FEMS Yeast Research, 2022, 22, 1–13
DOI: 10.1093/femsyr/foac020
Advance access publication date: 24 March 2022
Minireview

Response to leucine in *Schizosaccharomyces pombe* (fission yeast)

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One sentence summary: This minireview summarizes the latest leucine metabolism mechanism and starvation response along with identification of a gene, ilv3+, involved in leucine synthesis in the fission yeast *Schizosaccharomyces*.
Editor: Hyun Ah Kang

Abstract

Leucine (Leu) is a branched-chain, essential amino acid in animals, including humans. Fungi, including the fission yeast *Schizosaccharomyces pombe*, can biosynthesize Leu, but deletion of any of the genes in this biosynthesis leads to Leu auxotrophy. In this yeast, although a mutation in the Leu biosynthetic pathway, *leu1-32*, is clearly inconvenient for this species, it has increased its usefulness as a model organism in laboratories worldwide. Leu auxotrophy produces intracellular responses and phenotypes different from those of the prototrophic strains, depending on the growing environment, which necessitates a certain degree of caution in the analysis and interpretation of the experimental results. Under amino acid starvation, the amino acid-auxotrophic yeast induces cellular responses, which are conserved in higher organisms without the ability of synthesizing amino acids. This mini-review focuses on the roles of Leu in *S. pombe* and discusses biosynthetic pathways, contribution to experimental convenience using a plasmid specific for Leu auxotrophic yeast, signaling pathways, and phenotypes caused by Leu starvation. An accurate understanding of the intracellular responses brought about by Leu auxotrophy can contribute to research in various fields using this model organism and to the understanding of intracellular responses in higher organisms that cannot synthesize Leu.

Keywords: fission yeast, *Schizosaccharomyces pombe*, leucine, *leu1-32*, general amino acid control, TORC1

Introduction

Leucine (Leu) is one of the branched-chain amino acids (BCAAs) like isoleucine (Ile) and valine (Val). It is also one of the essential amino acids in animals, including nematodes, silkworms, and humans (Oda 2007, Le Couteur *et al.* 2020). It has important physiological functions in protein synthesis, metabolism, nutrient uptake, and control of aging (Le Couteur *et al.* 2020). As the most bioactive BCAA, Leu contributes to the maintenance of the level of cellular acetyl coenzyme A and the acetylation of cytoplasmic proteins during nutrient deprivation (Mariño *et al.* 2014, Son *et al.* 2020). Since BCAA biosynthetic pathways exist in plant, fungi, archaea, and bacteria, but not in animals, these pathways may also be targets for herbicides and antimicrobial compounds (Liu *et al.* 2016). Various studies have reported a relationship between the lifespan of organisms and the presence of essential amino acids including Leu (Fontana and Partridge 2015, Ohtsuka *et al.* 2019, Le Couteur *et al.* 2020), and an accurate understanding of this phenomenon will contribute to a wide range of life science fields, including antiaging.

Amino acid or protein restriction causes activation of general amino acid control and suppression of the target of rapamycin (TOR) pathway, leading to improved metabolic fitness, increased stress tolerance, and extended lifespan (Gallinetti *et al.* 2013). Restrictions on methionine and tryptophan have been shown to reduce the activity of nutrient-sensing pathways and extend the lifespan in mice (Fontana and Partridge 2015). In yeasts such as the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, cells can synthesize all amino acids, but a defect in the amino acid biosynthetic pathway leads to the loss of synthetic ability, resulting in activation of the general amino acid control and suppression of the TOR pathway, depending on the growth environment (Zaborske *et al.* 2009, Kamada 2017, Ohtsuka *et al.* 2019, Fukuda *et al.* 2021). In *S. cerevisiae*, restriction of methionine, asparagine, or glutamate (Glu) has been reported to extend the chronological lifespan, which is defined as the survival of a cell population after the stationary phase (Powers *et al.* 2006, Dilova *et al.* 2007, Ruckenstuhl *et al.* 2014, Fontana and Partridge 2015). Starvation of Leu, one of the BCAAs, has also been found to activate the general amino acid control and extend the chronological lifespan in *S. pombe* (Ohtsuka *et al.* 2019). This chronological lifespan extension by Leu starvation occurs in Leu auxotrophic cells but not in prototrophic cells (Ohtsuka *et al.* 2019).

In research using the model organism *S. pombe*, Leu auxotrophs have been used since the latter half of the twentieth century (Kohli *et al.* 1977, Beach and Nurse 1981). These auxotrophs have made it convenient to study this organism as described later in the text and made significant contributions to research in various fields (Seike *et al.* 2019, Imai *et al.* 2020, Fukuda *et al.* 2021). However, as Leu auxotrophy can cause intracellular responses differ-

Received: October 29, 2021. **Revised:** March 8, 2022. **Accepted:** March 22, 2022

ent from those of prototrophic cells, caution is needed when evaluating the results of studies using auxotrophic cells. For example, Leu starvation results in completely different chronological lifespan depending on whether cells are Leu auxotrophs or not (Ohtsuka *et al.* 2019). Additionally, the supplementation of Leu to the medium affects not only the growth of Leu auxotrophic cells but also the length of prototrophic cells (Petersen and Russell 2016). Notably, autophagy defects lead to a significantly reduced survival rate under nitrogen depletion in Leu auxotrophic cells, but not in arginine (Arg) or lysine (Lys) auxotrophic cells (Kohda *et al.* 2007), indicating that the phenotype differs dependent on the type of amino acid auxotrophy. Although Leu has had a strong influence on research using *S. pombe*, there are not many reports summarizing the relationships involved. This mini-review summarizes the Leu utilization and starvation responses that have been clarified to date in *S. pombe*. A clear understanding of the relationship between this model organism and Leu is expected to facilitate more accurate interpretations of the results of future studies using this organism.

Leu biosynthesis in *S. pombe*

Schizosaccharomyces pombe can biosynthesize Leu, as do other fungi, and half of Leu synthesis occurs by the same pathway as the other BCAAs Ile and Val (Fig. 1) (McDonald *et al.* 1974). Because all the enzymes that act in Ile and Val biosynthesis are also required for Leu biosynthesis, there are Leu auxotrophic strains but not auxotrophic strains that require only Ile or Val.

BCAAs including Leu are synthesized from pyruvate (Kondo *et al.* 2012, Liu *et al.* 2016). The enzyme acetolactate synthase (ALS), also known as acetohydroxy acid synthase, which catalyzes the first reaction of the BCAA biosynthetic pathway, converts two pyruvate molecules to (2*S*)-2-acetolactate ((2*S*)-2-hydroxy-2-methyl-3-oxobutanoate), which is required for Leu and Val synthesis, while converting pyruvate and 2-oxobutanoate (2-ketobutyrate) to (*S*)-2-aceto-2-hydroxybutanoate, which is required for Ile synthesis (Fig. 1) (Liu *et al.* 2016). ALS consists of a large subunit that acts as a catalytic core and a small subunit that acts as a regulatory subunit (Liu *et al.* 2016). In *S. pombe*, the former is encoded by *ilv1*$^+$, and the latter is predicted to be encoded by *ilv6*$^+$ as identified through sequence similarity (McDonald *et al.* 1974, Bekkaoui *et al.* 1993). ALS activity is strongly inhibited by feedback from the final product, Val, and is also less potently inhibited by Ile; however, the inhibitory activity of Leu is small (McDonald *et al.* 1973, 1974). It has also been reported that mercury reversibly weakens the inhibition of ALS activity by Val (McDonald *et al.* 1974). Full activity of this ALS requires flavin adenine dinucleotide and thiamine pyrophosphate (McDonald *et al.* 1973). In plants, ALS is transported to chloroplasts, but in Anheuser-Busch baker's yeast, it is transported to the mitochondria (Miflin 1974, Ryan and Kohlhaw 1974, Bekkaoui *et al.* 1993). In *S. cerevisiae*, both Ilv2, an ortholog of *S. pombe* Ilv1, and Ilv6, an ortholog of *S. pombe* Ilv6, have also been localized to mitochondria (Sickmann *et al.* 2003, Kondo *et al.* 2012). In *S. pombe*, both the products of *ilv1*$^+$ and *ilv6*$^+$ are localized in the mitochondria (Matsuyama *et al.* 2006), similar to baker's yeast and laboratory *S. cerevisiae*, suggesting that *S. pombe* ALS also acts in the mitochondria. The existence of an Ilv1 homolog has been confirmed as ILVBL in humans (Joutel *et al.* 1996), whereas no apparent homolog of the regulatory subunit Ilv6 has been confirmed in humans.

The (2*S*)-2-acetolactate and (*S*)-2-aceto-2-hydroxybutanoate produced by ALS are then converted to (2*R*)-2,3-dihydroxy-3-methylbutanoate and (2*R*,3*R*)-2,3-dihydroxy-3-methylpentanoate, respectively, by acetohydroxyacid reductoisomerase, which is predicted to be encoded by *ilv5*$^+$ (Fig. 1) (McDonald *et al.* 1974, Ryan and Kohlhaw 1974). Acetohydroxyacid reductoisomerase is also known as Mg-dependent isomeroreductase, and it has been confirmed that at least the reaction using (*S*)-2-aceto-2-hydroxybutanoate as a substrate requires Mg^{2+} in *S. pombe* (McDonald *et al.* 1974, Ryan and Kohlhaw 1974). The ortholog of this enzyme in *S. cerevisiae* is Ilv5, which uses NADPH as the redox cofactor for its catalytic reaction (Wess *et al.* 2019). Like ALS, the protein encoded by *ilv5*$^+$ is localized in the mitochondria (Matsuyama *et al.* 2006).

(2*R*)-2,3-dihydroxy-3-methylbutanoate and (2*R*,3*R*)-2,3-dihydroxy-3-methylpentanoate are then converted by dihydroxyacid dehydratase to 3-methyl-2-oxobutanoate and (*S*)-3-methyl-2-oxopentanoate, respectively (McDonald *et al.* 1974). The full activity of these reactions requires Mg^{2+} or Mn^{2+} (McDonald *et al.* 1974). In *S. pombe*, this enzyme is predicted to be encoded by SPAC17G8.06c, but this prediction has not been confirmed. Therefore, we investigated whether *S. cerevisiae ILV3* encoding dihydroxy-acid dehydratase can complement this gene (Fig. 2). The deletion mutant of SPAC17G8.06c can grow in yeast extract complete medium but cannot grow in Edinburgh minimal medium (EMM) with Leu. This is because this gene is involved in the synthesis of Leu as well as Ile and Val. However, even if Leu, Ile, and Val are added, this mutant cannot grow in EMM, whereas it can surprisingly grow in synthetic dextrose medium with these BCAAs. This observation suggests that this enzyme is involved in the biosynthesis of Leu, Ile, and Val and is also essential for the growth of *S. pombe* in EMM. The defective growth of the ΔSPAC17G8.06c mutant was complemented not only by the expression of *S. pombe* SPAC17G8.06c itself but also by the *S. cerevisiae ILV3*. This finding suggests that *S. pombe* SPAC17G8.06c is a functional ortholog of *S. cerevisiae ILV3* and encodes a dihydroxy-acid dehydratase. On this basis, we called SPAC17G8.06c *ilv3*$^+$. Like ALS and Ilv5, Ilv3 is also localized in the mitochondria (Matsuyama *et al.* 2006).

At this stage, Ile and Val are produced by BCAAs aminotransferase (BCAT), encoded by *eca39*$^+$, from (*S*)-3-methyl-2-oxopentanoate and 3-methyl-2-oxobutanoate, respectively (Fig. 1) (Eden and Benvenisty 1998). BCAT catalyzes the transamination of the amino group of Glu to Leu, Ile, and Val stereoselectively, with cofactor pyridoxal 5′-phosphate (PLP) (Bezsudnova *et al.* 2017). These reactions are reversible and not only act as the last steps in BCAA syntheses but also as the first steps in the BCAA catabolic pathways, which are important reactions in BCAA metabolism in all organisms (Eden and Benvenisty 1998, Bezsudnova *et al.* 2017). As in Anheuser-Busch baker's yeast, BCAT Eca39 has been confirmed to be present in various parts of *S. pombe* cells, including cytoplasm and mitochondria (Ryan and Kohlhaw 1974, Eden and Benvenisty 1998, Matsuyama *et al.* 2006). In *S. cerevisiae*, there are two BCATs, Bat1, which acts in mitochondria, and Bat2, which acts in cytosol (Wess *et al.* 2019). Humans also have two BCATs, BCAT1, which acts in cytosol, and BCAT2, which acts in mitochondria (Bledsoe *et al.* 1997).

Leu is synthesized from 3-methyl-2-oxobutanoate, which is also used in Val synthesis, through four step reactions (McDonald *et al.* 1974). Some biochemical pathways are thought to be derived from ancestral enzymes, which have broader substrate specificity than current enzymes, and the biosynthetic pathway from 3-methyl-2-oxobutanoate to Leu is similar to part of the biosynthetic pathway from 2-oxoglutarate to Lys (Fondi *et al.* 2007, Larson and Idnurm 2010). The final step in Leu biosynthesis, i.e. the transamination reaction, is catalyzed by BCAT Eca39, which is

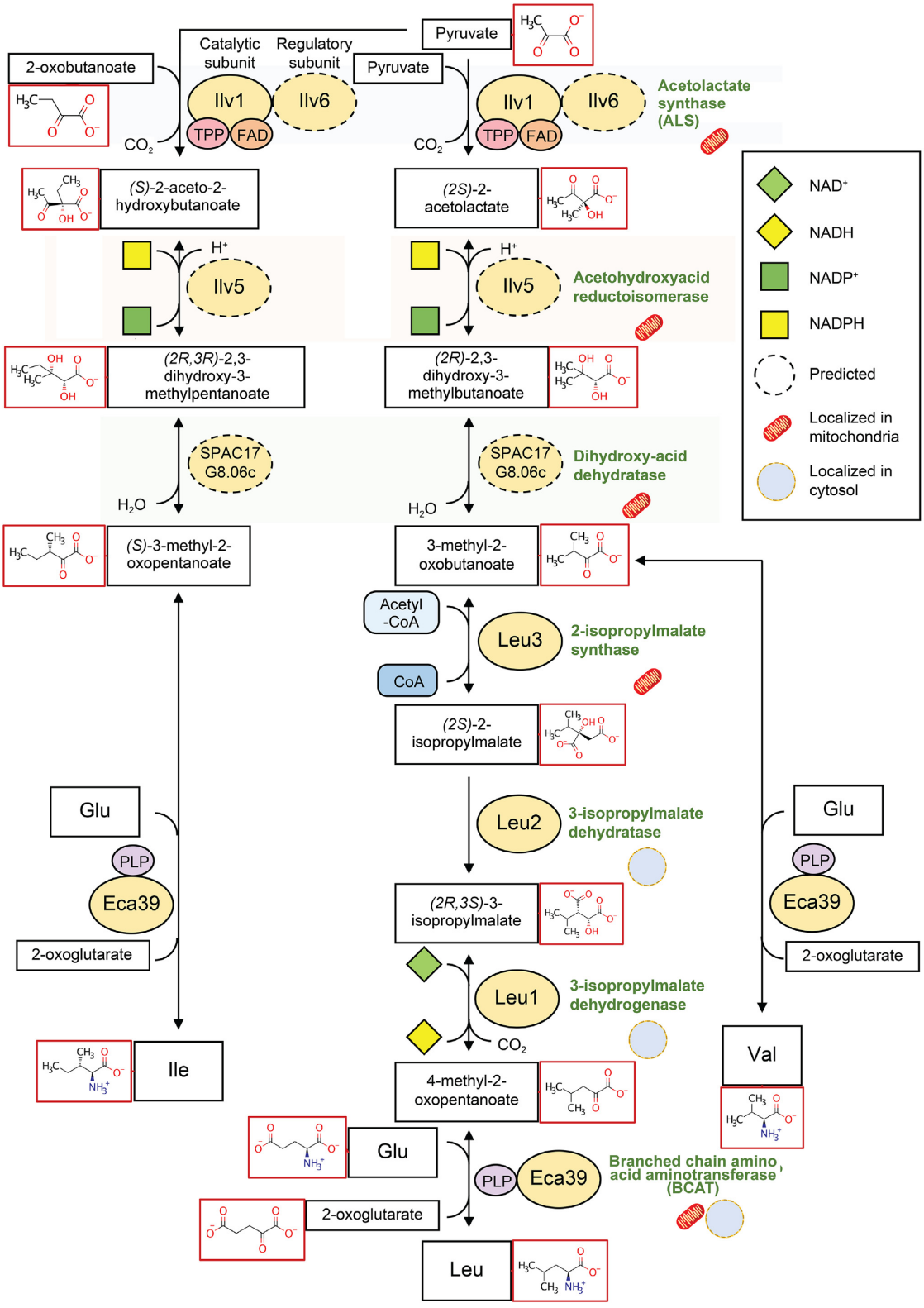

Figure 1. Leucine biosynthesis pathway in fission yeast, *Schizosaccharomyces pombe*. The details of each process are described in the text. TPP, thiamine pyrophosphate; FAD, flavin adenine dinucleotide; PLP, pyridoxal phosphate.

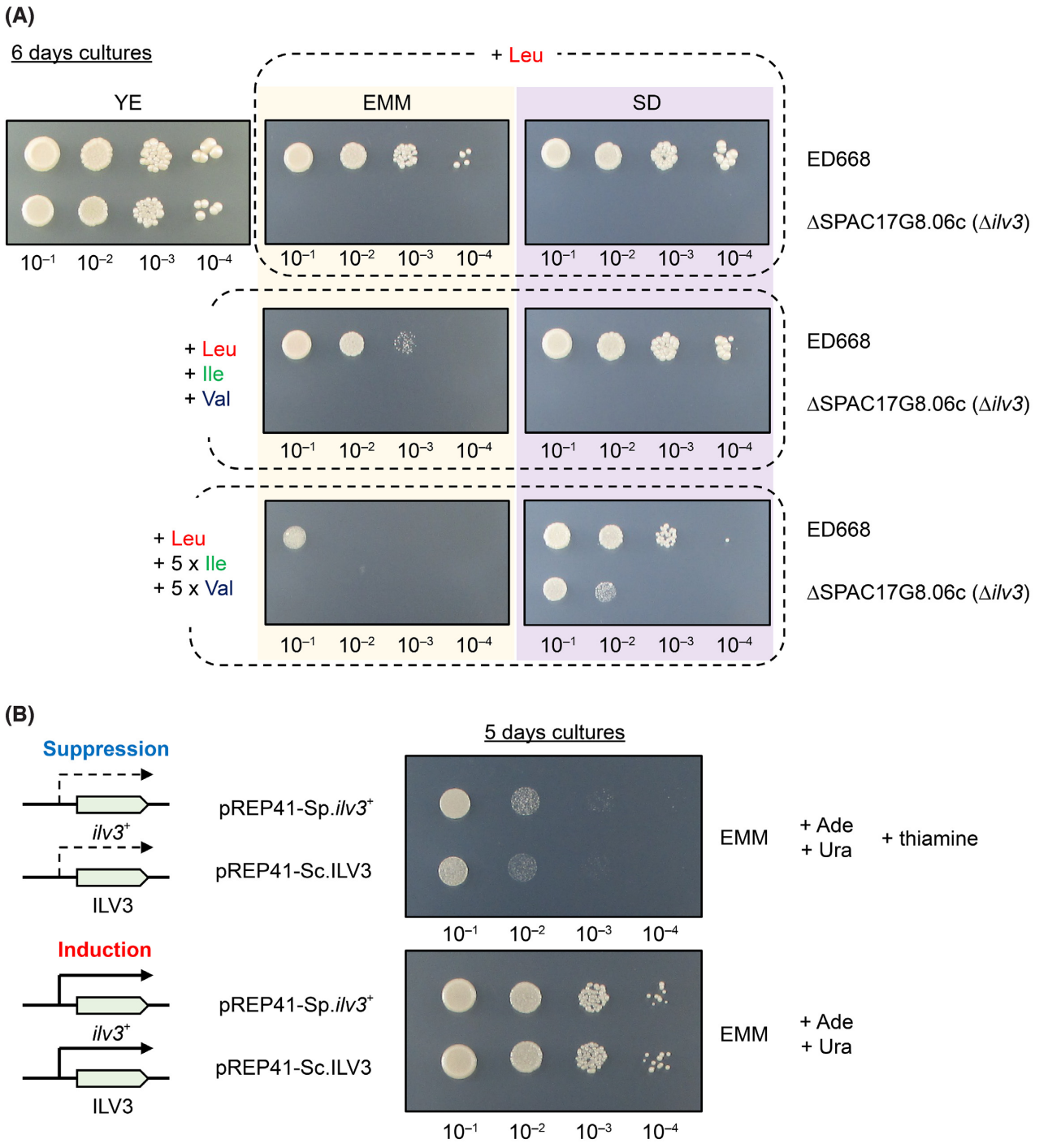

Figure 2. *Schizosaccharomyces pombe* SPAC17G8.06c (*ilv3*$^+$) encodes a dihydroxy-acid dehydratase. **(A)** Growth of ED668 (*h*$^+$ *ade6-M216 leu1-32 ura4-D18*) and ΔSPAC17G8.06c (from Bioneer) strains in complete (YE), synthetic dextrose (SD), and Edinburgh minimal (EMM) media with supplements (Ade, 40 μg/mL adenine; Leu, 60 μg/mL leucine; Ura, 20 μg/mL uracil; Ile, 40 μg/mL isoleucine; Val, 40 μg/mL valine; 5 × Ile, 200 μg/mL isoleucine; 5 × Val, 200 μg/mL valine). **(B)** Growth of ΔSPAC17G8.06c cells carrying pREP41-Sp.*ilv3*$^+$ (*S. pombe* SPAC17G8.06c inserted in pREP41) or pREP41-Sc.*ILV3* (*S. cerevisiae ILV3* inserted in pREP41) in EMM with supplements (Ade, 40 μg/mL adenine; Ura, 20 μg/mL uracil; 5 μg/mL thiamine). To make pREP41-Sp.*ilv3*$^+$ and pREP41-Sc.*ILV3*, DNA fragments of the *ilv3*$^+$ and *ILV3* were amplified from each genome of *S. pombe* and *S. cerevisiae* using following primers, respectively: TTAGCATATGATGTTCTGCAAGCTTCTCC and CCAGGATCCTATGCGCGCTTATAAAAGCATTG for *ilv3*$^+$, AGTACATATGGGCTTGTTAACGAAAGTTGC and ATAGGATCCTCGATTGGGGCCTATAATGC for *ILV3*. The amplified DNA fragments were digested with NdeI and BamHI and then cloned into the plasmid pREP41. The composition of SD medium is 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose. The composition of EMM is as previously described (Moreno *et al.* 1991).

the same enzyme as in Ile and Val syntheses, and the other three reactions are catalyzed by the enzymes encoded by *leu1*$^+$, *leu2*$^+$, and *leu3*$^+$ (Fig. 1) (Kikuchi *et al.* 1988, Eden and Benvenisty 1998, Larson and Idnurm 2010). A loss-of-function mutation of any of *leu1*$^+$, *leu2*$^+$, or *leu3*$^+$ leads to Leu auxotrophy (Kohli *et al.* 1977).

The gene *leu3*$^+$ encodes 2-isopropylmalate synthase, which uses acetyl-CoA to acetylate 3-methyl-2-oxobutanoate to produce (2*S*)-2-isopropylmalate, and is regulated by the general amino acid control (McDonald *et al.* 1974, Larson and Idnurm 2010, Tarumoto *et al.* 2013). Leu allosterically inhibits 2-isopropylmalate synthase (McDonald *et al.* 1974, Larson and Idnurm 2010), and this interaction can contribute to the maintenance of Leu homeostasis. Leu3 is localized to the mitochondria (Matsuyama *et al.* 2006). While in *S. pombe*, Leu3 is the only known 2-isopropylmalate synthase, in *S. cerevisiae*, Leu4 and Leu9 are paralogs with 2-isopropylmalate synthase activity (Wess *et al.* 2019). Among these paralogs, Leu4 accounts for about 80% of the total synthase activity (Kohlhaw 2003).

The enzyme 3-isopropylmalate dehydratase (isopropylmalate isomerase) converts (2*S*)-2-isopropylmalate to (2*R*,3*S*)-3-isopropylmalate (Kohlhaw 2003). In *S. pombe*, this enzyme is predicted to be encoded by *leu2*$^+$, which is regulated by the transcription factor Fil1 acting on the general amino acid control (Duncan *et al.* 2018). In *S. cerevisiae*, this enzyme is encoded by *LEU1* and is localized in cytosol (Hammer *et al.* 2020).

(2*R*,3*S*)-3-isopropylmalate is converted to 4-methyl-2-oxopentanoate by 3-isopropylmalate dehydrogenase (Kohlhaw 2003). This catalytic reaction requires NAD^+ and divalent cations (Mn^{2+} or Mg^{2+}) (Gráczer *et al.* 2011). In *S. pombe*, 3-isopropylmalate dehydrogenase is encoded by *leu1*$^+$, and *S. pombe leu1*$^+$ can complement the *Escherichia coli leuB* mutation and *S. cerevisiae leu2* mutation (Kikuchi *et al.* 1988). Leu1 is localized in the cytosol (Kikuchi *et al.* 1988, Matsuyama *et al.* 2006). The 4-methyl-2-oxopentanoate produced by Leu1 is reversibly converted to Leu by Eca39 (Eden and Benvenisty 1998).

The intracellular localizations of the enzymes involved in BCAA biosynthesis suggest that in *S. pombe* Ile and Val biosynthesis is completed in the mitochondria, but Leu biosynthesis involves reactions in both the cytosol and the mitochondria (Kikuchi *et al.* 1988, Kohlhaw 2003, Matsuyama *et al.* 2006).

Utilization of Leu auxotrophy in *S. pombe*

Schizosaccharomyces pombe Leu auxotrophs have been used in numerous studies. Among them, the Leu auxotroph created by the mutation of *leu1*$^+$ encoding 3-isopropylmalate dehydrogenase acting on Leu biosynthesis, mentioned above, is widely used. In addition, various plasmid vectors targeting Leu auxotrophy have been developed and used in research (Table 1).

The *leu1-32* mutation producing Leu auxotrophy has been widely used in research (Kiriya *et al.* 2017, Kurauchi *et al.* 2017, Fukuda *et al.* 2021, Jiménez-Saucedo *et al.* 2021). In the *leu1-32* mutation, guanine 137 is changed to adenine, which changes the amino acid sequence from glycine (Gly) 46 to Glu. The Gly of the *leu1-32* mutation is conserved in *E. coli* LeuB and *S. cerevisiae* Leu2 (Fig. 3A). The Gly46 is also conserved in a number of bacterial species including the hyperthermophilic bacterium *Thermus thermophilus* and the torula yeast *Cyberlindnera jadinii* (*Candida utilis*) (Wallon *et al.* 1997). This mutation site is relatively distant from the active center of the enzyme but is a helix-capping residue, which reportedly increases the stability of the adjacent α-helix (Fig. 3B) (Wallon *et al.* 1997, Aurora and Rose 1998). Therefore, the *leu1-32* mutation may make it difficult to maintain the conformation required for Leu1 to have proper catalytic activity, resulting in the loss of enzymatic activity and Leu auxotrophy.

In the latter half of the twentieth century, the Leu auxotroph of the *leu1-32* mutant was shown to be complemented by a plasmid carrying *S. cerevisiae LEU2* encoding 3-isopropylmalate dehydrogenase (Beach and Nurse 1981). This method has since been used, and various plasmids targeting Leu auxotrophic strains have been created (Table 1). Because *LEU2* complements the *leu1* mutant with high copy number, the number of copies of the plasmids carrying *LEU2* tends to be high (Siam *et al.* 2004, Kiriya *et al.* 2017). These plasmids, which can be used in Leu auxotrophic strains, are diverse; some are stably retained in cells, some aim to integrate the target sequence into the chromosome, some incorporate a promoter whose expression level can be adjusted, and some can be fused with epitope tags or fluorescent protein to target proteins.

Response to Leu starvation and uptake in *S. pombe*

Leu auxotrophic strains facilitate artificial genetic manipulation and are a powerful tool in various fields of research while simultaneously causing the Leu starvation response under some culture environments (Ohtsuka *et al.* 2019). Leu-starved cells activate the general amino acid control, suppress the TORC1 pathway, arrest the cell cycle at G1, reduce translational activity, and extend the chronological lifespan (Ohtsuka *et al.* 2019, Fukuda *et al.* 2021) (Fig. 4).

Activation of general amino acid control

The general amino acid control is important for cell survival in amino acid starvation conditions, and its activation stimulates the selective expression of genes involved in stress response and the biosynthesis of amino acids (Tarumoto *et al.* 2013). The general amino acid control is conserved from yeast to mammals and is called the amino acid response in mammals (Kilberg *et al.* 2012). Under amino acid starvation, uncharged tRNA binds to and activates protein kinase Gcn2 (general control nonderepressible 2), and then activated Gcn2 phosphorylates the α-subunit of eIF2 (eukaryotic initiation factor 2), resulting in the suppression of eIF2 and general repression of mRNA translation (Zaborske *et al.* 2009, Anda *et al.* 2017, González and Hall 2017). At this time, the translational activities of certain genes, including *fil1*$^+$ encoding a transcription factor required for amino acid starvation response, increase; this translational regulation is mediated by a 5′-leader sequence that contains multiple upstream ORFs (Duncan *et al.* 2018, Jiménez-Saucedo *et al.* 2021). The *S. pombe* Fil1 is a functional homolog of *S. cerevisiae* Gcn4 and ATF4 in mammals (Duncan *et al.* 2018). Thus, amino acid starvation activates the general amino acid control, and Leu starvation also activates the general amino acid control in *S. pombe* (Anda *et al.* 2017, Ohtsuka *et al.* 2019, 2021).

Induction of Ecl1 family proteins

In *S. pombe*, amino acid starvation increases Fil1 translation and induces significant expression of *ecl1*$^+$ and weaker expression of *ecl2*$^+$ (Ohtsuka *et al.* 2019). The proteins encoded by *ecl1*$^+$ and *ecl2*$^+$ are two of the three Ecl1 family proteins of *S. pombe*, and Ecl1 family proteins are conserved in fungi (Azuma *et al.* 2012, Ohtsuka and Aiba 2017). In *S. pombe*, Ecl1 is a zinc-binding protein, whose expression is induced by various stresses, including sulfur, amino acid, and magnesium starvation, and oxidative stress (Miwa *et al.* 2011, Shimasaki *et al.* 2014, 2017, 2020, Ohtsuka *et al.* 2021). This protein acts on several cellular responses, including autophagy, cell cycle, translation, sexual differentiation, and the regulation

(A)

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Sp_Leu1         -MCAKKIVVLPGDHIGPEIVASALEVLKVVEKKRPELKLEFEEHKIGGASIDAYGTPLTDETVKACLEADGVLLGAVGGPEWT--NPNCRPEQG-L 92
Sc_Leu2         MSAPKKIVVLPGDHVGQEITAEAIKVLKAISDVRSNVKFDFENHLIGGAAIDATGVPLPDEALEASKKADAVLLGAVGGPKWG--TGSVRPEQG-L 93
Ec_LeuB         MSKNYHIAVLPGDGIGPEVMTQALKVLDAVRN-RFAMRITTSHYDVGGAAIDNHGQPLPPATVEGCEQADAVLFGSVGGPKWEHLPPDQQPERGAL 95
                     :*.***** :* *: ::*:.**..: . *  :::  ..: :***:**  * **.  ::::. :**.**:*:****:*     . :**:* *

Sp_Leu1         LKLRKSMGVWANLRPCNFASKSLVKYSPLKPEIVE-GVDFCVVRELTGGCYFGERTEDNGSG---YAMDTWPYSLEEVSRIARLAAWLAETSNPPA 184
Sc_Leu2         LKIRKELQLYANLRPCNFASDSLLDLSPIKPQFAK-GTDFVVVRELVGGIYFGKRKEDDGDG---VAWDSEQYTVPEVQRITRMAAFMALQHEPPL 185
Ec_LeuB         LPLRKHFKLFSNLRPAKLYQG-LEAFCPLRADIAANGFDILCVRELTGGIYFGQPKGREGSGQYEKAFDTEVYHRFEIERIARIAFESARKRR--H 188
                * :** : :::****.:: .  *   .*::.::.  * *:  ****.** ***: .  :*.*    * *:  *   *:.**:*:*   *   .

Sp_Leu1         PVTLLDKANVLATSRLWRKTVAKIFKEEYPHLTLKNQLIDSAAMLLVKSPRTLNGVVLTDNLFGDIISDEASVIPGSLGLLPSASLSGVVGKSEEK 280
Sc_Leu2         PIWSLDKANVLASSRLWRKTVEETIKNEFPTLKVQHQLIDSAAMILVKNPTHLNGIIITSNMFGDIISDEASVIPGSLGLLPSASLASLPDKN--T 279
Ec_LeuB         KVTSIDKANVLQSSILWREIVNEIAT-EYPDVELAHMYIDNATMQLIKDPSQFD-VLLCSNLFGDILSDECAMITGSMGMLPSASLN------EQ 275
                 :  :****** :* ***: * :  . *:* : : :  **.*:* *:*.*  :: ::: .*:****:***.::*.**:*:******

Sp_Leu1         VHCLVEPIHGSAPDIAGKGIVNPVGTILSASLLLRYGLNAPKEAEAIEAAVRKVLDDTSIGGRGLYTRDLG---GEASTADITKAVVEELEKIL- 371
Sc_Leu2         AFGLYEPCHGSAPDLP-KNKVNPIATILSAAMMLKLSLNLPEEGKAIEDAVKKVLD------AGIRTGDLG---GSNSTTEVGDAVAEEVKKILA 364
Ec_LeuB         GFGLYEPAGGSAPDIAGKNIANPIAQILSLALLLRYSLDADDAACAIERAINRALE------EGIRTGDLARGAAAVSTDEMGDIIARYVAEGV- 363
                    . * **  *****:. *. .**:. *** :::*: .*:  . . *** *:.:.*:       *: * **. . ** :: . :.. : : :
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(B)

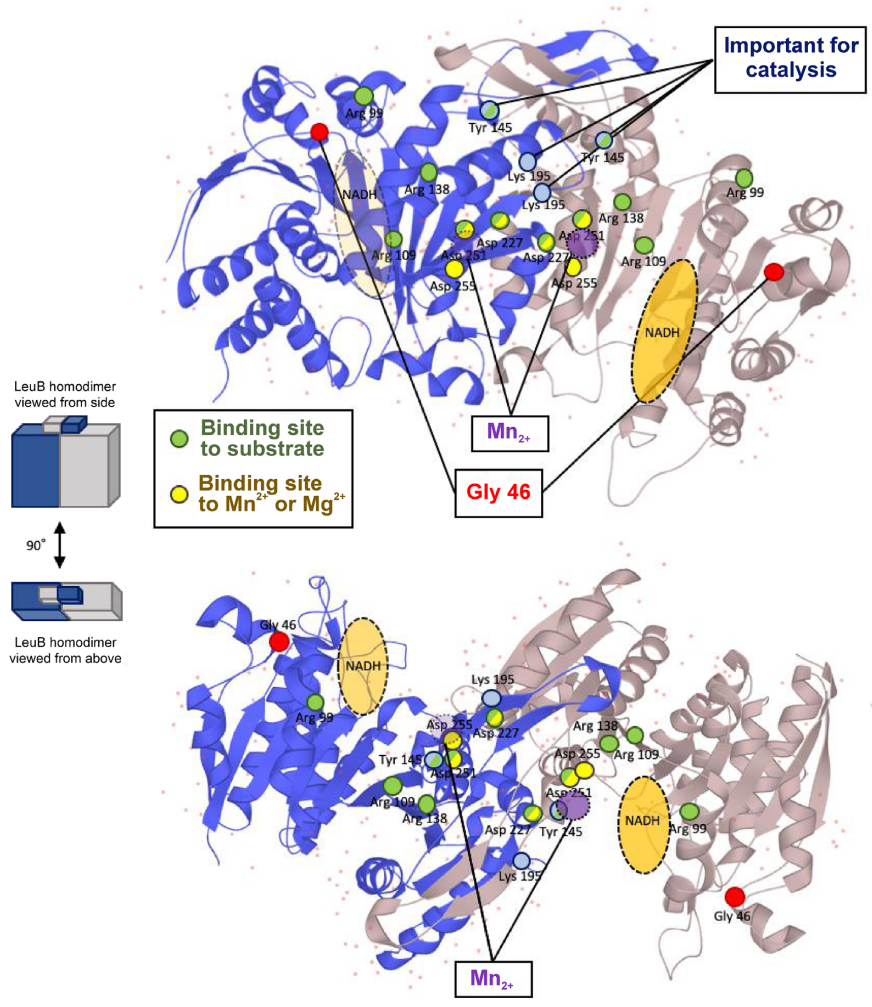

Figure 3. (A) *Schizosaccharomyces pombe* Leu1 (Sp_Leu1), *S. cerevisiae* Leu2 (Sc_Leu2), and *E. coli* LeuB (Ec_LeuB) amino acid sequences. The Gly 46 position corresponding to the *S. pombe leu1-32* mutation and the corresponding Sc_Leu2 and Ec_LeuB positions are shown in red. In the *leu1-32* mutation, this Gly is changed to Glu. **(B)** The three-dimensional structure of the 3-isopropylmalate dehydrogenase homodimer of *E. coli* (Ec_LeuB) shown from two different viewpoints (www.uniprot.org/uniprot/P30125). The mutation points of Gly corresponding to the *S. pombe leu1-32* mutation are shown as red dots. This enzyme binds NADH to the active site with a divalent cation (Mn^{2+} takes precedence over Mg^{2+} or other divalent cations) (Wallon *et al.* 1997, Gráczer *et al.* 2011). In the catalytic cycle, this enzyme performs domain closure, which is required for interactions between Mn^{2+} and the substrate (2*R*,3*S*)-3-isopropylmalate (Gráczer *et al.* 2011). Structural analysis using 3-isopropylmalate dehydrogenase of the bacterium *Thermus thermophilus* showed that Tyr139 and Lys185 are important for the catalytic function, which correspond to Tyr145 and Lys195 in *E. coli* LeuB and Tyr142 and Lys191 in *S. pombe* Leu1, respectively (Miyazaki and Oshima 1993, Palló *et al.* 2014). Structural analysis using *T. thermophilus* also showed that Mn^{2+} binds Asp 217 (second subunit of the dimeric enzyme), Asp241, and Asp 245, which correspond to Asp227, Asp251, and Asp255 in *E. coli* LeuB and Asp224, Asp249, and Asp253 in *S. pombe* Leu1, respectively (Palló *et al.* 2014). Furthermore, the substrate, (2*R*,3*S*)-3-isopropylmalate, binds *T. thermophilus* LeuB via the Lys94, Lys104, Lys132, Tyr139, Asp217 (second subunit of the dimeric enzyme via Mn^{2+}), and Asp241 (via Mn^{2+}), which correspond to Lys99, Lys109, Lys138, Tyr145, Asp227, and Asp251 in *E. coli* LeuB and Lys96, Lys106, Lys135, Tyr142, Asp224, and Asp249 in *S. pombe* Leu1, respectively (Palló *et al.* 2014).

Table 1. Plasmid vectors using leucine auxotrophy in *S. pombe*.

Plasmid	Replication origin	Marker	Promoter	Chromosome integration	Tag	References
pALSK$^+$	*ars1*	*ScLEU2*	–	–	–	(Tanaka *et al.* 2000)
pART1	*ars1*	*ScLEU2*	*adh1*	–	–	(McLeod *et al.* 1987)
pcL-X	*ars1*	*ScLEU2*	SV40	–	–	(Tanaka *et al.* 2000)
pCMVL	*ars1*	*ScLEU2*	CMV	–	–	(Igarashi *et al.* 1991)
pDB248X	Sc 2μm	*ScLEU2*	–	–	–	(Nischt *et al.* 1986)
pDUAL	*ars1*	*leu1*	–	–	–	
(pDUAL2)		*ura4*$^+$	*nmt1*$^+$	○	FLAG His GFP *ccdB*	(Matsuyama *et al.* 2004)
pIL2	–	*ScLEU2*	–	○	–	(Nakamura *et al.* 2001)
pIRT2	*ars1*	*ScLEU2*	–	–		(Hindley *et al.* 1987)
pJK13 pJK148	–	*leu1*	–	○	–	(Keeney and Boeke 1994)
pLB-Dblet	*ars3002*	*ScLEU2*	–	–	–	(Yamada *et al.* 1997)
pREP1					– HA	
pREP3					His	(Maundrell 1993)
pREP41 pREP81	*ars1*	*ScLEU2*	*nmt1*$^+$	–	Myc GFP Pk	(Craven *et al.* 1998)
		– *ura4*$^+$				
	–	*ScLEU2*	–		–	
pRGG	*ars1*	*Kan* *Nat* *hph* *bsd*	*nmt1*$^+$ *adh1* *urg1*	– ○	GFP mCherry CFP	(Kiriya *et al.* 2017)
pRIP1/s pRIP3/s	–	*ScLEU2* *sup3-5*	*nmt1*$^+$	○	–	(Maundrell 1993)

of the chronological lifespan (Ohtsuka and Aiba 2017, Shimasaki *et al.* 2020, Ohtsuka *et al.* 2021). Currently, the details of the relationships are not clear, but the cellular response evoked by the induction of Ecl1 family proteins is similar to that caused by the inhibition of TORC1 signaling.

Inhibition of TORC1 complex

TOR is a serine/threonine kinase which is highly conserved among eukaryotes and which regulates various AGC kinases and controls intracellular responses such as growth, autophagy, cell cycle, translation, sexual differentiation, and the maintenance of telomere length (Hidayat *et al.* 2003, Urban *et al.* 2007, Jacinto and Lorberg 2008, Kupiec and Weisman 2012, Otsubo *et al.* 2020). Two TOR complexes have been identified, namely, TORC1 and TORC2. In *S. pombe*, Tor1 is a component of rapamycin-insensitive TORC2, and Tor2 is a component of TORC1 (Petersen 2009). Tor1 can also reportedly act as TORC1 (Hartmuth and Petersen 2009). TORC1 normally comprises Mip1, Pop3, Tco89, Toc1, and Tor2, whereas TORC2 consists of Bit61, Pop3, Sin1, Ste20, and Tor1 (Hayashi *et al.* 2007, Otsubo and Yamamato 2008, Yanagida 2009). In mammals, Leu has been reported to regulate autophagy via acetylation of the TORC1 component raptor, a homolog of the *S. pombe* Mip1 (Son *et al.* 2020). Furthermore, in mammalian cells, the effect of TORC1 signaling, which is inactivated by amino acid starvation, depends on the type of amino acid. However, the withdrawal of Leu or Arg is nearly as effective in downregulating TORC1 signaling as the withdrawal of all amino acids (Avruch *et al.* 2009).

Rapamycin, a macrolide known to be an immunosuppressive and antiproliferative agent, forms an intracellular complex with immunophilin FKBP12 and suppresses TORC1 activity (Nakashima *et al.* 2010, Otsubo *et al.* 2017). Rapamycin does not inhibit the growth of *S. pombe* cells in complete medium but does inhibit the growth of Leu auxotrophic cells in minimal medium (Weisman *et al.* 2005). In an *in vitro* assay, it has been reported that rapamycin inhibits the fission yeast TORC1 kinase activity (Takahara and Maeda 2012).

In *S. pombe*, TORC1 is regulated primarily by three signaling pathways, namely, the tuberous sclerosis complex (TSC) complex, GAP activity toward the Rag GTPase 1 (GATOR1), and the general amino acid control pathway (Fukuda *et al.* 2021). Although Leu starvation activates TORC1 via the general amino acid control pathway, some perturbations in the other two signaling pathways can change the TORC1 activity and also affect the cellular phenotypes against Leu (van Slegtenhorst *et al.* 2005, Ma *et al.* 2013). In humans, inactivation of tuberous sclerosis protein TSC1 or TSC2 causes tuberous sclerosis (Matsumoto *et al.* 2002). In *S. pombe*, the TSC complex, which negatively regulates Rheb GTPase, consists of Tsc1 and Tsc2 (Matsumoto *et al.* 2002, Davie *et al.* 2015). In *S. pombe*, Rheb is encoded by *rhb1*$^+$ and activates TORC1 activity (Weisman *et al.* 2007, Murai *et al.* 2009, Fukuda *et al.* 2021). Each deletion of *tsc1*$^+$ or *tsc2*$^+$ or *rhb1* mutant decreases Leu import (Matsumoto *et al.* 2002, van Slegtenhorst *et al.* 2005, Weisman *et al.* 2007, Murai *et al.* 2009, Ma *et al.* 2013). GATOR1, consisting of Iml1, Npr2, and Npr3, suppresses TORC1 via Rag GTPase Gtr1 and Gtr2 (Chia *et al.* 2017). The deletion of *npr2*$^+$ also decreases Leu import (Ma *et al.* 2013). The general amino acid control pathway transmits the amino acid starvation signal to TORC1 (Fukuda *et al.* 2021). It has been reported that a decrease in the precursors of tRNA, which

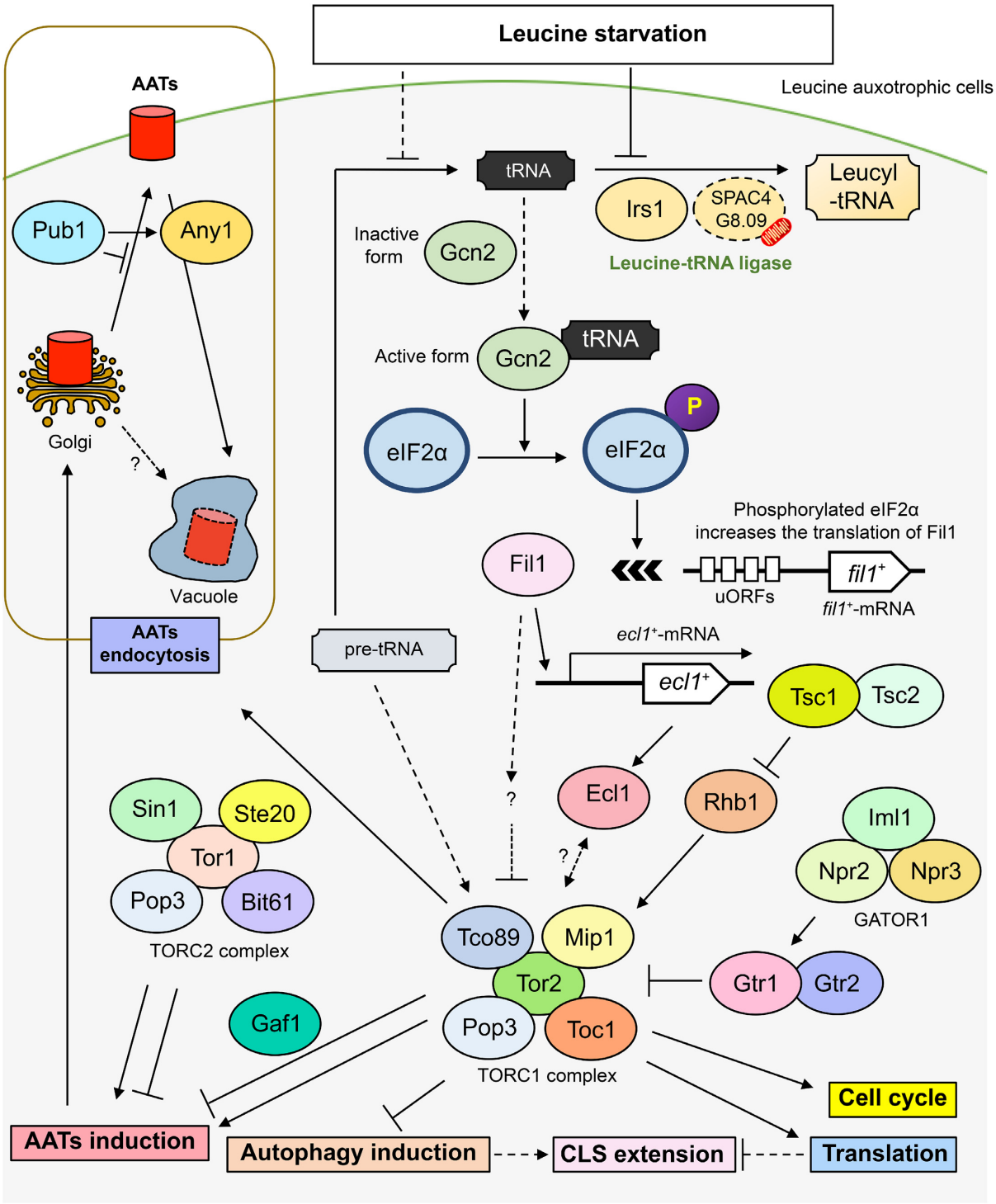

Figure 4. Response to leucine starvation in the fission yeast *Schizosaccharomyces pombe*. The details of each process are described in the text. AAT, amino acid transporter.

also affects the general amino acid control pathway, may suppress TORC1 activity (Otsubo *et al.* 2018, Otsubo *et al.* 2020). Thus, the Leu starvation signal suppresses TORC1 activity via the general amino acid control pathway, and appropriate intracellular responses appear to be caused through the various factors regulated TORC1, such as the AGC kinases Psk1, Sck1, and Sck2 (Nakashima *et al.* 2012, Otsubo *et al.* 2017).

Regulation of amino acid transporters

In *S. pombe*, TORC1 and TORC2 regulate the expression of amino acid transporters (AATs), in different directions (Ma *et al.* 2015). That is, suppression of TORC1 increases the expression of some AATs, whereas suppression of TORC2 decreases the expression of the AATs (Weisman *et al.* 2007, Krzyzanowski *et al.* 2012, Ma *et al.* 2015). In *S. pombe*, these reverse effects of TORC1 and TORC2 are

also observed in other phenotypes. Inactivation of TORC1 causes cell shortening and cell cycle arrest in G1, whereas deletion mutants of *tor1*$^+$ or its substrate *gad8*$^+$ cause cell elongation and inability to arrest in G1 (Martín and Lopez-Aviles 2018). Deletion mutants of *tsc1*$^+$ or *tsc2*$^+$ increase TORC1 activity and decrease the expression of AATs, including *isp5*$^+$, resulting in a decrease in the uptake of Arg and Leu (van Slegtenhorst *et al.* 2004, van Slegtenhorst *et al.* 2005, Weisman *et al.* 2007). The growth of Δ*tsc2* or Δ*npr2* mutants is strongly influenced by Leu auxotrophy (Ma *et al.* 2013), possibly due to poor Leu uptake resulting from increased TORC1 activity. However, some AATs are controlled in the opposite direction to the transporters described above. The loss of Tor1 decreases the expression of AAT genes including *isp5*$^+$, *per1*$^+$, *put4*$^+$, and *SPBPB2B2.01* but increases the expression of *cat1*$^+$, whereas inhibition of Tor2 increases *isp5*$^+$, *per1*$^+$, *put4*$^+$, and *SPBPB2B2.01* but decreases *cat1*$^+$ (Ma *et al.* 2015). It has been reported that the GATA transcription factor Gaf1 is involved in the regulation of expression of AAT by TORC1 (Ma *et al.* 2015).

The suppression of TORC1 by amino acid starvation regulates the localization of AATs, including the cationic amino acid transporter Cat1, to the cell membrane surface (Ma *et al.* 2013). Conversely, the upregulation of TORC1 activity by deletion of *tsc2*$^+$ causes mis-localization of Cat1 (Aspuria and Tamanoi 2008, Takahashi *et al.* 2012). Thus, in conditions of amino acid starvation, TORC1 is considered to regulate the amino acid uptake by regulating the transcription and localization of AATs.

The Spt-Ada-Gcn acetyltransferase (SAGA) complex is a multiprotein complex that modifies chromatin (Soffers and Workman 2020) and is thought to control AATs and regulate Leu uptake in *S. pombe* (Takahashi *et al.* 2012). Deletion of *gcn5*$^+$, which encodes a component of the SAGA complex, reduces the uptake of Leu, which depends on the amino acid permease Agp3 (Takahashi *et al.* 2012). *gcn5*$^+$ genetically interacts with *tor2*$^+$ and *tco89*$^+$, encoding TORC1 subunits, and with *tor1*$^+$, encoding a TORC2 subunit (Ryan *et al.* 2012, Laboucarié *et al.* 2017). *ngg1*$^+$ and *sgf29*$^+$, encoding other subunits of the SAGA complex, also genetically interact with *tco89*$^+$ (Ryan *et al.* 2012), and *taf12*$^+$, encoding another subunit of the SAGA complex, interacts with *tor1*$^+$ and *tor2*$^+$ (Laboucarié *et al.* 2017). TORC1 and TORC2 respond to starvation and regulate the phosphorylation status of Taf12 (Laboucarié *et al.* 2017).

In *S. pombe*, high-quality nitrogen sources such as NH_4^+ suppress the uptake of poor nitrogen sources such as amino acids (Takahashi *et al.* 2012). HECT-type ubiquitin-protein ligase E3, Pub1, negatively regulates Leu uptake by preventing the localization of AATs to the cell membrane, via the ubiquitination of AATs in the presence of NH_4^+ (Karagiannis *et al.* 1999, Nakase *et al.* 2012, Takahashi *et al.* 2012). AATs on the cell membrane are transported to the vacuole by endosomal sorting complex required for transport (Nakase *et al.* 2012). Endocytosis of the AAT Cat1 is regulated by Any1, which is an arrestin-related trafficking adaptor (Nakase *et al.* 2013, Nakashima *et al.* 2014). Pub1 interacts with and ubiquitinates Any1, leading to AAT endocytosis under the regulation of TSC-Rheb pathway (Nakase *et al.* 2013, Nakashima *et al.* 2014).

In addition, the AATs involved in Leu uptake in *S. pombe* may also be affected by pH. Leu auxotrophic *leu1-32* cells grow poorly under neutral to basic pH conditions, suggesting that the pH of the extracellular environment is important for Leu uptake (Arndt and Atkins 1996).

Regulation of the cell cycle, chronological lifespan, and sexual development

Leu starvation arrests the cell cycle at G1 in a manner dependent on the Ecl1 family proteins (Ohtsuka *et al.* 2019). This response may occur via TORC1 because both factors are associated with general amino acid control (Ohtsuka *et al.* 2019, Fukuda *et al.* 2021); however, the details are not known. Treatment with both rapamycin and caffeine has been shown to suppress TORC1 but does not induce G1 arrest (Takahara and Maeda 2012). Furthermore, sulfur depletion causes cell cycle arrest in an Ecl1 family protein-dependent manner, but the cell cycle arrest occurs at G2 phase (Ohtsuka *et al.* 2017, Ohtsuka *et al.* 2021a). Meanwhile, although the detailed relationship between Leu and the cell cycle is unknown, it has been reported that the deletion of G1 cyclin Pas1 decreases the uptake of Arg and Leu independently of the effect of TORC1 activation (van Slegtenhorst *et al.* 2005).

The chronological lifespan is assessed by the survival of cells entering the stationary phase (Takuma *et al.* 2013, Hibi *et al.* 2018, Legon and Rallis 2021). More than 100 genes have been reported to be involved in the lifespan extension, more than 30 drugs extend the lifespan, and starvation of various types of nutrients extend the lifespan in *S. pombe* (Matsui *et al.* 2021, Ohtsuka *et al.* 2021b,c, Romila *et al.* 2021). Leu starvation extends the chronological lifespan of *S. pombe* Leu auxotrophic cells but not prototrophic cells, in an Ecl1 family protein-dependent manner (Ohtsuka *et al.* 2019). Leu starvation also results in a decrease in intracellular ribosome levels, which has been suggested to be important for regulation of the chronological lifespan (Ohtsuka *et al.* 2021). Leu starvation induces autophagy, which is required for maintaining the chronological lifespan during Leu starvation (Corral-Ramos *et al.* 2021). Meanwhile, lifespan studies have been conducted not only on Leu auxotrophic cells, but also on other amino acid-auxotrophic cells. The restriction of Lys, one of the essential amino acids for mammals, in Lys-auxotrophic cells extends the chronological lifespan, similar to the restriction of Leu, which also extends the chronological lifespan (Ohtsuka *et al.* 2019). Although a strain with the *leu1-32* mutation that leads to Leu auxotroph tends to have a longer lifespan than a prototrophic strain (Ohtsuka *et al.* 2017, Hibi *et al.* 2018, Matsui *et al.* 2021), it is likely to be the same for Lys: the deletion of *lys7*$^+$, which is involved in Lys biosynthesis, reportedly extends the lifespan (Rallis *et al.* 2014, Ohtsuka *et al.* 2021b). The restriction of Arg, which is an essential amino acid for chicken or salmon, but not for humans (Oda 2007), in Arg-auxotrophic cells also extends the chronological lifespan (Ohtsuka *et al.* 2019). Although Leu restriction significantly affects the lifespan in an autophagy mutant with Leu auxotrophy, Arg or Lys restriction has no significant effect on the corresponding mutants with Arg or Lys auxotrophy (Corral-Ramos *et al.* 2021). However, in histidine (His)-auxotrophic strains, His starvation has been shown to shorten the lifespan (Ohtsuka *et al.* 2019).

Unlike nitrogen starvation, Leu starvation does not induce sexual development (Ohtsuka *et al.* 2019). It is not known whether the inability to synthesize Leu causes a deficiency in the synthesis of new proteins required for sexual development or whether Leu starvation induces intracellular signaling pathways that suppresses the sexual development response.

Conclusions

Like other fungi, *S. pombe* can synthesize Leu, but mutations in the Leu biosynthetic pathway can cause a Leu auxotrophic phenotype, sometimes resulting in different phenotypes of the prototrophic cells. However, Leu auxotrophy has markedly increased the usefulness of this organism as a model organism, and many plasmids that can only be used in Leu auxotrophic strains have been designed. Although the study on the Leu biosynthesis pathway in *S. pombe* is not as advanced as that in *S. cerevisiae*, it is sug-

gested that the protein encoded by SPAC17G8.06c is a functional ortholog of *S. cerevisiae* Ilv3 (Fig. 2). Among the Leu biosynthetic pathway, a mutation of *leu1*$^+$, *leu1-32*, is widely used, which causes Leu auxotrophy. The *leu1-32* mutation is a mutation that causes an amino acid substitution at the helix-capping residue, suggesting that this mutation cannot maintain a proper structure and loses its enzymatic activity.

Leu starvation leads to the induction of autophagy, a decrease in the number of ribosomes, and extension of the chronological lifespan through the induction of the general amino acid control and suppression of TORC1. Because some intracellular responses due to amino acid starvation are also observed in higher organisms, amino acid-auxotrophic yeast may be a useful model in studies analyzing the amino acid starvation responses in higher organisms. A full understanding of the effects of essential amino acids, including Leu, on organisms contributes to an accurate understanding of various physiological responses, including lifespan. In Leu auxotrophic cells of *S. pombe*, the cellular response activity to Leu starvation can be easily regulated by adjusting the amount of Leu in the medium. Changes in the amount of Leu supplementation significantly alter the expression of *ecl1*$^+$ that responds to Leu starvation. Moreover, Leu removal causes a variety of responses, such as increasing the activity of general amino acid control, suppressing TORC1 activity, extending the lifespan, and arresting G1 (Ohtsuka *et al.* 2019, Corral-Ramos *et al.* 2021, Fukuda *et al.* 2021, 2021). To study Leu starvation, the findings obtained from studies using Leu auxotrophic cells of *S. pombe* are expected to contribute to the understanding of not only *S. pombe* itself but also higher organisms that cannot synthesize Leu, because the factors involved in signal transduction pathway respond to Leu starvation, such as TORC1 signaling or general amino acid control, are conserved from yeast to mammals.

Consent to participate

Not applicable.

Availability of data and material

All the data have been presented in the manuscript.

Ethics approval

Not applicable.

Consent for publication

Not applicable.

Author contributions

HO has made major contributions to this study and toward writing the manuscript. TS performed experiments for characterization of *ilv3*$^+$. TS and HA have contributed to the factual and logical confirmation and revision of this manuscript.

Acknowledgments

We are grateful to the scientists whose work provided the basis for this review. We would also like to thank M Wada, K Hayashi, and K Tanaka for technical assistance and Enago (www.enago.jp) for the English language review.

Funding

This work was supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan [JP21K05363 to HO] and a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and [JP20H02898 to HA].

Conflict of interests statement. None declared.

References

Anda S, Zach R, Grallert B. Activation of Gcn2 in response to different stresses. Mata J (ed). *PLoS One* 2017;**12**:e0182143.

Arndt GM, Atkins D. pH sensitivity of *Schizosaccharomyces pombe*: effect on the cellular phenotype associated with lacZ gene expression. *Curr Genet* 1996;**29**:457–61.

Aspuria P-J, Tamanoi F. The Tsc/Rheb signaling pathway controls basic amino acid uptake via the Cat1 permease in fission yeast. *Mol Genet Genomics* 2008;**279**:441–50.

Aurora R, Rose GD. Helix capping. *Protein Sci* 1998;**7**:21–38.

Avruch J, Long X, Ortiz-Vega S *et al.* Amino acid regulation of TOR complex 1. *Am J Physiol Endocrinol Metab* 2009;**296**:E592–602.

Azuma K, Ohtsuka H, Murakami H *et al.* Extension of chronological lifespan by Scecl1 depends on mitochondria in *Saccharomyces cerevisiae*. *Biosci Biotechnol Biochem* 2012;**76**:1938–42.

Beach D, Nurse P. High-frequency transformation of the fission yeast *Schizosaccharomyces Pombe*. *Nature* 1981;**290**:140–2.

Bekkaoui F, Nadin-Davis SA, Crosby WL. Isolation and structure of an acetolactate synthase gene from *Schizosaccharomyces pombe* and complementation of the *ilv2* mutation in *Saccharomyces cerevisiae*. *Curr Genet* 1993;**24**:544–7.

Bezsudnova EY, Boyko KM, Popov VO. Properties of bacterial and archaeal branched-chain amino acid aminotransferases. *Biochem* 2017;**82**:1572–91.

Bledsoe RK, Dawson PA, Hutson SM. Cloning of the rat and human mitochondrial branched chain aminotransferases (BCATm). *Biochimica Et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* 1997;**1339**:9–13.

Chia KH, Fukuda T, Sofyantoro F *et al.* Ragulator and GATOR1 complexes promote fission yeast growth by attenuating TOR complex 1 through Rag GTPases. *Elife* 2017;**6**:1–21.

Corral-Ramos C, Barrios R, Ayté J *et al.* TOR and MAP kinase pathways synergistically regulate autophagy in response to nutrient depletion in fission yeast. *Autophagy* 2021;1–16.

Davie E, Forte GMA, Petersen J. Nitrogen regulates AMPK to control TORC1 signaling. *Curr Biol* 2015;**25**:445–54.

Dilova I, Easlon E, Lin SJ. Calorie restriction and the nutrient sensing signaling pathways. *Cell Mol Life Sci* 2007;**64**:752–67.

Duncan CDS, Rodríguez-López M, Ruis P *et al.* General amino acid control in fission yeast is regulated by a nonconserved transcription factor, with functions analogous to Gcn4/Atf4. *Proc Natl Acad Sci* 2018;**115**:E1829–38.

Eden A, Benvenisty N. Characterization of a branched-chain amino-acid aminotransferase from *Schizosaccharomyces pombe*. *Yeast* 1998;**14**:189–94.

Fondi M, Brilli M, Emiliani G *et al.* The primordial metabolism: an ancestral interconnection between leucine, arginine, and lysine biosynthesis. *BMC Evol Biol* 2007;**7 Suppl 2**:S3.

Fontana L, Partridge L. Promoting health and longevity through diet: from model organisms to humans. *Cell* 2015;**161**:106–18.

Fukuda T, Sofyantoro F, Tai YT *et al.* Tripartite suppression of fission yeast TORC1 signaling by the GATOR1-Sea3 complex, the TSC complex, and Gcn2 kinase. *Elife* 2021;**10**:1–22.

Gallinetti J, Harputlugil E, Mitchell JR Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR. *Biochem J* 2013;**449**:1–10.

González A, Hall MN. Nutrient sensing and TOR signaling in yeast and mammals. *EMBO J* 2017;**36**:397–408.

Gráczer É, Merli A, Singh RK *et al.* Atomic level description of the domain closure in a dimeric enzyme: *Thermus thermophilus* 3-isopropylmalate dehydrogenase. *Mol Biosyst* 2011;**7**: 1646–59.

Hammer SK, Zhang Y, Avalos JL. Mitochondrial compartmentalization confers specificity to the 2-ketoacid recursive pathway: increasing isopentanol production in *Saccharomyces cerevisiae*. *ACS Synthetic Biology* 2020;**9**:546–55.

Hartmuth S, Petersen J. Fission yeast Tor1 functions as part of TORC1 to control mitotic entry through the stress MAPK pathway following nutrient stress. *J Cell Sci* 2009;**122**:1737–46.

Hayashi T, Hatanaka M, Nagao K *et al.* Rapamycin sensitivity of the *Schizosaccharomyces pombe tor2* mutant and organization of two highly phosphorylated TOR complexes by specific and common subunits. *Genes Cells* 2007;**12**:1357–70.

Hibi T, Ohtsuka H, Shimasaki T *et al.* Tschimganine and its derivatives extend the chronological life span of yeast via activation of the Sty1 pathway. *Genes Cells* 2018;**23**:620–37.

Hidayat S, Yoshino K, Tokunaga C *et al.* Inhibition of amino acid-mTOR signaling by a leucine derivative induces G1 arrest in Jurkat cells. *Biochem Biophys Res Commun* 2003;**301**:417–23.

Hindley J, Phear G, Stein M *et al. suc1*$^{+}$ encodes a predicted 13-kilodalton protein that is essential for cell viability and is directly involved in the division cycle of *Schizosaccharomyces pombe*. *Mol Cell Biol* 1987;**7**:504–11.

Igarashi M, Nagata A, Jinno S *et al. wee1*$^{+}$-like gene in human cells. *Nature* 1991;**353**:80–3.

Imai Y, Shimasaki T, Enokimura C *et al. gas1* mutation extends chronological lifespan via Pmk1 and Sty1 MAPKs in *Schizosaccharomyces pombe*. *Biosci Biotechnol Biochem* 2020;**84**:330–7.

Jacinto E, Lorberg A. TOR regulation of AGC kinases in yeast and mammals. *Biochem J* 2008;**410**:19–37.

Jiménez-Saucedo T, Berlanga JJ, Rodríguez-Gabriel M. Translational control of gene expression by eIF2 modulates proteostasis and extends lifespan. *Aging* 2021;**13**:10989–1009.

Joutel A, Ducros A, Alamowitch S *et al.* A human homolog of bacterial acetolactate synthase genes maps within the CADASIL critical region. *Genomics* 1996;**38**:192–8.

Kamada Y. Novel tRNA function in amino acid sensing of yeast Tor complex1. *Genes Cells* 2017;**22**:135–47.

Karagiannis J, Saleki R, Young PG. The *pub1* E3 ubiquitin ligase negatively regulates leucine uptake in response to NH_4^+ in fission yeast. *Curr Genet* 1999;**35**:593–601.

Keeney JB, Boeke JD. Efficient targeted integration at *leu1-32* and *ura4-294* in *Schizosaccharomyces pombe*. *Genetics* 1994;**136**:849–56.

Kikuchi Y, Kitazawa Y, Shimatake H *et al.* The primary structure of the *leu1*$^{+}$ gene of *Schizosaccharomyces pombe*. *Curr Genet* 1988;**14**:375–9.

Kilberg MS, Balasubramanian M, Fu L *et al.* The transcription factor network associated with the amino acid response in mammalian cells. *Adv Nutr* 2012;**3**:295–306.

Kiriya K, Tsuyuzaki H, Sato M. Module-based systematic construction of plasmids for episomal gene expression in fission yeast. *Gene* 2017;**637**:14–24.

Kohda TA, Tanaka K, Konomi M *et al.* Fission yeast autophagy induced by nitrogen starvation generates a nitrogen source that drives adaptation processes. *Genes Cells* 2007;**12**:155–70.

Kohlhaw GB. Leucine biosynthesis in fungi: entering metabolism through the back door. *Microbiol Mol Biol Rev* 2003;**67**:1–15, table of contents.

Kohli J, Hottinger H, Munz P *et al.* Genetic mapping in *Schizosaccharomyces pombe* by mitotic and meiotic analysis and induced haploidization. *Genetics* 1977;**87**:471–89.

Kondo T, Tezuka H, Ishii J *et al.* Genetic engineering to enhance the Ehrlich pathway and alter carbon flux for increased isobutanol production from glucose by *Saccharomyces cerevisiae*. *J Biotechnol* 2012;**159**:32–7.

Krzyzanowski MK, Kozlowska E, Kozlowski P. Identification and functional analysis of the *erh1*$^{+}$ gene encoding enhancer of rudimentary homolog from the fission yeast *Schizosaccharomyces pombe*. *PLoS One* 2012;**7**:e49059.

Kupiec M, Weisman R. TOR links starvation responses to telomere length maintenance. *Cell Cycle* 2012;**11**:2268–71.

Kurauchi T, Hashizume A, Imai Y *et al.* Identification of a novel protein kinase that affects the chronological lifespan in fission yeast. *FEMS Microbiol Lett* 2017;**364**:fnw257.

Laboucarié T, Detilleux D, Rodriguez-Mias RA *et al.* TORC1 and TORC2 converge to regulate the SAGA co-activator in response to nutrient availability. *EMBO Rep* 2017;**18**:2197–218.

Larson EM, Idnurm A. Two origins for the gene encoding alpha-isopropylmalate synthase in fungi. *PLoS One* 2010;**5**:e11605.

Le Couteur DG, Solon-Biet SM, Cogger VC *et al.* Branched chain amino acids, aging and age-related health. *Ageing Res Rev* 2020;**64**:101198.

Legon L, Rallis C. Genome-wide screens in yeast models towards understanding chronological lifespan regulation. *Brief Funct Genomics* 2021;1–9.

Liu Y, Li Y, Wang X. Acetohydroxyacid synthases: evolution, structure, and function. *Appl Microbiol Biotechnol* 2016;**100**:8633–49.

Ma N, Liu Q, Zhang L *et al.* TORC1 signaling is governed by two negative regulators in fission yeast. *Genetics* 2013;**195**:457–68.

Ma Y, Ma N, Liu Q *et al.* Tor signaling regulates transcription of amino acid permeases through a GATA transcription factor Gaf1 in fission yeast. *PLoS One* 2015;**10**:e0144677.

Mariño G, Pietrocola F, Eisenberg T *et al.* Regulation of autophagy by cytosolic acetyl-coenzyme A. *Mol Cell* 2014;**53**:710–25.

Martín R, Lopez-Aviles S. Express yourself: how PP2A-B55^{Pab1} helps TORC1 talk to TORC2. *Curr Genet* 2018;**64**:43–51.

Matsui K, Okamoto K, Hasegawa T *et al.* Identification of ksg1 mutation showing long-lived phenotype in fission yeast. *Genes Cells* 2021;**26**:967–78.

Matsumoto S, Bandyopadhyay A, Kwiatkowski DJ *et al.* Role of the Tsc1-Tsc2 complex in signaling and transport across the cell membrane in the fission yeast *Schizosaccharomyces pombe*. *Genetics* 2002;**161**:1053–63.

Matsuyama A, Arai R, Yashiroda Y *et al.* ORFeome cloning and global analysis of protein localization in the fission yeast *Schizosaccharomyces pombe*. *Nat Biotechnol* 2006;**24**:841–7.

Matsuyama A, Shirai A, Yashiroda Y *et al.* pDUAL, a multipurpose multicopy vector capable of chromosomal integration in fission yeast. *Yeast* 2004;**21**:1289–305.

Maundrell K. Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene* 1993;**123**:127–30.

McDonald RA, Satyanarayana T, Kaplan JG. Biosynthesis of branched-chain amino acids in *Schizosaccharomyces pombe*: properties of acetohydroxy acid synthetase. *J Bacteriol* 1973;**114**: 332–40.

McDonald RA, Satyanarayana T, Kaplan JG. Biosynthesis of branched-chain amino acids in *Schizosaccharomyces pombe*:

regulation of the enzymes involved in isoleucine, valine, and leucine synthesis. *Can J Biochem* 1974;**52**:51–9.

McLeod M, Stein M, Beach D. The product of the *mei3*$^+$ gene, expressed under control of the mating-type locus, induces meiosis and sporulation in fission yeast. *EMBO J* 1987;**6**:729–36.

Miflin BJ. The location of nitrite reductase and other enzymes related to amino acid biosynthesis in the plastids of root and leaves. *Plant Physiol* 1974;**54**:550–5.

Miwa Y, Ohtsuka H, Naito C *et al.* Ecl1, a regulator of the chronological lifespan of *Schizosaccharomyces pombe*, is induced upon nitrogen starvation. *Biosci Biotechnol Biochem* 2011;**75**:279–83.

Murai T, Nakase Y, Fukuda K *et al.* Distinctive responses to nitrogen starvation in the dominant active mutants of the fission yeast Rheb GTPase. *Genetics* 2009;**183**:517–27.

Nakamura T, Nakamura-Kubo M, Hirata A *et al.* The *Schizosaccharomyces pombe spo3*$^+$ gene is required for assembly of the forespore membrane and genetically interacts with *psy1*$^+$-encoding syntaxin-like protein. *Mol Biol Cell* 2001;**12**:3955–72.

Nakase M, Nakase Y, Chardwiriyapreecha S *et al.* Intracellular trafficking and ubiquitination of the *Schizosaccharomyces pombe* amino acid permease Aat1p. *Microbiology* 2012;**158**:659–73.

Nakase Y, Nakase M, Kashiwazaki J *et al.* The fission yeast β-arrestin-like protein Any1 is involved in TSC-Rheb signaling and the regulation of amino acid transporters. *J Cell Sci* 2013;**126**:3972–81.

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

Nakashima A, Otsubo Y, Yamashita A *et al.* Psk1, an AGC kinase family member in fission yeast, is directly phosphorylated and controlled by TORC1 and functions as S6 kinase. *J Cell Sci* 2012;**125**:5840–9.

Nakashima A, Sato T, Tamanoi F. Fission yeast TORC1 regulates phosphorylation of ribosomal S6 proteins in response to nutrients and its activity is inhibited by rapamycin. *J Cell Sci* 2010;**123**:777–86.

Nischt R, Thüroff E, Käufer NF. Molecular cloning of a ribosomal protein gene from the fission yeast *Schizosaccharomyces ponbe*. *Curr Genet* 1986;**10**:365–70.

Oda H. Essential amino acids and nonessential amino acids in evolution. *Nippon Eiyo Shokuryo Gakkaishi* 2007;**60**:137–49.

Ohtsuka H, Aiba H. Factors extending the chronological lifespan of yeast: Ecl1 family genes. *FEMS Yeast Res* 2017;**17**:fox066.

Ohtsuka H, Kato T, Sato T *et al.* Leucine depletion extends the lifespans of leucine-auxotrophic fission yeast by inducing Ecl1 family genes via the transcription factor Fil1. *Mol Genet Genomics* 2019;**294**:1499–509.

Ohtsuka H, Kobayashi M, Shimasaki T *et al.* Magnesium depletion extends fission yeast lifespan via general amino acid control activation. *Microbiologyopen* 2021;**10**:e1176.

Ohtsuka H, Shimasaki T, Aiba H. Extension of chronological lifespan in *Schizosaccharomyces pombe*. *Genes Cells* 2021c;**26**:459–73.

Ohtsuka H, Shimasaki T, Aiba H. Genes affecting the extension of chronological lifespan in *Schizosaccharomyces pombe* (fission yeast). *Mol Microbiol* 2021b;**115**:623–42.

Ohtsuka H, Shimasaki T, Aiba H. Response to sulfur in *Schizosaccharomyces pombe*. *FEMS Yeast Res* 2021a;**21**:1–9.

Ohtsuka H, Takinami M, Shimasaki T *et al.* Sulfur restriction extends fission yeast chronological lifespan through Ecl1 family genes by downregulation of ribosome. *Mol Microbiol* 2017;**105**:84–97.

Otsubo Y, Kamada Y, Yamashita A. Novel links between TORC1 and traditional non-coding RNA, tRNA. *Genes* 2020;**11**:956.

Otsubo Y, Matsuo T, Nishimura A *et al.* tRNA production links nutrient conditions to the onset of sexual differentiation through the TORC1 pathway. *EMBO Rep* 2018;**19**:1–12.

Otsubo Y, Nakashima A, Yamamoto M *et al.* TORC1-dependent phosphorylation targets in fission yeast. *Biomolecules* 2017;**7**:50.

Otsubo Y, Yamamato M. TOR signaling in fission yeast. *Crit Rev Biochem Mol Biol* 2008;**43**:277–83.

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

Petersen J. TOR signalling regulates mitotic commitment through stress-activated MAPK and Polo kinase in response to nutrient stress. *Biochem Soc Trans* 2009;**37**:273–7.

Powers RW, Kaeberlein M, Caldwell SD *et al.* Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes Dev* 2006;**20**:174–84.

Rallis C, López-Maury L, Georgescu T *et al.* Systematic screen for mutants resistant to TORC1 inhibition in fission yeast reveals genes involved in cellular ageing and growth. *Biology Open* 2014;**3**:161–71.

Romila CA, Townsend S, Malecki M *et al.* Barcode sequencing and a high-throughput assay for chronological lifespan uncover ageing-associated genes in fission yeast. *Microbial Cell (Graz, Austria)* 2021;**8**:146–60.

Ruckenstuhl C, Netzberger C, Entfellner I *et al.* Lifespan extension by methionine restriction requires autophagy-dependent vacuolar acidification. *PLos Genet* 2014;**10**:e1004347.

Ryan CJ, Roguev A, Patrick K *et al.* Hierarchical modularity and the evolution of genetic interactomes across species. *Mol Cell* 2012;**46**:691–704.

Ryan ED, Kohlhaw GB. Subcellular localization of isoleucine-valine biosynthetic enzymes in yeast. *J Bacteriol* 1974;**120**:631–7.

Seike T, Maekawa H, Nakamura T *et al.* The asymmetric chemical structures of two mating pheromones reflect their differential roles in mating of fission yeast. *J Cell Sci* 2019;**132**:1–8.

Shimasaki T, Ohtsuka H, Naito C *et al.* Ecl1 is a zinc-binding protein involved in the zinc-limitation-dependent extension of chronological life span in fission yeast. *Mol Genet Genomics* 2017;**292**:475–81.

Shimasaki T, Ohtsuka H, Naito C *et al.* Ecl1 is activated by the transcription factor Atf1 in response to H_2O_2 stress in *Schizosaccharomyces pombe*. *Mol Genet Genomics* 2014;**289**:685–93.

Shimasaki T, Okamoto K, Ohtsuka H *et al.* Sulfur depletion induces autophagy through Ecl1 family genes in fission yeast. *Genes Cells* 2020;**25**:825–30.

Siam R, Dolan WP, Forsburg SL. Choosing and using *Schizosaccharomyces pombe* plasmids. *Methods* 2004;**33**:189–98.

Sickmann A, Reinders J, Wagner Y *et al.* The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc Natl Acad Sci* 2003;**100**:13207–12.

Soffers JHM, Workman JL. The SAGA chromatin-modifying complex: the sum of its parts is greater than the whole. *Genes Dev* 2020;**34**:1287–303.

Son SM, Park SJ, Stamatakou E *et al.* Leucine regulates autophagy via acetylation of the mTORC1 component raptor. *Nat Commun* 2020;**11**:1–13.

Takahara T, Maeda T. TORC1 of fission yeast is rapamycin-sensitive. *Genes Cells* 2012;**17**:698–708.

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

Takuma K, Ohtsuka H, Azuma K *et al.* The fission yeast *php2* mutant displays a lengthened chronological lifespan. *Biosci Biotechnol Biochem* 2013;**77**:1548–55.

Tanaka K, Yonekawa T, Kawasaki Y *et al.* Fission yeast Eso1p is required for establishing sister chromatid cohesion during S phase. *Mol Cell Biol* 2000;**20**:3459–69.

Tarumoto Y, Kanoh J, Ishikawa F. Receptor for activated C-kinase (RACK1) homolog Cpc2 facilitates the general amino acid control response through Gcn2 kinase in fission yeast. *J Biol Chem* 2013;**288**:19260–8.

Urban J, Soulard A, Huber A *et al.* Sch9 is a major target of TORC1 in *Saccharomyces cerevisiae*. *Mol Cell* 2007;**26**:663–74.

van Slegtenhorst M, Carr E, Stoyanova R *et al.* *tsc1*$^+$ and *tsc2*$^+$ regulate arginine uptake and metabolism in *Schizosaccharomyces pombe*. *J Biol Chem* 2004;**279**:12706–13.

van Slegtenhorst M, Mustafa A, Henske EP. Pas1, a G1 cyclin, regulates amino acid uptake and rescues a delay in G1 arrest in Tsc1 and Tsc2 mutants in *Schizosaccharomyces pombe*. *Hum Mol Genet* 2005;**14**:2851–8.

Wallon G, Kryger G, Lovett ST *et al.* Crystal structures of *Escherichia coli* and *Salmonella typhimurium* 3-isopropylmalate dehydrogenase and comparison with their thermophilic counterpart from *Thermus thermophilus*. *J Mol Biol* 1997;**266**:1016–31.

Weisman R, Roitburg I, Nahari T *et al.* Regulation of leucine uptake by *tor1*$^+$ in *Schizosaccharomyces pombe* is sensitive to rapamycin. *Genetics* 2005;**169**:539–50.

Weisman R, Roitburg I, Schonbrun M *et al.* Opposite effects of *tor1* and *tor2* on nitrogen starvation responses in fission yeast. *Genetics* 2007;**175**:1153–62.

Wess J, Brinek M, Boles E. Improving isobutanol production with the yeast *Saccharomyces cerevisiae* by successively blocking competing metabolic pathways as well as ethanol and glycerol formation. *Biotechnol Biofuels* 2019;**12**:1–15.

Yamada H, Ohmiya R, Yamamoto E *et al.* Characterization of multicopy suppressor genes that complement a defect in the Wis1-Sty1 MAP kinase cascade involved in stress responses in *Schizosaccharomyces pombe*. *J Gen Appl Microbiol* 1997;**43**:209–15.

Yanagida M. Cellular quiescence: are controlling genes conserved? *Trends Cell Biol* 2009;**19**:705–15.

Zaborske JM, Narasimhan J, Jiang L *et al.* Genome-wide analysis of tRNA charging and activation of the eIF2 kinase Gcn2p. *J Biol Chem* 2009;**284**:25254–67.