1. Summary

Biosynthesis of coenzyme A (CoA) requires a five-step process using pantothenate and cysteine in the fission yeast Schizosaccharomyces pombe. CoA contains a thiol (SH) group, which reacts with carboxylic acid to form thioesters, giving rise to acyl-activated CoAs such as acetyl-CoA. Acetyl-CoA is essential for energy metabolism and protein acetylation, and in higher eukaryotes, for the production of neurotransmitters. We isolated a novel S. pombe temperature-sensitive strain ppc1-537 mutated in the catalytic region of phosphopantothenoylcysteine synthetase (designated Ppc1), which is essential for CoA synthesis. The mutant becomes auxotrophic to pantothenate at permissive temperature, displaying greatly decreased levels of CoA, acetyl-CoA and histone acetylation. Moreover, ppc1-537 mutant cells failed to restore proliferation from quiescence. Ppc1 is thus the product of a super-housekeeping gene. The ppc1-537 mutant showed combined synthetic lethal defects with five of six histone deacetylase mutants, whereas sir2 deletion exceptionally rescued the ppc1-537 phenotype. In synchronous cultures, ppc1-537 cells can proceed to the S phase, but lose viability during mitosis failing in sister centromere/kinetochore segregation and nuclear division. Additionally, double-strand break repair is defective in the ppc1-537 mutant, producing fragile broken DNA, probably owing to diminished histone acetylation. The CoA-supported metabolism thus controls the state of chromosome DNA.

2. Introduction

Coenzyme A (CoA) is a ubiquitous, essential cofactor that plays a central role in the metabolism of carboxylic acids and lipids [1]. About 4 per cent of all known enzymes use CoA as an obligate cofactor. Therefore, CoA is involved in over 100 different reactions of intermediary metabolism [2,3]. CoA was discovered through a study on amino group acetylation of small molecules [4,5]. CoA was subsequently shown to be composed of adenosine 5’-phosphate, pantothenate and a sulphhydryl moiety. Acylation (thioesterification) of CoA at the sulphhydryl group by various carboxylic acids results in the production of many important acylated CoAs, including acetyl-CoA. Given that acetyl-CoA
acts as the donor of acetyl group to numerous proteins, including histones by protein acetyltransferases, and protein acetylation is one of the principal post-translational protein modifications [6], the biosynthesis of CoA may be important for a number of cell regulations, including nutrient metabolism. In higher organisms, choline acetyltransferase produces the neurotransmitter acetylcholine by combining acetyl-CoA and choline.

The CoA synthetic pathway, present in prokaryotes, fungi, plants and animals, consists of five steps, requiring four molecules of nucleotide triphosphate (ATP or CTP) [7–10] (figure 1a). Pantothenate is a specific precursor for CoA that is phosphorylated by pantothenate kinase (PANK) to 4'-phosphopantetheine as the initial step. In the next step, phosphopantetheinylcysteine synthetase (PPCS) catalyses the condensation reaction of 4'-phosphopantetheinate and cysteine [11]. The third reaction involves decarboxylation reaction to 4'-phosphopantetheine [12]. Finally, the AMP moiety is added to form dephospho-CoA, which is subsequently phosphorylated on the 3'-OH of the ribose to yield CoA [13].

In this study, we report the isolation and characterization of novel temperature-sensitive (ts) mutant strains of fission yeast with defects in mitosis and chromosome segregation, which turned out to contain substitution mutations in the PCS gene (encoded by SPCC4B3.18 and hereafter designated ppc1Δ). Schizosaccharomyces pombe has proved to be an excellent model system to study the underlying mechanisms of cell division and cycle control, mitosis, meiosis, heterochromatin formation and cellular quiescence, by using powerful genetic approaches [14–22]. Metabolic control can be investigated by metabolomic analysis using mass spectrometry [23–25]. In this study, we measured the level of CoA and provided direct evidence for a considerable decrease in CoA level in mutant cells. Pleiotropic phenotypes observed at molecular and cellular levels are interpreted based on metabolomic results. Our results show that the biosynthetic enzyme for CoA is a super-housekeeping enzyme [26], essential for both proliferation and cellular quiescence. The incidence of breaks in mutant chromosome DNA suggests the requirement of CoA for genome stability and centromere/kinetochore-mediated mitotic progression. Furthermore, as expected from its involvement in the fatty acid biosynthesis and energy metabolism, CoA plays a role in proper maintenance of lipid droplets, the organelles for lipid storage.

3. Results

3.1. Nuclear division defects observed in ts-537 mutant cells

One thousand and fifteen ts strains of S. pombe were previously isolated, their phenotypes characterized, and some of the genes essential for mitosis, cell growth, cellular quiescence maintenance, glucose metabolism and gene silencing were determined through phenotypic characterizations followed by gene identification and gene product analyses [26–30]. Around 10 per cent of the mutant strain ts-537 cells, compared with less than 1 per cent of the wild-type (WT), showed the phenotypes of mitotic progression and chromosome segregation defects at the restrictive temperature (36°C), as shown below. Auxotrophy and DNA double-strand break (DSB) damage sensitivity were found at the permissive temperature (26°C). Owing to the unique combination of these chromosomal and nutrient-related phenotypes among so-far isolated mitotic mutants of this organism, we decided to investigate the mutant in this study by using the metabolome approach combined with cellular and molecular analyses.

Staining with the fluorescent 4',6-diamidino-2-phenylindole (DAPI) revealed the nuclear DNA in fluorescence micrographs of S. pombe WT and mutant ts-537 cells (figure 1b). The mutant cells showed an undivided nucleus in one of the separated cytoplasmic spaces (indicated by arrowheads), which were presumably due to temporal cell cycle arrest at mitotic metaphase followed by the displacement of the undivided nucleus and septum formation. Such a phenotype is rarely observed in WT cells [31,32]. Furthermore, an unequal segregation phenotype displaying large and small daughter nuclei (indicated by arrows) was observed, similar to that previously reported in mutants with functional defects in mitotic centromere/kinetochores [27].

3.2. Timing of lethal mitotic phenotypes produced after shift to 36°C

For time-dependent determination of defective phenotype appearance, WT and mutant cells were grown at 26°C and then shifted to 36°C. As shown in figure 1c(i), the assay for the cell number indicated that the majority of mutant cells (red line) ceased division, whereas the WT cell (blue line) continued to increase. The cell viability of mutant cells (red line, figure 1c(ii)) decreased to 20 per cent after 8 h. The highest frequency of the mitotic defects with the displaced nucleus was reached at around 4 h, whereas mitotic defects in the WT were negligible (figure 1c(iii)). The appearance of the large and small daughter nuclear phenotype in mutant cells appeared delayed when compared with that of the displaced nucleus, consistent with the fact that the centromeric missegregation phenotype is often observed in mitosis once mutant cells pass the G1-S phase at the restrictive temperature [27].

3.3. Segregation defects of sister centromeres

For monitoring the segregation patterns of mitotic sister centromeres, DNA was visualized by green fluorescent protein (GFP) bound to the peri-centromeric regions in the genetically engineered S. pombe genome [33]. The GFP-tagged Lac repressor protein was expressed in WT and in the ts-537 mutant. In these strains, repeats of the Lac operator DNA sequence that binds to the repressor had been chromosomally integrated at the peri-centromere of chromosome I. While the two sister centromere signals were always separated in the daughter nuclei in the WT cells (figure 1d(i)), the centromere signals were asymmetrically segregated, displaying occasional (approx. 10%) absence in one of the two daughter nuclei in ts-537 mutant cells after incubation at 36°C (figure 1d(ii)).

3.4. Loss of minichromosome Ch10-CN2 at 26°C

To quantify the loss rate of an artificial linear minichromosome Ch10-CN2 [34–36] in ts-537 at 26°C, we used a colony colour assay as shown in figure 1c(i). Ch10-CN2, containing the centromere DNA of chromosome III, is stably maintained as an extra chromosome in WT. The colony colour marker
Figure 1. ts-537 strain is defective in the accurate chromosome segregation. (a) The biosynthetic pathway of CoA from pantothenate. (b) Light micrographs of WT and ts mutant ts-537, which were cultured at 36°C for 4 h. Cells were fixed and stained with DAPI. Scale bar, 10 μm. The arrows and arrowheads indicate cells with the defective phenotypes of unequal segregation and displaced nucleus, respectively. (c) The WT and mutant ts-537 cells exponentially grown at 26°C in the complete YPD medium were transferred to 36°C for 0–8 h. The cell number and cell viability percentage were scored at 1-h intervals under microscope and by plating. Aliquots of cells were fixed and stained with DAPI, and the frequency of aberrant mitosis was scored under the microscope. See text. (d) WT and ts-537 strains chromosomally integrated with GFP-tagged Lac repressor that was bound to the peri-centromeric region of chromosome I (§5) were cultured at 36°C for 4 h, then fixed with methanol. The peri-centromeric GFP signals are shown together with DAPI staining. Scale bar, 10 μm. (e) WT and ts-537 strains carrying an artificial minichromosome Ch10-CN2 were cultured in YPD medium at 26°C for 10 generations and plated on the complete medium. The colonies with high frequencies of minichromosome loss showed red–orange colour. The frequencies of minichromosome loss rates were scored as the frequency of Ade− red colonies. (f) Additive defects were observed for the double mutants between ts-537 and mis6, mis12 or mis16 (see text).
The cell number of WT started increasing around 8 h, but that of ts-537 did not (figure 2(i)). Mutant cells producing the earlier-mentioned aberrant mitotic phenotypes were observed after 7–14 h (figure 1b(iii)). Despite high cell viability of the WT, ts-537 viability decreased after 7 h at 36°C (figure 2r(i)), suggesting that aberrant mitosis in mutant cells might be a lethal event. The septation index of the ts-537 mutant started to increase around 6–7 h, about 1 h later than in the WT (figure 2r(ii)). Taken together, in ts-537 at 36°C, the cell viability remained high during DNA synthesis, but decreased in the course of mitosis and septum formation. Cell division/cytokinesis was blocked, suggesting an inhibition of cytokinesis onset owing to aberrant mitosis.

3.8. ts-537 is the mutant of phosphopantothenoylcysteine synthetase

Plasmids that fully rescued the ts phenotype in ts-537 were isolated using S. pombe genomic DNA sequence-containing plasmid library as described previously [27]. The subcloned DNA sequences capable of promoting ts-537 colony formation at 36°C contained the single gene SPCC4B3.18 that was designated ppc1+ as it encodes PPCS, an intermediate enzyme in the biosynthetic pathway to produce CoA from pantothenate. Genetic analysis by tetrad dissection confirmed that ts-537 was linked (6.2 cM) to the sti1+ locus that is situated 120 kb apart from the Ppc1/SPCC4B3.18 locus. We then isolated the ppc1+ gene from the genomic DNA of the ts-537 mutant and its DNA sequence was determined. Only a single nucleotide change was found in the mutant ppc1 gene that corresponded to the amino acid substitution T48I in the amino-terminal region. This site is highly conserved among human, fly and budding yeast (figure 3a(i)). We hence concluded that ts-537 is a mutant of the PPCS/ppc1+/SPCC4B3.18 gene and designated it ppc1-537.

In addition, the other mutant ts-88 was also isolated based on its suppression by the ppc1+ gene-carrying plasmid. The mutant showed a mitotic phenotype similar to that of ppc1-537, as shown in electronic supplementary material, figure S1. Subcloned plasmid carrying the ppc1+ gene suppressed the ts phenotype of ts-88. The single nucleotide alteration found corresponded to the M209T substitution near the carboxy terminal region (figure 3a(ii)). The hydrophobic nature of M209 is conserved in the corresponding genes of other organisms. The phenotype of ts-88 (hereafter designated ppc1-88) was less severe than that of ppc1-537, so it was not a target for in-depth investigation.

Schizosaccharomyces pombe PPCS/Ppc1 contains 316 amino acids. It is similar to human PPCS (amino acid identity 42%) and budding yeast Cab2 (identity 45%). Two mutation sites in the three-dimensional structure of PPCS/Ppc1 based on the human PPCS [40] are shown in figure 3b. The mutation site T48I in ppc1-537 resides closely to the central catalytic domain, whereas the M209T site in ppc1-88 is located at the periphery of molecule.

3.9. Pantothenate auxotrophy of ppc1-537 at 26°C

The synthetic medium EMM2 contains pantothenate (1 mg l−1). WT S. pombe can form colonies regardless of the
presence of pantothenate at 26°C. However, colony formation was somewhat retarded at 36°C in the absence of pantothenate (figure 3c). By contrast, ppc1-537 failed to produce colonies at 26°C and 36°C in the absence of pantothenate, whereas the ts phenotype arose in the presence of pantothenate. Therefore, ppc1-537 turns auxotrophic for pantothenate even at 26°C. The mutant enzyme thus seemed to require a higher concentration of pantothenate to produce sufficient amounts of CoA. A lis1 mutant that is defective in the S. pombe pantothenate uptake was previously shown to require the addition of pantothenate [41]. The phenotypes of lis1 were slow growth, mitotic defects in the presence of hydroxyl urea, and delayed cytokinesis.

### 3.10. In ppc1-537 extracts acetyl-CoA usable for the histone acetyltransferase assay is scarce

Acetyl-CoA acts as an acetyl group donor for protein acetylation in metabolism. To address whether the level of acetyl-
CoA that can be used for the acetyltransferase reaction is low in the ppc1-537 mutant, a histone acetyltransferase (HAT) enzyme assay was conducted, using S. pombe cell extracts as the supplier for acetyl-CoA. WT and ppc1-537 extracts were incubated with histone H4 peptide and recombinant mamalian P300/CBP-associated factor (PCAF), which possesses acetyltransferase activity [42]. Acetylated H4 peptide was detected by anti-acetyllysine antibody. As shown in figure 3d, HAT activity was obtained without acetyl-CoA addition to the WT cell extracts from cultures incubated at 26°C and 36°C, whereas ppc1-537 was cultured only in 26°C extract. The PCAF HAT activity was decreased fourfold for ppc1-537 at 36°C and was restored by the addition of pure acetyl-CoA in the HAT assay. The level of acetyl-CoA usable for the histone acetylation assay was thus deficient in the mutant extracts prepared from cells cultured at 36°C.
3.11. The accumulation of 4'-phosphopantothenate in ppc1-537

To characterize mutant phenotypes, the metabolomic analysis recently developed for S. pombe [23–25] was applied to assay the level of CoA and acetyl-CoA present in WT and mutant cell extracts, using an LTQ Orbitrap mass spectrometer. In S. pombe, using our extraction and detection conditions, the levels of CoA and acetyl-CoA were relatively low, nearly 1/100-fold in the peak areas in comparison with other abundant metabolites such as ATP, ADP and AMP (figure 4a). However, we could detect reproducible peaks of ionized CoA, acetyl-CoA and their precursor compounds in repeated experiments [24]. The identity of
CoA and acetyl-CoA peaks was verified using purchased standard compounds.

WT and ppc1-537 mutant cells were grown at 26°C, then transferred to 36°C and collected after 4 h. In the mutant cell extracts, the levels of CoA and acetyl-CoA detected were low (figure 4b; the numbers indicate normalized peak areas). By contrast, the level of 4'-phosphopantothenate, the substrate of Ppc1 enzyme in the pathway, was detected at 26°C and sharply increased at 36°C. However, 4'-phosphopantothenate was barely detected in the WT at both 26 and 36°C, indicating that 4'-phosphopantothenate might be rapidly metabolized as an intermediate for the synthesis of CoA in WT cells.

Next, we examined whether 4'-phosphopantothenate level decreased following the introduction of WT ppc1+ gene under the native promoter into mutant cells using plasmids. As shown in figure 4c, the level of 4'-phosphopantothenate became negligible upon ppc1+ gene introduction into ppc1-537 cells followed by incubation at 26°C. Taken together, Ppc1 appeared to act at the predicted step of the CoA synthetic process. If Ppc1 was defective, the precursor compound 4'-phosphopantothenate was highly accumulated as a consequence of blockage of the responsible enzymatic step.

3.12. Detection of Ppc1 protein and its whole cellular localization

To identify the Ppc1 protein in S. pombe cells, the ppc1+ gene was tagged at the carboxy terminus with GFP by chromosomal integration and expressed under the native promoter. For comparison, the genes for SPBC4B4.01c/Ptk1 (designated Ptk1, PANK) and SPCC191.02c (designated Acs1, acetyl-CoA synthetase that forms acetyl-CoA from acetate and CoA) were GFP-tagged and chromosomally integrated under the native promoter. Figure 4d depicts these three proteins detected by immunoblot using an antibody against GFP in the extracts of growing WT S. pombe cells. Single bands were detected at the expected MWs. Ptk1/SPBC4B4.01c and particularly Acs1/SPCC191.02c were much more abundant than Ppc1.

The intracellular localization of Ppc1, Ptk1 and Acs1 was determined using these integrated GFP-tagged strains expressed under the native promoter. The GFP signals of both Ppc1 and Ptk1 were observed in the whole cell (figure 4e), whereas Acs1 was enriched in the nuclear chromatin. These localization results are similar to those reported [44].

3.13. Hypersensitivity to double-strand break-causing phleomycin and bleomycin

We found that ppc1-537 was hypersensitive to certain DNA-damaging agents at the permissive temperature. As shown in figure 5a, ppc1-537 was sensitive to 2.5 μg ml⁻¹ DNA-breaking phleomycin at 26°C. It was moderately sensitive to 4 mM hydroxyurea (DNA replication inhibitor), but hardly sensitive to 5 μM camptothecin and to 90–100 J m⁻² ultraviolet (UV) ray. The rad3 deletion (Rad3 is an ATR-like checkpoint kinase [45]) is the control strain for DNA damage sensitivity.

It was also found that ppc1-537 was sensitive to bleomycin that cleaves double-strand DNA. As shown in figure 5b, ppc1-537 was sensitive to 4 mU ml⁻¹ bleomycin at 26°C.

The sensitivity was comparable to that of Δrad3. At 30°C (semi-permissive temperature), ppc1-537 could produce colonies in the absence of the drug, but failed to produce colonies in the presence of 2 μM ml⁻¹ bleomycin, showing that ppc1 mutant was more sensitive than Δrad3 to bleomycin at this temperature. The electronic supplementary material, figure S2 shows that the sensitivity of ppc1-537 to bleomycin at 26°C was restored by plasmid pPPC1 carrying the ppc1+ gene, but not by vector plasmid.

To monitor the occurrence of DSB in vivo for chromosomal DNA when the DSB-causing drug was added to the culture medium, pulsed field gel electrophoresis (PFGE) was used (§5). In the presence of 3 μM ml⁻¹ bleomycin at 26°C for 3 h, the intensity of the three intact chromosome bands (I, II, III) greatly decreased in ppc1-537 (figure 5c, right; the WT control pattern is shown at left).

The time course disappearance of the three chromosomes I, II and III was examined at 26°C in mutant cells in the presence of 3 μM ml⁻¹ bleomycin at 26°C (figure 5d). In the WT control, the three bands were still intact at 180 min. In ppc1-537 mutant, however, broken DNA appeared already after 10 min. On the other hand, most chromosomal DNA had been broken about 60 min following 3 μM ml⁻¹ bleomycin addition at 26°C.

3.14. ppc1-537 is UV-sensitive in G0 quiescent cells

While ppc1-537 was insensitive to UV irradiation in the growth culture medium, we found that the mutant cells became sensitive to 100 J m⁻² UV while residing in the quiescent G0 phase, as shown in figure 5e. The ppc1-537 mutant cells were cultured for 24 h at 26°C in the absence of a nitrogen source. Cells were then irradiated by UV (0–150 J m⁻²) at 26°C. The difference of sensitivity between the WT and ppc1-537 was about tenfold in the G0 phase, while the sensitivity in the WT did not differ significantly from mutant cells during vegetative phase. The nitrogen-starvation-induced G0 phase entry of S. pombe was previously shown to be hypersensitive to DNA-damaging agents as the damage repair through homologous recombination was largely missing due to the absence of post-replicative DNA [46].

3.15. Greatly diminished histone H3 and H4 acetylation in ppc1-537 cells

Because both levels of CoA and also acetyl-CoA were greatly diminished in ppc1-537 mutant cells even under permissive temperature, in the next step we examined whether histone acetylation decreased in mutant cells. Immunoblot analysis was done using antibodies against acetylated histones. Cell extracts were prepared from three strains: WT, histone deacetylase mutant clr6-1 [47] and ppc1-537. The cells were shifted from 26°C to 36°C for 0–12 h, and three specific antibodies against acetylated histone H3 or H4 (AcH4, AcH3K9, AcH3K14) were used to detect histone acetylation. For the loading control, antibodies against the carboxy terminus of histone H3 (H3) and Cdc2 (PSTAIRE) were used (§5).

In clr6-1 mutant cells, the level of histone H3 and H4 acetylation moderately increased (figure 6a), as reported [48]. By contrast, the levels of histone acetylation detected by antibodies against acetylated histones H4 AcH4
were all diminished in \( \text{ppc1-537} \) for 0–12 h at 36°C, while the histone H3 protein level did not change. These results were consistent with a notion that histone acetylation was diminished in \( \text{ppc1-537} \) owing to the deficiency of CoA and acetyl-CoA.

3.16. Genetic interactions of \( \text{ppc1-537} \) with histone acetyltransferase and deacetylase mutants

To obtain information about genetic interactions with HAT, double mutants containing two mutations of HAT \( \text{mst1-} \)
L344S [50] and Δhat1 [51] were constructed. Mst1, a homologue of budding yeast Esa1 [52], is required for damage response and chromosome segregation. Hat1, a homologue of budding yeast Hat1, is required for histone H4 acetylation. Mis16 is an essential histone H4 chaperone, and its ts mutant is defective in chromosome segregation and centromere histone H3 (CENP-A) loading to the centromere [27]. As shown in figure 6b,c,ii, ppc1-537 caused synthetic lethality when combined with mst1-L344S but not with Δhat1. Note that ppc1-537 was synthetically defective with mis16 mutant, as described in figure 1f.

Schizosaccharomyces pombe has six histone deacetylases (HDACs). We constructed double mutants of ppc1-537 with these and examined their ts phenotypes (figure 6b,c). The additive defects were found in five double mutants. The severe additive phenotypes were found in the type 1 and type 2 HDACs, clr6, clr3 and phd1. However, double mutants with NAD-dependent HDACs ppc1 hst2 and ppc1 hst4 only

### Figure 6

**Figure 6.** Histone acetylation is diminished in ppc1-537 that strongly interacts with histone acetyltransferase and deacetylase mutants. (a) WT, clr6-1 and ppc1-537 mutant strains were first grown at 26°C, then transferred to 36°C for 0, 4, 8 and 12 h, and harvested. Their extracts were prepared and immunoblotted using antibodies against acetylated histone H3 and H4 as indicated and antibodies against histone H3. Cdc2 antibody PSTAIRE was the loading control. (b) Results of the double mutants between ppc1-537 and histone acetyltransferase Δhst1 and mst1-L344S, and six deacetylase mutants. Additive defects were observed except for the case of double mutant with Δsir2, which was rescued, and with Δhat1, which showed no effect. (c) The single and double mutants of ppc1-537 and acetyltransferase (hat1, mst1) and deacetylase (hst2, hst4, sir2, clr6, clr3, phd1) mutants. Cells grown exponentially were diluted, spotted on YPD plate (two spots for the double mutant), and then incubated at 26°C, 30°C or 33°C. (d) Protein and (e) mRNA transcript levels of FLAG-tagged Ppc1 in the genetic background of WT, Δhst2, Δhst4 and Δsir2. The level of Ppc1-FLAG chromosomally integrated and expressed under the native promoter was determined by anti-FLAG antibody. (f) The levels of acetylated histone H4 K12 in WT, Δsir2, ppc1-537 and the double mutant were determined by antibody against acetylated H4K12. Anti-tubulin antibody TAT1 was determined as the loading control.

La table suivante résume les résultats des tests de fonctionnalité des protéines Ppc1-FLAG chromosomiquement intégrées et exprimées sous le promoteur naturel. Pour chaque test, la température maximale atteinte est indiquée en degrés Celsius (°C) et la durée de croissance en heures (h). Les protéines sont testées pour leur fonctionnalité par immuno-blotting et sont identifiées par leur acétylation. Les résultats montrent une diminution significative de l’acétylation de l’histone H4 K12 dans les mutants ppc1-537, par rapport aux protéines WT et Δhat1. Nota bene que ppc1-537 est synthétiquement déficitaire avec mis16, comme indiqué dans la figure 1f.

Schizosaccharomyces pombe a six histones déacétylases (HDACs). Nous avons construit des mutants doubles de ppc1-537 avec ces dernières et examiné leurs phénomènes ts (figure 6b,c). Les défauts additifs ont été observés dans cinq mutants doubles. Les phénomènes additifs sévères ont été trouvés dans les types 1 et 2 HDACs, clr6, clr3 et phd1. Cependant, des mutants doubles avec des HDACs NAD-dépendants ppc1 hst2 et ppc1 hst4 n'ont que...
Figure 7. Lipid homeostasis is impaired in ppc1-537 mutant. (a) WT and ppc1-537 cells grown at 26°C and at 36°C for 4 h were stained by nile red [53] to visualize lipid droplets. The number indicates the relative abundance of lipid droplets in WT and ppc1-537 cells. Scale bar, 10 μm. (b) Recovery of lipid droplets stained by nile red upon the transformation of ppc1-1 gene. Scale bar, 10 μm. (c) ppc1-537 mutant was sensitive to TSA at 26°C. The sensitivity was similar to that of clr6-1 mutant at 26°C. (ii) ppc1-537 was sensitive to nicotinamide (NA) at 26–30°C, while the WT cells normally grew at the same temperature.

showed a weak additive defect at both 26°C and 30°C. Interestingly, ppc1-537 combined with Δsir2 showed a clear rescue of the ts phenotype at 30°C and 33°C. Sir2 belongs to the group of HDACs dependent on NAD for their activity.

As the degree of rescue for the double mutant ppc1 Δsir2 was strong, we performed further experiments. It was noticed that the protein and mRNA levels of Ppc1 significantly increased in Δsir2 deletion mutant. The levels were higher than those of WT cells (figure 6f). The level of acetylated histone H4 K12 was low in ppc1-537, but restored in the ppc1 Δsir2 double mutant cells at semi-permissive temperature (33°C; figure 6f). Histone H4 K12 is not a direct target of fission yeast Sir2. This recovery of histone acetylation might occur through the upregulation of Ppc1 by the deletion of Sir2.

3.17. The number of lipid droplets decreased in ppc1-537

The neutral lipids (triacylglycerol and cholesteryl esters) serve as an energy reserve, and these molecules are stored in lipid droplets. The neutral lipids (triacylglycerol and cholesteryl esters) serve as an energy reserve, and these molecules are stored in lipid droplets. The number of lipid droplets decreased significantly in ppc1-537 mutants (figure 7a). The number of lipid droplets was already relatively low in ppc1-537 mutant at the permissive temperature. At restrictive temperature, the number further decreased, while the number did not change in WT cells. Introduction of the plasmid carrying the ppc1+ gene into the mutant cells restored the number of lipid droplets (figure 7b). These results suggest that lipid droplet homeostasis requires proper CoA biosynthesis.

3.18. ppc1-537 is sensitive to the histone deacetylase inhibitors trichostatin A and nicotinamide

Schizosaccharomyces pombe chromosome segregation is affected by inhibitors acting against HDACs. HDAC inhibitor trichostatin A (TSA) [54] is a potent inhibitor, and promotes normal and abnormal sister chromatid separation by affecting APC/cyclosome and adherin. As shown in figure 7c(i), despite normal growth of the WT strain, ppc1-537 mutant was hypersensitive to TSA (12.5 μg ml⁻¹), similar to the clr6-1 mutant at 26°C. Additionally, mutant cells were sensitive to 25–50 mM nicotinamide, which inhibits NAD-dependent HDACs such as Sir2 [55,56]. Again, WT cells were equally unaffected by this drug concentration, as shown in figure 7c(ii). Thus, ppc1-537 was sensitive to two distinct types of HDAC inhibitors.

4. Discussion

The name of pantothenate or pantothenic acid derives from the Greek word *pan* or *pantos*, meaning all or universal. This compound is present in all known organisms as the specific precursor of CoA, a fundamentally essential coenzyme in cellular metabolism. The enzyme PANK produces phosphopantothenate by phosphorylation, and PPCS subsequently forms phosphopantethenoylcytochrome by the ligation reaction with cysteine and CTP or ATP. The following three reactions lead to the synthesis of CoA. These reactions are conserved and absolutely essential for life [57–61]. While fungi such as S. pombe produce pantothenate from pyruvate and beta-alanine [41], animals do not, taking pantothenate entirely from the food. Therefore, pantothenate is also called vitamin B5. In this study, we isolated two $S. \text{pombe}$ ts mutant strains (ppc1-537, ppc1-88) defective in PPCS through gene cloning by transformation and sequencing. In addition, one ts mutant strain (ptk1-201) defective in PANK was isolated (T. Nakamura 2010, unpublished result). Mass spectrometry analysis established that the levels of CoA in these mutant cell extracts are greatly diminished, and that the level of phosphopantothenate is decreased in ptk1-201 owing to the lack of phosphorylation of pantothenate, while it is increased in ppc1-537 owing to the defect in the subsequent reaction. Our results support the conclusion...
that the cellular phenotypes of these mutants are due to the loss of the activities of PANK and PPCS, which causes the diminished level of CoA.

After CoA is synthesized, it can be acylated at the thiol group (SH) by various compounds, giving rise to thioester forms. Acetyl, acetoacetyl, succinyl, glutaryl, malonyl, hydroxymethylglutaryl, coumaronyl, crotonyl, propionyl are examples. Differently acylated CoAs are produced in different places within cells for different physiological purposes. Among acylated CoAs, acetyl-CoA is the most important, as it is essential in so many crucial metabolic pathways. Pyruvate dehydrogenase complex catalyses the reaction of CoA with pyruvate, the end product of glycolysis and produces acetyl-CoA. Acetyl-CoA is then required to start the TCA (Krebs) cycle by citrate synthase that catalyses the reaction acetyl-CoA + oxaloacetic acid + H₂O → citrate + CoA – SH.

In other words, citrate synthase is a major consumer of acetyl-CoA when TCA cycle is fully active. This reaction occurs in mitochondria.

Acetyl-CoA is also required for the synthesis of fatty acids by fatty acid synthase through its association with an acyl-carrier protein in the very large fatty acid synthase complex. The fatty acid synthesis occurs in cytoplasm. In contrast, fatty acids are oxidized by the process called beta-oxidation and produce acetyl-CoA in mitochondria. Acetyl-CoA is required for the synthesis of cholesterol. Furthermore, acetyl-CoA is required for acetylation of numerous proteins having various functions. As these represent only a portion of whole metabolic events that require acetyl-CoA and other acyl-CoAs, pleiotropic defective phenotype of the mutants deficient in the CoA synthesis may well be expected. Hence, the phenotypes of the reduction of lipid droplets and the defects in cell division cycle and cellular quiescence may be explained owing to the diminished TCA and sugar catabolic processes, and/or fatty acid synthesis. CoA is also required for the super-housekeeping metabolism required for two distinct cell states under acid synthesis. CoA is also required for the super-housekeeping metabolism required for two distinct cell states under acid synthesis. CoA and acetyl-CoA were exogenously added to the samples prior to extractions from cells, the levels sharply increased.

The relationship between Ppc1 and HDACs remains to be clarified. The ppc1 mutant cells are hypersensitive to DNA DSB agents such as bleomycin and phleomycin, suggesting that the mutant chromosome DNA is fragile in the presence of these DSB agents. This may be caused by the decrease in DSB repair efficiency in ppc1 mutant owing to the hypo-acetylation of histones. A previous report showed that hypoacetylation of histones leads to the defect in DNA damage repair. In Saccharomyces cerevisiae [63], the HAT Esa1 is required for non-homologous end-joining repair. In higher eukaryotic cells, similar damage repair defects are related to protein acetylation–deacetylation [64,65]. In S. pombe, Mst1 similar to Esa1 is related to both DNA damage response and chromosome segregation [50]. We show in this study the synthetic defective phenotype of the double mutant pcc1 mst1: Ppc1 may support the DSB repair through the supply of CoA, and concomitantly acetyl-CoA.

The most unexpected findings in the present study are the specific mutant phenotypes that revealed chromosome mis-segregation in mitosis. The mutant cells did not lose viability during the S phase. The link between the paucity of CoA and the error in chromosome segregation is not immediately grasped with ease. The chromosome segregation defects observed in the CoA biosynthesis mutants are not coincidental to these S. pombe mutants, as the isolated mutants show similar segregation defects. It was reported that the fly PPCS mutant caused aberrant mitotic chromosomes and hyper-sensitivity to ionizing radiation [57,58]. Similar mitotic defects were thus observed in the distant organisms. In S. pombe, the fatty acid synthesis mutant cut6 defective in acetyl-CoA carboxylase showed a severe defect in the equal nuclear division [62], indicating that the defect in the fatty acid synthesis metabolism through acetyl-CoA decarboxylation could profoundly affect the mode of spindle dynamics, chromosome segregation and nucleolar division.

The biosynthesis of CoA is closely related to two fundamental aspects of cellular metabolism: the energy production and the sugar/lipid catabolism and synthesis. In this study, we show that the paucity of CoA actually strongly affects the state of chromosomes and also the mode of chromosome segregation in mitosis. While full mechanistic understanding of the relationship between CoA and chromosome must be addressed in a future study, we will discuss several aspects of the relationship revealed in the present study.

We show that ppc1 mutant is hypersensitive to DNA DSB agents such as bleomycin and phleomycin, suggesting that the mutant chromosome DNA is fragile in the presence of these DSB agents. This may be caused by the decrease in DSB repair efficiency in ppc1 mutant owing to the hypo-acetylation of histones. A previous report showed that hypoacetylation of histones leads to the defect in DNA damage repair. In Saccharomyces cerevisiae [63], the HAT Esa1 is required for non-homologous end-joining repair. In higher eukaryotic cells, similar damage repair defects are related to protein acetylation–deacytation [64,65]. In S. pombe, Mst1 similar to Esa1 is related to both DNA damage response and chromosome segregation [50]. We show in this study the synthetic defective phenotype of the double mutant pcc1 mst1: Ppc1 may support the DSB repair through the supply of CoA, and concomitantly acetyl-CoA.
5. Material and methods

5.1. Strains, media and plasmids

The genomic DNA library was used to transform mutant strains ts-88 and ts-537, in order to obtain plasmids that suppressed the mutant strains. Plasmids were recovered from resulting transformants. The sequences at the end of the inserts in plasmids defined the inserted genomic sequences. Subcloning established that the SPCC4B3.18 gene was responsible for suppression of ts-88 and ts-537. Genetic linkage analysis was done by tetrad analysis using the marker locus. The result verified the conclusion that the subcloned gene was actually the mutant gene. The amino acid substitutions were confirmed for each mutant gene by sequencing of the mutant genes. The GFP or FLAG tag sequence was inserted at the C terminus of ppc1 and pkl1 and aes1 genes, and followed by the drug-resistant kanMX6 marker. These DNA fragments were introduced into endogenous loci, and transformants were selected by the resistance to G418. Finally, correct integrations were verified by PCR.

5.2. Gene cloning and construction of yeast strains

The stability of minichromosome Ch10-CN2 in fission strains was determined as described [35]. Briefly, cells were harvested byOmnipore membrane filter (Millipore) and washed with 1 ml Mill-Q water. The filter was plunged into 1.5 ml of cold 50 per cent methanol (−40°C) containing 10 μl of 0.4 mM internal standard piperezine-N,N'-bis(2-ethanesulphonic acid) (PIPES), then frozen in liquid nitrogen followed by thawing on ice. Cells were resuspended by brief vortex and the membrane filter was removed. The suspension was centrifuged at −20°C. The supernatant was collected and 1 ml of cold 50 per cent methanol (−40°C) was added to the pellet and cells were resuspended by brief vortex. The suspension was centrifuged at −20°C, and both supernatants were pooled. Supernatants were centrifugally filtered through an Amicon 5-kDa cut-off filter (Millipore). The filtrate was evaporated and dissolved in 40 μl of 50 per cent acetonitrile.

5.3. In vitro histone acetyltransferase assay

In vitro

5.4. Light microscopy

5.5. Minichromosome stability assay

The stability of minichromosome Ch10-CN2 in fission yeast strain was determined as described [35]. Briefly, cells were cultured in the selective minimal medium at 26°C, then diluted and transferred to non-selective-rich medium and cultured at 26°C. The ratio of Ade + to total cells was obtained after the transfer to the rich medium by plating cells and counting red colonies.

5.6. Cell viability assay in the G0 state

Cells were first grown in the minimal medium (EMM2) at 26°C, and then transferred to the nitrogen-deficient EMM2-N medium at 26°C for 24 h. These cultures were split into two and transferred to 26°C and 36°C. Portions of the cultures were taken at each time point and plated on complete YPD medium plates at 26°C. Cell viability was calculated as a percentage of the number of formed colonies against the number of plated cells.

5.7. Extraction of CoA and acetyl-CoA for mass spectrometry analysis

Schizosaccharomyces pombe cells were grown in the EMM2 medium, and 40 ml (5 × 10^6 cells ml⁻¹) of the culture was used for the metabolite extraction using the method described previously [24], with modifications. Cells were harvested by Omnipore membrane filter (Millipore) and washed with 1 ml Mill-Q water. The filter was plunged into 1.5 ml of cold 50 per cent methanol (−40°C) containing 10 μl of 0.4 mM internal standard piperezine-N,N'-bis(2-ethanesulphonic acid) (PIPES), then frozen in liquid nitrogen followed by thawing on ice. Cells were resuspended by brief vortex and the membrane filter was removed. The suspension was centrifuged at −20°C. The supernatant was collected and 1 ml of cold 50 per cent methanol (−40°C) was added to the pellet and cells were resuspended by brief vortex. The suspension was centrifuged at −20°C, and both supernatants were pooled. Supernatants were centrifugally filtered through an Amicon 5-kDa cut-off filter (Millipore). The filtrate was evaporated and dissolved in 40 μl of 50 per cent acetonitrile.

5.8. Liquid chromatography—mass spectrometry analysis

LC–MS data were obtained using a Paradigm MS4 HPLC system (Michrom Bioresources) coupled to an LTQ Orbitrap hybrid ion-trap/Fourier transform mass spectrometer (Thermo Fisher Scientific). LC separation was performed on a ZIC-pHILIC column (Merck SeQuant; 150 × 2.1 mm, 5 μm particle size). Acetonitrile (solvent A) and 10 mM ammonium carbonate, pH 9.3 (solvent B) were used as a mobile phase, with a gradient elution from 20 per cent B to 80 per cent B in 30 min with the 100 μl min⁻¹ flow rate. For MS detection, an electro spray ionization source was used and operated in negative ionization mode. Spray voltage was set to 2.5 kV, capillary temperature to 350°C. N₂ was used as sheath gas. Mass spectrometer was operated in full scan mode with a 100–1000 m/z scan range. Raw data were analysed using the MZmine 2 software [43]. Metabolite peak areas were normalized by the peak areas of the internal standard (PIPES). Peaks of CoA and acetyl-CoA were identified by their accurate m/z values, and identification was verified using the pure standard CoA and acetyl-CoA.

5.9. Pulse-field gel electrophoresis

Schizosaccharomyces pombe cells (1 × 10^6) were harvested and chromosome DNA samples were prepared as described [72]. Chromosomal DNA embedded in 1 per cent agarose
plug was run on 1 per cent SeaKem Gold Agarose (Lonza) gel in 1× TAE buffer using CHEF Mapper (Bio-Rad). Electrophoresis was carried out at 14°C with 2 V cm⁻¹ voltage. Block 1 was done for 8 h with 96° angle and 1200 s switching time. Block 2 was done for 8 h with 100° angle and 1500 s switching time. Block 3 was done for 8 h with 106° angle and 1800 s switching time. The DNA was stained with EtBr.

5.10. Detection of histone acetylation

Antibodies specific for acetylated histone (Millipore Upstate 06-866, 07-450, 07-353 and 06-1352) were used to monitor histone acetylation in cell extracts. Antibody specific for histone H3 (GeneTec GTX21791) was used to detect the amount of histone H3.

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