I/*Bgl*II sites to yield MTP3114 and MTP3131, respectively. The plasmid used for the C-terminal tagging of mNG-Strep tag II (MTP3139) was constructed in the same manner: a DNA fragment encoding single mNG fused with the Strep II tag (WSHPQFEK) by a spacer sequence (ELYKGSA) was synthesized and then replaced with the GFP of pYM25.

Media and cell culture

Synthetic medium optimized for the growth of the BY strain was prepared as described by Hanscho et al. [48]. Growth assays in solid and liquid media used optimized synthetic media lacking glutamate, phenylalanine, serine, and threonine. Yeast nitrogen base without amino acids, but with ammonium sulfate (cat. # Q30009) was purchased from Invitrogen. Yeast nitrogen base without amino acids and ammonium sulfate (cat. # CYN0501) was purchased from FORMEDIUM. Rich medium was standard YPD medium composed of 1% (w/v) yeast extract (BD, cat. # 212750), 2% (w/v) bacto-peptone (BD, cat. # 211677), and 2% (w/v) D-glucose. Glucose (cat. # 049-31165), adenine sulfate (cat. # 018-10613), hypoxanthine (cat. # 086-03403), 1, 6-hexanediol (cat. # 087-00432), and digitonin (cat. # 043-2137) were purchased from FUJIFILM Wako. Cells were grown to the mid-log phase at 30°C before imaging unless otherwise noted. To replace medium containing adenine with medium lacking adenine, cells were collected in a microtube by brief centrifugation and suspended in medium lacking adenine. This washing procedure was repeated at least twice. Rapamycin was purchased from LC Laboratories (cat. # R-5000).

Microscopy

Cells expressing a GFP-fusion protein were concentrated by centrifugation and sandwiched between a slide and coverslip (No. 1.5 thickness, Matsunami, Osaka,

Japan). Immobilized cells were imaged using an inverted fluorescent microscope

(Eclipse Ti2-E, Nikon, Tokyo, Japan) equipped with a CFI Plan Apoλ 100× Oil

DIC/NA1.45 objective lens and CMOS image sensor (DS-Qi2, Nikon). The fluorescent

signal was collected from stacks of 11 z-sections spaced by 0.5 μm, and the maximum

projections of the optical sections were shown unless otherwise noted. A CFI Plan Apoλ

60× Oil Ph3 DM/NA1.40 or CFI Plan Apoλ 100× Oil Ph3 DM/NA1.45 objective lens

was used for phase contrast imaging. Images of cells were acquired from several fields

of view for each experimental condition. All digital images were processed with the Fiji

image platform[49].

Quantitative analysis of intracellular fluorescent condensation

Intracellular fluorescent condensation (*i.e.*, Ade4-GFP foci or Ade4-mNG granules) was

automatically detected and analyzed by running Fiji macros

(https://github.com/masaktakaine/FP-foci-quantification and

https://github.com/masaktakaine/FP-granules-quantification). Briefly, these macro

extract cell outlines from phase-contrast images and detect fluorescent condensation

inside the cell boundary using the *FindMaxima* function of Fiji software.

To monitor temporal changes in the number of intracellular Ade4-mNG granules by

time-lapse imaging, the number of maxima of green fluorescence intensity in the field

was counted in each time frame and divided by the total cell number by running a Fiji

macro (https://github.com/masaktakaine/Counting-FP-granules-in-time-lapse).

In cases in which phase-contrast images were unavailable, we manually examined

condensation.

Spot growth assay

Cells were grown to the mid-log phase at 30°C in synthetic medium, and cell density

(cells/ml) was measured by an automated cell counter (TC20, Bio-Rad). The culture

was diluted by medium lacking nitrogen and carbon sources to a density of $1×10^6$

cells/ml, serially diluted 5-fold, and spotted on plates. Plates were incubated for 2-3

days at 30°C and then imaged by an image scanner (GT-X830, EPSON).

Measurement of growth curves in a liquid culture

Cell growth in liquid media was monitored in a 96-well flat-bottomed plate using a

microplate reader (iMark, Bio-Rad). Cells were grown to the mid-log phase and then

diluted to OD_{600} = 0.1. Each well was filled with 300 µl of the cell dilution and OD_{595}

was measured at 30°C every 10 min.

Immunoblot analysis

A denatured whole-cell extract was prepared according to the method reported by von der

Haar (2007) [50]. Briefly, mid-log cells were harvested from 2–3 ml of the culture by

centrifugation. The cell pellet was resuspended in a small volume (<100 µl) of lysis buffer

(0.1 M NaOH, 50 mM EDTA, 2% (w/v) SDS, and 2% (v/v) β-mercaptoethanol) and then

incubated at 90°C for 10 min. The sample was neutralized by the addition of a one-tenth

volume of 1 M acetic acid, incubated at 90°C for 10 min, and mixed with a one-third

volume of 4×Laemmli protein sample buffer (cat. # 1610747, Bio-Rad). Following

centrifugation at 20,000×*g* at 4°C for 10 min, the upper 90% of the supernatant was

collected as the clarified whole-cell extract and stored at -30°C until used. Proteins were

resolved by SDS-PAGE using a pre-cast gel containing 10% (w/v) acrylamide

(SuperSep™ Ace, cat. # 195-14951, FUJIFILM Wako) and then transferred onto a PVDF

membrane (Immobilon-P, Millipore) using a protein transfer system (Trans-Blot Turbo

Transfer System, Bio-Rad). HA-tagged Ade4 proteins were detected using an anti-HA

antibody (mouse monoclonal (12CA5), cat. # GTX16918, Funakoshi) at a 1:1000 dilution.

GFP-tagged proteins were detected using an anti-GFP antibody (mouse monoclonal

(1E10H7), cat. #66002-1-Ig, ProteinTech) at a 1:1000 dilution. GAPDH was detected

using an anti-GAPDH antibody (mouse monoclonal (1E6D9), cat. # 60004-1-Ig,

ProteinTech) at a 1:1000 dilution. PGK1 was detected using an anti-PGK1 antibody

(mouse monoclonal (22C5D8), cat. # ab113687, Abcam) at a 1:1000 dilution. An HRP-

conjugated secondary antibody (from sheep, cat. #NA931V, GE Healthcare) was probed

at a 1:10000 dilution. Immunoreactive bands were luminescent in a chemiluminescent

substrate (Western BLoT Quant HRP substrate, cat. # T7102A, TaKaRa) and imaged

using an image analyzer (LAS4000, GE Healthcare).

Protein purification from yeast cells

Ade4-mNG, Ade4ΔIDR-mNG, and mNG were C-terminally tagged with a single Strep-

tag II, expressed in budding yeast cells, and purified as follows. Yeast cells expressing

the tagged protein were grown in liquid YPD medium supplemented with 0.02 mg/ml

adenine sulfate at 30°C for at least 20 h. The pre-culture was diluted by 100–1000-fold in

100 ml of fresh YPD plus adenine medium, and cells were further grown at 30°C to 4×10^7

cells/ml. Cells were harvested by centrifugation at 2,000×*g* and washed twice with 1 ml

of extraction buffer (EB) (0.15 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 40 mM

Tris-HCl, pH 7.5) supplemented with 1 mM PMSF and cOmplete protease inhibitor
cocktail (EDTA-free) (Roche, cat. # 11836170001). One gram of glass beads with a
diameter of 0.5 mm (YASUI KIKAI, cat. # YGBLA05) was mixed with the cell
suspension, and cells were disrupted by vigorous shaking using a beads beater (YASUI
KIKAI, MB901). The crude cell extract was collected by brief centrifugation, and glass
beads were washed with a small volume of EB (<0.5 ml). The crude extract and washings
were combined and clarified by centrifugation twice at 20,000×g at 4℃ for 10 min. To
block endogenous biotin, the cleared lysate was supplemented with 0.2 mg/ml neutralized
avidin (FUJIFILM Wako, cat. # 015-24231), incubated on ice for 30 min, and clarified
by centrifugation at 20,000×g at 4℃ for 10 min. The supernatant was loaded onto a
handmade open column packed with 0.5 ml of Strep-Tactin Sepharose (IBA GmbH, cat.
2-1201-002). After washing with 10 column volumes of EB, bound proteins were eluted
with 6 column volumes of EB plus 2.5 mM D-desthiobiotin (IBA GmbH, cat. # 2-1000-
025). The protein amounts and compositions of elution fractions were analyzed by SDS-
10% PAGE and stained with FastGene Q-stain (Nippon Genetics, cat. # NE-FG-QS1).
Peak fractions were pooled and concentrated using an Amicon Ultra-0.5 centrifugal filter
device with a molecular weight cut-off of 30 or 10 kDa (Merck, cat. # UFC503024 or
UFC501008). D-desthiobiotin in the peak fraction was diluted more than 100-fold by a
repeating dilution with EB and the centrifugal concentration. The concentration of the
purified protein was assessed by densitometry using bovine serum albumin (TaKaRa, cat.
T9310A) as a standard following SDS-PAGE and staining with the Q-stain. Purified
Ade4-mNG and Ade4ΔIDR-mNG were supplemented with 5 and 10 mM DTT and stored
on ice.
625
In vitro condensation assay
The purified protein was diluted to the indicated concentration in assay buffer (EB plus
5 mM MgCl$_2$) and supplemented with 0–15% (w/v) PEG 8000 (FUJIFILM Wako, cat. #
596-09755). In the experiments shown in Fig. 6S1C, samples were spiked with 10%
(w/v) PEG of the indicated molecular size. The mixture was immediately loaded into an
assay chamber composed of a glass slide and coverslip attached by double-sided tape.
Fluorescent condensation was imaged using the Nikon inverted fluorescent microscope
(see above). Images of fluorescent condensation were analyzed by running a Fiji macro
(https://github.com/masaktakaine/In-vitro-condensates-quantification). Briefly, the
macro automatically detects fluorescent condensation and quantifies its area and
fluorescence intensity.
637

MD simulation

The crystal structure of *E. coli* PPAT (PDB ID: 1ECC) was obtained from the Protein Data Bank and used for all MD simulations [39]. All water molecules and ions were removed before simulations. To build the parameters of ligands (NH_3, PRPP, and glutamic acid), their restrained electrostatic potential charges were calculated based on gas-phase HF/6-31(d) quantum mechanics calculations using Gaussian 16. A set of parameters were generated with a general AMBER force field using the antechamber from AMBER tools [51].

The Amber 14SB force field was employed for all MD simulations [52]. Missing hydrogen atoms were added by LEaP from AMBER tools. Proteins were solvated with the TIP3P water box [53]. The system was neutralized by replacing water with Na^+ or Cl^- ions. The particle-mesh Ewald (PME) method was employed for the Coulomb interaction [54]. Amber-formatted input files were converted for GROMACS using acpype [55]. All MD simulations were performed using GROMACS 2019.6 [56]. Before the production run, three-step equilibrations were performed for each system. Energy minimization was performed until 10,000 steps. This was followed by 100-ps *NVT* equilibration with the V-rescale thermostat [57] at 300 K under 1 atm. A 100-ps *NPT* equilibration was then conducted with Berendsen coupling [58] at 300 K under 1 atm. A run was performed under the *NPT* condition. The time step of each simulation was 2 fs with the SHAKE [59] and SETTLE [60] algorithms, and the snapshot was recorded every 100 ps during the production run. To examine the results of MD simulations, a Python library MDanalysis was used [61].

Data analysis

Numerical data were plotted using R studio software (R ver. 4.2.0) [62]. Means, SDs, and *p* values were calculated using Excel software (Microsoft, WA, US) and the R studio. The significance of differences between two sets of data was tested using the unpaired two-tailed Welch's *t*-test unless otherwise noted and was indicated by an asterisk or *p* value. The horizontal bar in the dot plot indicates the average of each population. All measurements were repeated at least twice to confirm reproducibility.

Acknowledgments

The authors thank the IMCR-JURSC in Gunma University for sharing research

equipment. This work was supported by research grants from the Gout and Uric Acid

Foundation of Japan and by JSPS KAKENHI Grant Number JP22K06216 to M.T. This

study used the computational resources of Cygnus provided by the Multidisciplinary

Cooperative Research Program at the Center for Computational Sciences (Project Code:

MOLBIO), University of Tsukuba.

Author Contributions

MT conceived and supervised the study, MT and RM designed the experiments, MT

performed the experiments, RM performed MD simulations, MT and RM analyzed the

data, MT wrote the manuscript draft with input from RM, and MT and RM revised the

manuscript.

Declaration of Interests

The authors declare no competing interests.

References

687   1.   Yamaoka, T. *et al.* Amidophosphoribosyltransferase Limits the Rate of Cell Growth-linked de
688   Novo Purine Biosynthesis in the Presence of Constant Capacity of Salvage Purine Biosynthesis.
689   *Journal of Biological Chemistry* **272**, 17719-17725 (1997).

690   2.   Daignan-Fornier, B. & Pinson, B. Yeast to Study Human Purine Metabolism Diseases. *Cells* **8**
691   (2019).

692   3.   Pareek, V., Pedley, A.M. & Benkovic, S.J. Human de novo purine biosynthesis. *Crit Rev Biochem*
693   *Mol Biol*, 1-16 (2020).

694   4.   Smith, J.L. *et al.* Structure of the allosteric regulatory enzyme of purine biosynthesis. *Science* **264**,
695   1427-1433 (1994).

696   5.   Messenger, L.J. & Zalkin, H. Glutamine phosphoribosylpyrophosphate amidotransferase from
697   Escherichia coli. Purification and properties. *J Biol Chem* **254**, 3382-3392 (1979).

698   6.   Meyer, E. & Switzer, R.L. Regulation of Bacillus subtilis glutamine phosphoribosylpyrophosphate
699   amidotransferase activity by end products. *J Biol Chem* **254**, 5397-5402 (1979).

700   7.   Yamaoka, T. *et al.* Feedback inhibition of amidophosphoribosyltransferase regulates the rate of
701   cell growth via purine nucleotide, DNA, and protein syntheses. *J Biol Chem* **276**, 21285-21291
702   (2001).

703   8.   Mantsala, P. & Zalkin, H. Glutamine nucleotide sequence of Saccharomyces cerevisiae ADE4
704   encoding phosphoribosylpyrophosphate amidotransferase. *J Biol Chem* **259**, 8478-8484 (1984).

705   9.   Jimenez, A., Santos, M.A., Pompejus, M. & Revuelta, J.L. Metabolic engineering of the purine
706   pathway for riboflavin production in Ashbya gossypii. *Appl Environ Microbiol* **71**, 5743-5751
707   (2005).

708   10.   Park, J.O. *et al.* Metabolite concentrations, fluxes and free energies imply efficient enzyme usage.
709   *Nat Chem Biol* **12**, 482-489 (2016).

710   11.   Prouteau, M. & Loewith, R. Regulation of Cellular Metabolism through Phase Separation of
711   Enzymes. *Biomolecules* **8** (2018).

712   12.   Cereghetti, G. *et al.* Reversible amyloids of pyruvate kinase couple cell metabolism and stress
713   granule disassembly. *Nat Cell Biol* **23**, 1085-1094 (2021).

714   13.   Saad, S. *et al.* Reversible protein aggregation is a protective mechanism to ensure cell cycle restart
715   after stress. *Nature Cell Biology* **19**, 1202 (2017).

716   14.   Noree, C., Monfort, E., Shiau, A.K. & Wilhelm, J.E. Common regulatory control of CTP synthase
717   enzyme activity and filament formation. *Molecular Biology of the Cell* **25**, 2282-2290 (2014).

718   15.   Azzam, G. & Liu, J.L. Only one isoform of Drosophila melanogaster CTP synthase forms the
719   cytoophidium. *PLoS Genet* **9**, e1003256 (2013).

720   16.   Chen, K. *et al.* Glutamine analogs promote cytoophidium assembly in human and Drosophila cells.
721   *J Genet Genomics* **38**, 391-402 (2011).

722   17.   Ingerson-Mahar, M., Briegel, A., Werner, J.N., Jensen, G.J. & Gitai, Z. The metabolic enzyme
723   CTP synthase forms cytoskeletal filaments. *Nat Cell Biol* **12**, 739-746 (2010).

724   18.   Lynch, E.M. *et al.* Human CTP synthase filament structure reveals the active enzyme conformation.
725   *Nat Struct Mol Biol* **24**, 507-514 (2017).

726   19.   Habrian, C. *et al.* Inhibition of Escherichia coli CTP Synthetase by NADH and Other
727   Nicotinamides and Their Mutual Interactions with CTP and GTP. *Biochemistry* **55**, 5554-5565
728   (2016).

729   20.   Narayanaswamy, R. *et al.* Widespread reorganization of metabolic enzymes into reversible
730   assemblies upon nutrient starvation. *Proc Natl Acad Sci U S A* **106**, 10147-10152 (2009).

731   21.   An, S., Kumar, R., Sheets, E.D. & Benkovic, S.J. Reversible compartmentalization of de novo
732   purine biosynthetic complexes in living cells. *Science* **320**, 103-106 (2008).

733   22.   Noree, C. *et al.* A quantitative screen for metabolic enzyme structures reveals patterns of assembly
734   across the yeast metabolic network. *Mol Biol Cell* **30**, 2721-2736 (2019).

735   23.   Zacharias, D.A., Violin, J.D., Newton, A.C. & Tsien, R.Y. Partitioning of lipid-modified
736   monomeric GFPs into membrane microdomains of live cells. *Science* **296**, 913-916 (2002).

737   24.   Kroschwald, S., Maharana, S. & Simon, A. Hexanediol: a chemical probe to investigate the
738   material properties of membrane-less compartments. *Matters* (2017).

739   25.   Uversky, V.N. Intrinsically disordered proteins in overcrowded milieu: Membrane-less organelles,
740   phase separation, and intrinsic disorder. *Curr Opin Struct Biol* **44**, 18-30 (2017).

741   26.   Jones, D.T. & Cozzetto, D. DISOPRED3: precise disordered region predictions with annotated
742   protein-binding activity. *Bioinformatics* **31**, 857-863 (2015).

743   27.   Delarue, M. *et al.* mTORC1 Controls Phase Separation and the Biophysical Properties of the
744   Cytoplasm by Tuning Crowding. *Cell* **174**, 338-349.e320 (2018).

745   28.   Heitman, J., Movva, N.R. & Hall, M.N. Targets for cell cycle arrest by the immunosuppressant
746   rapamycin in yeast. *Science* **253**, 905-909 (1991).

747   29.   Lempiainen, H. *et al.* Sfp1 interaction with TORC1 and Mrs6 reveals feedback regulation on TOR
748   signaling. *Mol Cell* **33**, 704-716 (2009).

749   30.   Fingerman, I., Nagaraj, V., Norris, D. & Vershon, A.K. Sfp1 plays a key role in yeast ribosome
750   biogenesis. *Eukaryot Cell* **2**, 1061-1068 (2003).

751   31.   Pertschy, B. *et al.* Diazaborine Treatment of Yeast Cells Inhibits Maturation of the 60S Ribosomal
752   Subunit. *Molecular and Cellular Biology* **24**, 6476-6487 (2004).

753   32.   Ferreira, T., Brethes, D., Pinson, B., Napias, C. & Chevallier, J. Functional analysis of mutated
754   purine-cytosine permease from Saccharomyces cerevisiae. A possible role of the hydrophilic
755   segment 371-377 in the active carrier conformation. *J Biol Chem* **272**, 9697-9702 (1997).

756   33.   Jayaram, H.N. *et al.* Metabolites of alanosine, an antitumor antibiotic. *Biochem Pharmacol* **28**,
757   3551-3566 (1979).

34.   Zhou, G., Smith, J.L. & Zalkin, H. Binding of purine nucleotides to two regulatory sites results in
synergistic feedback inhibition of glutamine 5-phosphoribosylpyrophosphate amidotransferase. *J*
Biol Chem **269**, 6784-6789 (1994).

35.   Chen, S. *et al.* Mechanism of the synergistic end-product regulation of Bacillus subtilis glutamine
phosphoribosylpyrophosphate amidotransferase by nucleotides. *Biochemistry* **36**, 10718-10726
(1997).

36.   Rebora, K., Desmoucelles, C., Borne, F., Pinson, B. & Daignan-Fornier, B. Yeast AMP pathway
genes respond to adenine through regulated synthesis of a metabolic intermediate. *Mol Cell Biol*
21, 7901-7912 (2001).

37.   Mantsala, P. & Zalkin, H. Glutamine amidotransferase function. Replacement of the active-site
cysteine in glutamine phosphoribosylpyrophosphate amidotransferase by site-directed
mutagenesis. *J Biol Chem* **259**, 14230-14236 (1984).

38.   Mei, B. & Zalkin, H. A cysteine-histidine-aspartate catalytic triad is involved in glutamine amide
transfer function in purF-type glutamine amidotransferases. *Journal of Biological Chemistry* **264**,
16613-16619 (1989).

39.   Krahn, J.M. *et al.* Coupled formation of an amidotransferase interdomain ammonia channel and a
phosphoribosyltransferase active site. *Biochemistry* **36**, 11061-11068 (1997).

40.   Holmes, E.W., McDonald, J.A., McCord, J.M., Wyngaarden, J.B. & Kelley, W.N. Human
glutamine phosphoribosylpyrophosphate amidotransferase. Kinetic and regulatory properties. *J*
Biol Chem **248**, 144-150 (1973).

41.   Satyanarayana, T. & Kaplan, J.G. Regulation of the purine pathway in bakers yeast: activity and
feedback inhibition of phosphoribosyl-pyrophosphate amidotransferase. *Arch Biochem Biophys*
142, 40-47 (1971).

42.   Castellana, M. *et al.* Enzyme clustering accelerates processing of intermediates through metabolic
channeling. *Nature Biotechnology* **32**, 1011-+ (2014).

43.   Rodwell, V.W., Bender, D., Botham, K.M., Kennelly, P.J. & Weil, P.A. *Harpers Illustrated*
Biochemistry 30th Edition. (McGraw Hill LLC, 2015).

44.   Takahara, T., Amemiya, Y., Sugiyama, R., Maki, M. & Shibata, H. Amino acid-dependent control
of mTORC1 signaling: a variety of regulatory modes. *Journal of Biomedical Science* **27**, 87 (2020).

45.   Ben-Sahra, I., Hoxhaj, G., Ricoult, S.J.H., Asara, J.M. & Manning, B.D. mTORC1 induces purine
synthesis through control of the mitochondrial tetrahydrofolate cycle. *Science* **351**, 728-733 (2016).

46.   Hunkeler, M. *et al.* Structural basis for regulation of human acetyl-CoA carboxylase. *Nature* **558**,
470-474 (2018).

47.   Janke, C. *et al.* A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins,
more markers and promoter substitution cassettes. *Yeast* **21**, 947-962 (2004).

48.   Hanscho, M. *et al.* Nutritional requirements of the BY series of Saccharomyces cerevisiae strains

for optimum growth. *FEMS Yeast Res* **12**, 796-808 (2012).

49.   Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**,
676-682 (2012).

50.   von der Haar, T. Optimized protein extraction for quantitative proteomics of yeasts. *PLoS One* **2**,
e1078 (2007).

51.   D.A. Case, H.M.A., K. Belfon, I.Y. Ben-Shalom, J.T. Berryman, S.R. Brozell, D.S. Cerutti, T.E.
Cheatham, III, G.A. Cisneros, V.W.D. Cruzeiro, T.A. Darden, R.E. Duke, G. Giambasu, M.K.
Gilson, H. Gohlke, A.W. Goetz, R. Harris, S. Izadi, S.A. Izmailov, K. Kasavajhala, M.C. Kaymak,
E. King, A. Kovalenko, T. Kurtzman, T.S. Lee, S. LeGrand, P. Li, C. Lin, J. Liu, T. Luchko, R.
Luo, M. Machado, V. Man, M. Manathunga, K.M. Merz, Y. Miao, O. Mikhailovskii, G. Monard,
H. Nguyen, K.A. O'Hearn, A. Onufriev, F. Pan, S. Pantano, R. Qi, A. Rahnamoun, D.R. Roe, A.
Roitberg, C. Sagui, S. Schott-Verdugo, A. Shajan, J. Shen, C.L. Simmerling, N.R. Skrynnikov, J.
Smith, J. Swails, R.C. Walker, J Wang, J. Wang, H. Wei, R.M. Wolf, X. Wu, Y. Xiong, Y. Xue,
D.M. York, S. Zhao, and P.A. Kollman   (University of California, San Francisco.; 2022).

52.   Maier, J.A. *et al.* ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters
from ff99SB. *J Chem Theory Comput* **11**, 3696-3713 (2015).

53.   Kiss, P.T. & Baranyai, A. Sources of the deficiencies in the popular SPC/E and TIP3P models of
water. *The Journal of Chemical Physics* **134**, 054106 (2011).

54.   Pippig, M. & Mercuri, F. Efficient evaluation of Coulomb interactions in kinetic Monte Carlo
simulations of charge transport. *The Journal of Chemical Physics* **152**, 164102 (2020).

55.   Sousa da Silva, A.W. & Vranken, W.F. ACPYPE - AnteChamber PYthon Parser interfacE. *BMC*
Res Notes **5**, 367 (2012).

56.   M.J. Abraham, D.v.d.S., E. Lindahl, B. Hess, and the GROMACS development team   (2019).

57.   Bussi, G., Donadio, D. & Parrinello, M. Canonical sampling through velocity rescaling. *J Chem*
Phys **126**, 014101 (2007).

58.   Berendsen, H.J.C., Postma, J.P.M., van Gunsteren, W.F., DiNola, A. & Haak, J.R. Molecular
dynamics with coupling to an external bath. *The Journal of Chemical Physics* **81**, 3684-3690
(1984).

59.   Ryckaert, J.-P., Ciccotti, G. & Berendsen, H.J.C. Numerical integration of the cartesian equations
of motion of a system with constraints: molecular dynamics of n-alkanes. *Journal of*
Computational Physics **23**, 327-341 (1977).

60.   Miyamoto, S. & Kollman, P.A. Settle: An analytical version of the SHAKE and RATTLE
algorithm for rigid water models. *Journal of Computational Chemistry* **13**, 952-962 (1992).

61.   Michaud-Agrawal, N., Denning, E.J., Woolf, T.B. & Beckstein, O. MDAnalysis: A toolkit for the
analysis of molecular dynamics simulations. *Journal of Computational Chemistry* **32**, 2319-2327
(2011).

62.   R Core Team   (R Foundation for Statistical Computing, Vienna, Austria; 2017).

63.   Chen, S., Burgner, J.W., Krahn, J.M., Smith, J.L. & Zalkin, H. Tryptophan fluorescence monitors

multiple conformational changes required for glutamine phosphoribosylpyrophosphate

amidotransferase interdomain signaling and catalysis. *Biochemistry* **38**, 11659-11669 (1999).

Figure Legends

Figure 1. The yeast PPAT Ade4 concentrates in fine granules

A. Bright field and GFP images of cells expressing Ade4 tagged C-terminally with the indicated green fluorescent protein. Cells were grown in medium containing 0.02 mg/ml adenine, washed with medium lacking adenine, and then incubated in medium without adenine for 30 min before imaging. The intensity of GFP is represented by pseudo-colors in the upper panels.

B. Quantification of the mean fluorescence intensity of Ade4 condensates observed in cells as shown in (A). Data are represented by box and dot plots. Cross marks indicate the population mean. *P* values versus mGFP are shown (Steel's multiple comparison test).

C. A higher resolution image of Ade4-mNG granules. Granules were imaged at an *xy* resolution of 34 pixels/μm and z-spacing of 0.5 μm. Z-positions are represented by a color code (left). A grayscale image of granules (middle) and bright field image of cells (right) are also shown. Scale bar = 5 μm.

D. Time-lapse imaging of the assembly of Ade4-mNG granules by adenine depletion. Cells were grown in medium containing 0.02 mg/ml adenine and washed with medium lacking adenine at t = 0 min. Scale bar = 5 μm.

E. Time-lapse imaging of the disassembly of Ade4-mNG granules by adenine supplementation. Cells were grown in the absence of adenine and 0.02 mg/ml adenine was supplemented at t = 0 min. Scale bar = 5 μm.

F, G. Panels F and G show the quantification of data in (D) and (E), respectively. The number of intracellular Ade4-mNG granules was counted as the number of maxima of green fluorescence intensity per cell. Data are the mean over 10-12 independent fields of view, and approximately 70 cells were examined per field. The shaded area indicates ± 1SD.

H. Schematic of *de novo* purine synthesis

Figure 1S1. Additional data regarding the intracellular localization of Ade4. Related to Figure 1

A. Bright field and GFP images of cells expressing Ade4 tagged C-terminally with the indicated green fluorescent protein. Cells were grown in medium containing 0.02 mg/ml adenine and imaged.

B. Phase and RFP images of cells expressing Ade4 tagged C-terminally with the red fluorescent protein mKate2. Scale bar = 5 μm.

C. A control experiment of time-lapse imaging of the assembly of Ade4-mNG granules by adenine depletion shown in Fig. 1D. Cells were grown in medium containing 0.02

mg/ml adenine and washed with medium containing the same concentration of adenine

at t = 0 min. Scale bar = 5 μm.

D. A control experiment of time-lapse imaging of the disassembly of Ade4-mNG granules

by adenine supplementation shown in Fig. 1E. Cells were grown in the absence of adenine

and sterile water was supplemented in place of adenine at t = 0 min. Scale bar = 5 μm.

Figure 2. Ade4 granules have liquid-like properties

A. The aliphatic alcohol 1,6–hexanediol dissolves Ade4 granules. Cells harboring Ade4-

mNG granules in the absence of purine bases were treated with 2 μg/ml digitonin ± 10%

(w/v) 1,6-hexanediol for 10 min and then imaged. Scale bar = 5 μm.

B. Quantification of data shown in (A). The percentage of cells with Ade4-mNG granules

per field of view (containing 361 ± 159 cells) was plotted. Data were pooled from three

independent experiments. Bars and error bars indicate the mean ± 1SD. *P* values were

calculated using the two-tailed Steel-Dwass multiple comparison test.

C. Domain structure of the budding yeast Ade4. The bottom graph shows disorder

predictions by the DISOPRED algorithm.

D. The C-terminal IDR of Ade4 is required for granule assembly. Cells expressing the

mutant Ade4 lacking the C-terminal IDR tagged with mNG were incubated in the absence

of purine bases for 40 min and imaged.

Figure 2S1. The C-terminal IDR is conserved in PPATs of Ascomycota

Heatmap showing the disorder scores of PPAT amino acid sequences of 81 ascomycota.

Figure 2S2. The IDR is conserved in PPATs of various species

Heatmap showing the disorder scores of PPAT amino acid sequences of 100 organisms,

including 19 ascomycota. Phylogenetic groups are color-coded on the left.

Figure 2S3. Amino acid sequences of IDRs vary across species

A-B. Multiple amino acid sequence alignment of the C-terminal region of PPAT. In (A),

three fungi and 13 representative organisms from the following groups are selected:

archaea, bacteria, protozoa, plants, nematodes, insects, echinoidea, fish, amphibians,

mammals, reptiles, birds, and ascidians. In (B), 13 species are selected from ascomycota.

Figure 3. Formation of Ade4 granules requires active TORC1 and ribosome

biogenesis

A. Wild-type and *fpr1Δ* cells expressing Ade4-GFP were grown in medium containing

adenine and incubated in medium without adenine in the presence of 0.02% (v/v) DMSO
or 0.02% DMSO plus 2 µg/ml rapamycin for 30 min before imaging.

B. Rapamycin suppresses the assembly of Ade4-mNG granules. Wild-type cells
expressing Ade4-mNG were treated with rapamycin and imaged as described in (A).

C. Quantification of data shown in (B). The percentage of cells with Ade4-mNG granules
per field of view (containing 443 ± 138 cells) was plotted. Data were pooled from two
independent experiments. Bars and error bars indicate the mean ± 1SD. P values were
calculated using the two-tailed Mann-Whitney U test.

D. The assembly of Ade4-mNG granules was significantly suppressed in *sfp1Δ* and *sit4Δ*
cells.

E. Quantification of data shown in (C). The percentage of cells with Ade4-mNG granules
per field of view (containing 517 ± 123 cells) was plotted. Data were pooled from three
independent experiments. Bars and error bars indicate the mean ± 1SD. P values versus
wt were calculated using the two-tailed Steel's multiple comparison test.

F. The inhibition of ribosome synthesis suppresses the assembly of Ade4 condensates.
Cells expressing Ade4-GFP were grown in medium containing adenine and then
incubated in medium without adenine in the presence of 0.05% (v/v) DMSO or 0.05%
DMSO plus 5 µg/ml diazaborine for 30 min before imaging. The percentage of cells with
Ade4-GFP foci per field of view (containing 780 ± 214 cells) was plotted. Data were
pooled from two independent experiments. Bars and error bars indicate the mean ± 1SD.
P values were calculated using the two-tailed Mann-Whitney U test. Representative cell
images are shown in Figure 3S1C. Scale bar = 5 µm.

Figure 3S1. Formation of Ade4 granules in TORC1-related mutants. Related to
Figure 3.

A. Assembly of Ade4-GFP foci in single gene deletion mutants of the upstream regulator
and downstream effector of TORC1 signaling. Cells of the indicated genotype expressing
Ade4-GFP were grown in medium containing adenine and then incubated in medium
without adenine for 30 min before imaging.

B. Summary of the percentage of cells showing Ade4-GFP foci in the wild-type and 37
single-gene deletion mutants. The percentage of cells with Ade4-GFP foci per field of
view was plotted. Data were pooled from 2-3 independent experiments. Bars and error
bars indicate the mean ± 1SD.

C. The inhibition of ribosome synthesis suppressed the assembly of Ade4-GFP foci.
Related to Figure 3F.

D. The inhibition of ribosome synthesis suppressed the assembly of Ade4-mNG granules.

Cells expressing Ade4-mNG were grown in medium containing adenine and then

incubated in medium without adenine in the presence of 0.05% (v/v) DMSO or 0.05%

DMSO plus 5 µg/ml diazaborine for 30 min before imaging. Scale bar = 5 µm.

Figure 4. Intracellular derivatives of purine nucleotides prevent the formation of

Ade4 condensates

A. *De novo* and salvage pathways for purine synthesis in budding yeast.

B. Assembly of Ade4-GFP foci in purine metabolism mutants. Cells having Ade4-GFP

foci in the absence of purine bases (mock) were treated with the same medium or medium

containing 20 µg/adenine (+adenine) or 25 µg/ml hypoxanthine (+hypoxanthine) for 20

min and then imaged. Scale bar = 5 µm.

C-E. Quantification of data shown in (B). The percentage of cells with foci per field of

view was quantified and plotted. Bars and error bars indicate the mean ± 1SD. Data were

pooled from 2-3 independent experiments, and more than 16 fields of view were imaged

for each condition. Triple asterisks indicate p values less than 10^{-10} versus the mock

(Steel's multiple comparison test, two-tailed). The single asterisk indicates a p value less

than 0.01 versus the mock. NS, not significant (p value >0.05).

Figure 4S1. Alanosine inhibits the dissolution of Ade4 foci in the presence of

hypoxanthine. Related to Figure 4.

A. Alanosine inhibits the adenylosuccinate synthetase ADE12.

B. Assembly of Ade4-GFP foci in the presence of alanosine. Cells expressing Ade4-GFP

were incubated in medium lacking adenine in the presence of 0 (mock) or 30 µg/ml

alanosine (+alanosine) for 40 min to allow the assembly of Ade4-GFP foci and were then

imaged (SC). Cells were further treated with the same medium supplemented with 24

µg/ml hypoxanthine for 15 min and then imaged again (SC + hyp). Scale bar = 5 µm.

C. Quantification of data shown in (B). The percentage of cells with foci per field of view

(containing 182 ± 46 cells) was quantified and plotted. Data were pooled from two

independent experiments. Bars and error bars indicate the mean ± 1SD. The significance

of differences was tested using the unpaired one-tailed Welch's *t*-test. *P* values versus the

mock are shown.

Figure 5. The interaction of purine nucleotides with Ade4 is implicated in granule

assembly

A. Interaction between ADP and specific amino acid residues in *B. subtilis* PPAT (pdb

file: 1ao0). The dotted line indicates a hydrogen bond.

B. Amino acid residues involved in the interaction with purine nucleotides are well
conserved among species.
C-E. Examination of the Ade4-VQQ mutant. Ade4-mNG and Ade4-VQQ-mNG cells
were incubated in medium without purine bases for 40 min to allow for the assembly of
Ade4 granules and then imaged. The percentage of cells with granules per field of view
(containing 265 ± 80 cells) was quantified and plotted in (C). The number of granules
detected in the cell were averaged over the field of view and plotted in (D). The mean
fluorescence intensity of granules was box plotted in (E). Data were pooled from three
independent experiments. Bars and error bars indicate the mean $\pm$ 1SD. The significance
of differences was tested using the unpaired two-tailed Welch's *t*-test (C) or Mann-
Whitney U test (D, E). *P* values versus wt are shown.
990

Figure 6. The Ade4 protein condensates into particles under molecular crowding
conditions *in vitro*
A. Pure Ade4-mNG condensated into fine particles in a PEG concentration-dependent
manner. Following the supplementation of 0.15 µM Ade4-mNG with PEG8000 at the
indicated concentration (%, w/v), images were taken after a 1-min incubation. Inverted
grayscale images are shown. Scale bar = 5 µm.
B-C. Quantification of data shown in (A). The mean fluorescence intensities of Ade4-
mNG particles were box plotted in (B). Cross marks indicate the population mean. The
integrated fluorescence intensities of particles were summed per field and plotted in (C).
Bars and error bars indicate the mean $\pm$ 1SD. Seven to 10 fields of view were imaged for
each condition and an average of 390 particles per field were examined.
D. 1,6-Hexanediol attenuated the *in vitro* condensation of Ade4-mNG. Following the
supplementation of 0.15 µM Ade4-mNG with 10% PEG8000 in the presence of 0 or 10%
(w/v) 1,6-hexanediol, images were taken after a 1-min incubation. Fluorescence intensity
is represented by pseudo-colors. Scale bar = 5 µm.
E-F. Quantification of data shown in (D). The mean fluorescence intensities of particles
were box plotted in (E). Cross marks indicate the population mean. The integrated
fluorescence intensities of particles were summed per field and plotted in (F). Bars and
error bars indicate the mean $\pm$ 1SD. Ten fields of view were imaged for each condition,
and an average of 1197 particles per field were examined. *P* values were calculated using
the two-tailed Mann-Whitney U test (E) or Welch's *t*-test (F).
G. Ade4ΔIDR-mNG assembled into granules less effectively than Ade4-mNG *in vitro*.
Following the supplementation of 0.2 µM Ade4-mNG or Ade4ΔIDR-mNG with 10%
PEG8000, images were taken after a 1-min incubation. Fluorescence intensity is

represented by pseudo-colors. Scale bar = 5 μm.

H-I. Quantification of data shown in (G). The mean fluorescence intensities of particles

were box plotted in (H). Cross marks indicate the population mean. The integrated

fluorescence intensities of particles were summed per field and plotted in (I). Bars and

error bars indicate the mean ± 1SD. Twenty-eight fields of view were imaged for each

condition, and an average of 1341 particles per field were examined. *P* values were

calculated using the two-tailed Mann-Whitney U test.

1022

Figure 6S1. Purification and basic characterization of the Ade4 protein *in vitro*

A. Purified proteins used in the present study. Proteins were resolved by SDS-PAGE and

stained with FastGene™ Q-stain.

B. The pure mNG protein did not condensate into particles even under molecular

crowding conditions. Following the supplementation of 0.14 μM mNG with 0 or 10%

PEG8000, images were taken after a 1-min incubation. Scale bar = 5 μm.

C. The *in vitro* condensation of Ade4-mNG depends on the size of PEG. Following the

supplementation of 0.15 μM Ade4-mNG with 10% PEG of the indicated average

molecular weight, images were taken after a 1-min incubation. Scale bar = 5 μm.

D-E. Quantification of data shown in (C). The mean fluorescence intensities of Ade4-

mNG particles were box plotted in (D). Cross marks indicate the population mean. The

integrated fluorescence intensities of particles were summed per field and plotted in (E).

Bars and error bars indicate the mean ± 1SD. Seven to 8 fields of view were imaged for

each condition, and an average of 399 particles per field were examined.

F. Condensation of Ade4-mNG into particles with crowding agents other than PEG.

Between 0.13 and 0.14 μM Ade4-mNG was supplemented with the indicated crowding

agents and images were taken after a 1-min incubation. Scale bar = 5 μm.

1040

Figure 6S2. Estimation of the intracellular concentration of Ade4.

Data regarding the protein abundance of the budding yeast Ade4 were downloaded from

the   Saccharomyces   Genome   Database

(https://www.yeastgenome.org/locus/S000004915/protein). Data were derived from

twenty quantitative genome-wide proteomic studies and normalized to a unit of molecules

per cell. These values were converted to micromoles assuming that the average

cytoplasmic volume of the haploid yeast cell is 42 fℓ (Jorgensen et al., 2002). Data were

classified according to the condition of cells (unstressed or stressed), measuring methods

(flow cytometry, quantitative fluorescence microscopy, and immunoblotting), and the

type of medium (rich or synthetic) and then plotted. Bars and error bars indicate the mean

± 1SD.

1052

Figure 7. Purine nucleotides attenuate the assembly of Ade4 particles *in vitro*

A. Effects of purine nucleotides on the *in vitro* assembly of Ade4 particles. Following the

supplementation of 0.2 μM Ade4-mNG with 10% PEG8000 in the presence of the

indicated purine nucleotide, images were taken after a 2-min incubation. The integrated

fluorescence intensities of particles were summed per field and plotted. Bars and error

bars indicate the mean ± 1SD. Between 23 and 28 fields of view were imaged for each

condition from two independent preparations, and an average of 1323 particles per field

were examined. *P* values were calculated using the two-tailed Tukey-Kramer's multiple

comparison test. ***, *p* <0.001; NS, not significant (*p* value >0.05).

B-D. The supplementation of 0.2 μM Ade4-mNG with 10% PEG8000 was performed in

the presence of the indicated concentration of AMP (B), ADP (C), and ATP (D). The

integrated fluorescence intensities of particles were summed per field, normalized to the

mean at 0 μM, and plotted. Bars and error bars indicate the mean ± 1SD. Between 28 and

55 fields of view were imaged for each condition from 2 or 3 independent preparations,

and an average of 1200 particles per field were examined.

E. Effects of AMP on the *in vitro* assembly of Ade4 and Ade4ΔIDR particles. The

supplementation of 0.2 μM Ade4-mNG (FL) or Ade4ΔIDR-mNG (ΔIDR) with 10%

PEG8000 was performed in the presence of 0 or 500 μM AMP. The integrated

fluorescence intensities of particles were summed per field, normalized to the mean of

FL at 0 μM AMP, and plotted. Bars and error bars indicate the mean ± 1SD. Between 40

and 45 fields of view were imaged for each condition from 3 independent preparations.

P values were calculated using the two-tailed Welch's *t*-test.

F. Schematic model showing the regulation of Ade4 condensation proposed in the present

study. See the text for details.

1077

Figure 7S1. Additional data regarding the assembly of Ade4 particles *in vitro*, related

to Fig. 7.

A, B. Effects of purine base derivatives on the *in vitro* assembly of Ade4 particles.

Following the supplementation of 0.2 μM Ade4-mNG with 10% PEG8000 in the

presence of the indicated compound, images were taken after a 2-min incubation. Glucose

was used as a negative control. The integrated fluorescence intensities of particles were

summed per field and plotted in (A). The mean fluorescence intensities of Ade4-mNG

particles were averaged per field and plotted in (B). Bars and error bars indicate the mean

± 1SD. Twenty fields of view were imaged for each condition from two independent

preparations, and an average of 1452 particles per field were examined. *P* values were
calculated using the two-tailed Steel's multiple comparison test. NS, not significant (*p*
value >0.05).
C. Effects of purine nucleotides on the *in vitro* assembly of Ade4 particles. Data were
from the same experiment shown in Figure 7A. The mean fluorescence intensities of
Ade4-mNG particles were averaged per field and plotted. Bars and error bars indicate the
mean ± 1SD. *P* values were calculated using the two-tailed Tukey-Kramer's multiple
comparison test. ***, *p* <0.001; NS, not significant (*p* value >0.05).
D. The supplementation of 0.2 μM Ade4-mNG with 10% PEG8000 was performed in the
presence of the indicated concentration of GMP. The integrated fluorescence intensities
of particles were summed per field, normalized to the mean at 0 μM, and plotted. Bars
and error bars indicate the mean ± 1SD. Between 25 and 30 fields of view were imaged
for each condition from two independent preparations, and an average of 1200 particles
per field were examined.
1101
Figure 8. The formation of Ade4 granules is important for efficient *de novo* purine
synthesis during cell growth
A. Growth of ADE4-3HA and ADE4ΔIDR-3HA cells on solid media. Cells of each strain
were serially diluted (five-fold), spotted on 2% glucose medium containing the indicated
nitrogen source, and then grown at 30°C for 2-3 days.
B. Immunoblot analysis of the protein level of Ade4. Total cell extracts were prepared
from cells expressing the indicated Ade4 construct. Ade4-3HA and Ade4ΔIDR-3HA were
detected as approximately 60-kDa bands by an anti-HA antibody. The bands of GAPDH
were shown as a loading control.
C. Growth curves of Ade4-3HA and Ade4ΔIDR-3HA cells in liquid media without
adenine. Cells were grown at 30°C for 40 h in medium containing the indicated nitrogen
source in the absence of adenine. Data are the mean of 6 replicates, and the shaded area
indicates ± 1SD.
D. Quantification of data shown in (C). The time to half-maximum density
(corresponding to normalized OD_{595} = 0.5) was calculated and plotted. Bars and error
bars indicate the mean ± 1SD. *P* values were calculated using the two-tailed Welch's *t*-
test.
1119
Figure 8S1. Cell growth assay in the presence of external purine bases, related to Fig.
8.
A. Growth curves of Ade4-3HA and Ade4ΔIDR-3HA cells in liquid media containing

adenine. Cells were grown at 30°C for 40 h in medium containing the indicated nitrogen
source in the presence of adenine. Data are the mean of 6 replicates, and the shaded area
indicates ± 1SD.
B. Quantification of data shown in (A). The time to half-maximum density
(corresponding to normalized OD_{595} = 0.5) was calculated and plotted. Bars and error
bars indicate the mean ± 1SD. P values were calculated using the two-tailed Welch's t-
test.
C. Reaction formulae corresponding to the glutamine-dependent PRA synthesis (Gln-
PRA activity) and NH_3-dependent PRA synthesis (NH_3-PRA activity) of PPAT.
D. Relationship between ammonia concentrations and the two activities of PPAT.
1133

Figure 9. MD calculations predict the potential leakage of glutamine-derived NH_3
from PPAT molecules
(A-C) Time series for the dihedral angle of the Phe260 side chain and the distance of NH_3
from the initial structure (A: holo; B: semi-holo; and C: R333A mutant). Moving averages
are plotted in bold lines. Under each condition, 100-ns simulations were performed ten
times individually. The blue backgrounds indicate the open state of the Phe260 sidechain.
(D) The hydrophobic (orange) and front (red) channels of *E. coli* PPAT. (E) A structural
snapshot around the Arg gate. Hydrogen bonds are shown as red dotted lines. (F) The
reaction mechanism suggested in a previous study. (G) The reaction mechanism
suggested in the present study. (H) Intramolecular and intermolecular channeling of NH_3.
1144

Figure 9S1. Possible enzymatic reaction pathways of PPAT
A. Intramolecular channeling of glutamine-derived NH_3 proposed by Krahn et al.[39].
B. Mechanism of NH_3-dependent activity proposed by Chen et al.[63]. External NH_3 enters
the channel from the glutamine site irrespective of glutamine binding.
C. The MD simulation in the present study suggests that glutamine-derived NH_3 leaks
through the front channel following the attack of exogenous NH_3.
D. Condensation-dependent activation of Ade4 proposed by the present study. Glutamine-
derived NH_3 leaked from the front channel rapidly diffuses away. In the Ade4 condensate,
the opportunity for NH_3 to be captured by other molecules and further reacted with PRPP
will increase. This activation may be described as intermolecular NH_3 channeling. PRPP
is not depicted in the schema.

Supplemental Information

Captions for supplementary movies

Movie S1

Time-lapse imaging of the assembly of Ade4-mNG granules by adenine depletion. Cells were grown in medium containing 0.02 mg/ml adenine and washed with medium lacking adenine at t = 0 min. The three cells in the upper right are shown in Fig. 1D. Scale bar = 5 μm.

Movie S2

Another example of cells showing the assembly of Ade4-mNG granules by adenine depletion. Experimental conditions are the same as in Movie S1. Scale bar = 5 μm.

Movie S3

A control experiment of time-lapse imaging of the assembly of Ade4-mNG granules by adenine depletion. Cells were grown in medium containing 0.02 mg/ml adenine and washed with medium containing the same concentration of adenine at t = 0 min. The rightmost cell is shown in Fig. 1S1C. Scale bar = 5 μm.

Movie S4

Time-lapse imaging of the disassembly of Ade4-mNG granules by adenine supplementation. Cells were grown in the absence of adenine and 0.02 mg/ml adenine was supplemented at t = 0 min. The four cells in the upper left are shown in Fig. 1E. Scale bar = 5 μm.

Movie S5

A control experiment of time-lapse imaging of the disassembly of Ade4-mNG granules by adenine supplementation. Cells were grown in the absence of adenine and sterile water was supplemented in place of adenine at t = 0 min. The two cells in the center are shown in Fig. 1S1D. Scale bar = 5 μm.

Movie S6

Another example of cells showing Ade4-mNG granules after a mock treatment with adenine. Experimental conditions are the same as in Movie S5. Scale bar = 5 μm.

Figure 1: The yeast PPAT Ade4 concentrates in fine granules

A
(-) purine base
Ade4-GFP   Ade4-mGFP   Ade4-mNG
GFP
GFP + BF

B

C
Ade4-mNG   BF
-2.5 µm   +2.5 µm

H
Pentose phosphate pathway
↓
PRPP
PPAT (Ade4)
AMP ◄--- IMP ---► GMP

D
+ adenine → − adenine
3′ 7′ 11′ 15′ 19′ 23′
27′ 31′ 35′ 39′ 43′ 47′
Ade4-mNG + phase

E
− adenine → + adenine
−3′ −1′ 1′ 3′ 5′ 7′
9′ 11′ 13′ 15′ 17′ 19′
Ade4-mNG + phase

F

G

+ade → −ade   +ade → +ade   −ade → −ade   −ade → +ade

Figure 1S1: Additional data regarding the intracellular localization of Ade4. Related to Figure 1

A

B

C

+ adenine → + adenine

Ade4-mNG + phase

D

– adenine → – adenine

Ade4-mNG + phase

Figure 2: Ade4 granules have liquid-like properties

A (−) purine base

untreated   +digitonin   +digitonin/1,6-HD

Ade4-mNG + phase

B % cells with Ade4-mNG granules

NS
<0.001
<0.001

untreated digitonin 1,6-HD
n = 25   n = 25   n = 25

C

1   68-168   287-401

GATase   PRTase   IDR

Disorder score

Residue

D (−) purine base

Ade4ΔIDR-mNG   BF

Figure 2S1: The C-terminal IDR is conserved in PPATs of Ascomycota

Figure 2S2: The IDR is conserved in PPATs of various species

Figure 2S3: Amino acid sequences of IDRs vary across species

A

B

Figure 3: Formation of Ade4 granules requires active TORC1 and ribosome biogenesis

A

Phase + Ade4-GFP

wt | lst7Δ | pib2Δ | ego2Δ

sfp1Δ | sit4Δ | gln3Δ

B

Strain (y-axis, top to bottom): sfp1, sit4, ego2, gln3, lst7, pib2, sea3, gtr2, gtr1, kap120, sea2, tpd3, npr2, tco89, sea4, lst4, sap155, npr3, seh1, gat1, ypk3, gcn4, tod6, ego3, atg13, cdc55, rts1, ego1, wt, sap190, rtg2, sap4, gcn2, rim15, maf1, mks1, sch9

x-axis: % cells with Ade4-GFP foci (0, 25, 50, 75, 100)

signaling:
- upstream (green)
- downstream (orange)
- others (blue)
- wild-type (magenta)

C

DMSO | Diazaborine

Ade4-GFP + phase

D

DMSO | Diazaborine

Ade4-mNG

BF

Figure 4: Intracellular derivatives of purine nucleotidse prevent the formation of the Ade4 condensates

Ade4-GFP + phase

A

alanosine

AMP ←—*ADE13*—← S-AMP ←—*ADE12*—← IMP —→ XMP —→ GMP

HPT1

hypoxanthine

B

SC ——————————→ SC + hyp

mock

+ alanosine

Ade4-GFP + phase

C

% cells with Ade4-GFP foci

0.02   9e-10

Treatment
● mock
● alanosine

SC   SC+hyp

Figure 5: The interaction of purine nucleotides with Ade4 is implicated in the granule assembly

A

K316

R318

D293

ADP

B

A_mississippiensis	VPESATPAAL	LCKNRYVGRT
G_gallus	VPESATPAAL	LCKNRYVGRT
X_laevis	VPESATPAAL	LCKNRYVGRT
H_sapiens	VPESATPAAL	LCKNRYVGRT
D_rerio	VPESATPAAL	LCKNRYVGRT
S_purpuratus	VPESATPAAL	LTKNRYVGRT
D_melanogaster	VPESGTAAAH	LCKNRYVGRT
C_intestinalis	VPESATPAAF	LAKNRYIGRT
C_elegans	VPDSSLSAAI	LHRNSYVGRS
D_discoideum	VPDSSLPAAI	LTKNRYIHRT
A_thaliana	VPDSGTVAAL	LLRSHYAKRT
B_subtilis	vpdssisaai	liknryvgrt
M_smithii	VPDSSIPAAI	LIKNRYVGRT
C_albicans	VPDTARTSAF	FVKNRYIGRT
S_cerevisiae	VPDTARTCAL	FVKNRYVGRT
S_pombe	VPDSARTSAL	FIKNRYIGRT
E_coli	IPETSCDIAL	FVKNRYVGRT
Consensus	VPESATPAAL	LCKNRYVGRT

C

% cells with Ade4-mNG granules

0.0086

wt   VQQ
n = 18   n = 22

D

Mean numbers of Ade4-mNG granules/cell/field

0.00030

wt   VQQ
n = 18   n = 22

E

Mean intensity of Ade4-mNG granules/field

1.2e-05

wt   VQQ
n = 18   n = 22

Figure 6: The Ade4 protein condensates into particles under molecular crowding conditions *in vitro*

Figure 6S1: Purification and basic characterization of the Ade4 protein *in vitro*

A

1: Ade4-mNG (86.2kD), 0.6 µg

2: Ade4ΔIDR-mNG (83.3kD), 0.6 µg

3: mNG (29.2kD), 0.3 µg

B

Pure mNG, inverted grayscale

0% PEG8K

10% PEG8K

C

Pure Ade4-mNG + 10% (w/v) PEG, inverted grayscale

0 (no PEG)   0.4 kDa   1 kDa

4 kDa   8 kDa   20 kDa

D

E

F

2% Lysozyme   10% Ficoll-400

Pure Ade4-mNG, inverted grayscale

Figure 7: Purine nucleotides attenuate the assembly of the Ade4 particles *in vitro*

A

B

C

D

Figure 8S1: Cell growth assay in the presence of external purine bases , related to Fig. 8

A

B

C

Gln-PRA activity:
$PRPP + Gln + H_2O \rightarrow PRA + Glu + PP_i$

NH$_3$-PRA activity:
$PRPP + NH_3 \rightarrow PRA + PP_i$

D

	Gln-PRA	NH$_3$-PRA
Low NH$_3$	Up	Down
High NH$_3$	Down	Up

Figure 9: MD calculations predict the potential leakage of glutamine-derived NH₃ from PPAT molecules

A

Glutamine-dependent activity *Krahn et al., 1997*

Intramolecular NH$_3$ channeling

▲ glutamine ● GATase
■ NH$_3$
● PRA ● PRTase

B

NH$_3$-dependent activity *Chen et al., 1999*

(i) exogenous NH$_3$ entry into the channel (glutamine-unloaded)

(ii) exogenous NH$_3$ entry into the channel (glutamine-loaded)

C

MD simulation *This study*

(i) exogenous NH$_3$ directly attacks PRPP

(ii) leakage of Gln-derived NH$_3$ from the front channel

D

Condensation-dependent activation *This study*

Intermolecular NH$_3$ channeling in the Ade4 condensates

(i) via intramolecular channel

(ii) directly to the PRPP