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Original Research Article

A systematic study of regulating inorganic polyphosphates production in *Saccharomyces cerevisiae*

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ABSTRACT

Inorganic polyphosphate (polyP), a linear polymer of orthophosphate residues, plays critical roles in diverse biological processes spanning blood coagulation, immunomodulation, and post-translational protein modifications in eukaryotes. Notably, long-chain polyP (>100 phosphate units) exhibits distinct biological functionalities compared to shorter-chain counterparts. While *Saccharomyces cerevisiae* serves as a promising microbial platform for polyP biosynthesis, the genetic regulatory mechanisms underlying polyP metabolism remain poorly elucidated. Here, we systematically investigated the genetic determinants governing intracellular polyP levels and chain length dynamics in yeast. Through screening a library of 55 single-gene knockout strains, we identified six mutants (*Δddp1*, *Δvip1*, *Δppn1*, *Δppn2*, *Δecm33*, and *Δccr4*) exhibiting elevated polyP accumulation, whereas deletions of *vtc1*, *kcs1*, *vma22*, *vma5*, *pho85*, *vtc4*, *vma2*, *vma3*, *ecm14*, and *vph2* resulted in near-complete polyP depletion. Subsequent combinatorial deletions in the *Δppn1* background revealed that the *Δppn1Δvip1* double mutant achieved synergistic enhancement in both polyP concentration (53.01 mg-P/g-DCW) and chain length, attributable to increased ATP availability and reduced polyphosphatase activity. Leveraging CRISPR/Cas9-mediated overexpression in *Δppn1Δvip1*, we engineered strain PP2 (*vtc4* overexpression), which demonstrated a 2-fold increase in polyP yield (62.6 mg-P/g-DCW) relative to wild-type BY4741, with predominant synthesis of long-chain species. Mechanistically, qRT-PCR analysis confirmed that PP2 exhibited 46-fold up-regulation of *vtc4* coupled with down-regulation of polyphosphatases encoding genes, *ppn2*, *ddp1*, and *ppx1*. This study performed a systematic study of regulating inorganic polyphosphates production in yeast and provides a synthetic biology strategy to engineer high-yield polyP-producing strains, advancing both fundamental understanding and biotechnological applications.

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1. Introduction

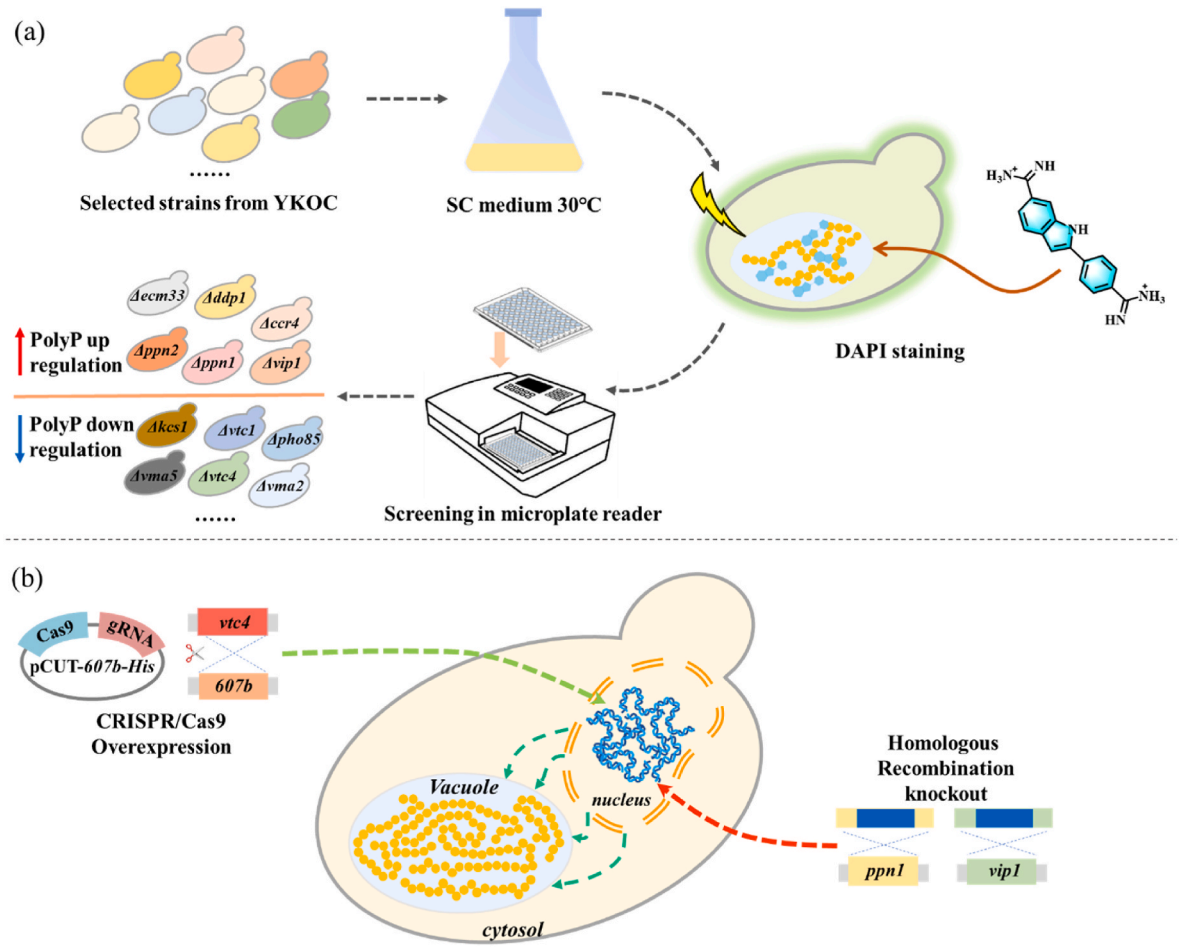
Inorganic polyphosphate (polyP) is an ancient biomacromolecule composed of orthophosphate units linked via phosphodiester bonds and it is present in all life forms, ranging from prokaryotic microorganisms to human [1]. Polyphosphate has significant applications across various fields, including food, chemistry, medicine and engineering [2–4], and it also plays a crucial role in cellular functions [4,5]. In the bloodstream, long-chain polyP accelerates blood clotting by acting as a cofactor for several key proteins [6]. Within the cytosol, it serves as a protein scaffold and immunomodulator, maintaining homeostasis and preventing cellular disorders [7–10]. Polyphosphate is also involved in post-translational modifications (PTM), the Saiardi group discovered that polyP can modify lysine residues of the PASK region of proteins through a non-enzyme-catalyzed reaction [11,12]. Downey and co-workers identified numerous protein targets that interacting with polyP, laying the groundwork for studying of polyP-protein interaction [13,14]. The Jia group demonstrated that polyP is involved in the modification and regulation of histidine area of protein [15]. Additionally, the Bossmann group screened a proteome microarray to identify several protein targets capable of binding with polyP [16]. The properties of polyP, which are much like other inorganic and organic polymers, are profoundly influenced by its chain length. In general, long-chain polyP with a chain length of more than 100 phosphate residues has biological functions that are not present in shorter chain species [17]. Some eukaryotic cells accumulate long-chain polyphosphate consists of more than 100 phosphate residues [11], which allows for potential folding and spatial structure, thereby conferring unique functions. For instance, long-chain polyP can be used as a high-quality energy source to promote bone repair [18]. As a polyanionic compound, long-chain polyP has been reported to possess cytoprotective and antiviral properties, potentially impairing SARS-CoV-2 infection [19,20]. Long-chain species were also more efficient than short-chain species in ATP regeneration using polyP as a substrate [17]. Our previous research indicated that long-chain polyP could induce phase separation of positively charged protein at lower concentration than short-chain polyP [21]. Recently, long-chain polyP has been implicated in enhancing ionizing radiation resistance in protocell [22]. Therefore, long-chain polyP plays a significant role in cellular metabolic processes. Therefore, acquiring long-chain polyP is crucial for researchers to explore the function of this biomolecule. Although polyP of various chain lengths can be obtained using chemical synthesis methods, the high-temperature operation (>600 °C) in this method is energy-consuming and requires high-purity phosphate source [23]. Microbial synthesis provides an alternative pathway whereby microorganisms can synthesize polyP using a low concentration, low purity phosphate source, which can be achieved in an environment close to room temperature [4,24].

Baker's yeast, *Saccharomyces cerevisiae*, serves as a crucial microbial cell factory for production of numerous natural products [25]. As a typical eukaryotic microbiological chassis, yeast offers several advantages, including a well-characterized genetic background, high safety, substantial biomass and comprehensive toolkit for genetic modification [26,27]. Yeast as a microbial cell factory can be used to synthesize polyP using a low purity, low concentration phosphate source. Industrially synthesizing polyP using high temperature methods requires a high purity phosphate feedstock. Using yeast to synthesize polyP reduces energy consumption and expands the range of phosphorus sources utilized. Yeast has been reported as a cellular factory for the synthesis of polyP. Blank and colleagues have made important contributions to the hyperaccumulation of yeast polyP [24]. They succeeded in accumulating 28 % polyP per gram of yeast dry weight [28], corresponding to the highest production of polyP content in yeast, by adjusting the medium composition and yeast growth conditions, satisfying food-grade requirements [29,30]. Meanwhile, significant progress has been made in understanding the enzymes and genes involved in polyP metabolism in yeast, which point us in the direction of research. The vacuolar transporter chaperone (VTC) complex, identified as the polyP synthetase by multiple researchers [31–33], has been elucidated through high-resolution dynamic cryo-EM structure by the Liu and Ye groups [34,35]. Additionally, polyphosphatases have been characterized, providing further insights into polyP metabolism [36]. Proteins involved in phosphate uptake and transport are linked to ATP metabolism and play a crucial role in polyP accumulation due to their association with the structural motifs and direct substrates of polyP [37–39]. Systematic investigations into genes associated with phosphorus metabolism and their relationship with intracellular polyphosphate level remain relatively underexplored. Specifically, there is still remained unknown regarding how the presence or absence of key phosphate metabolism genes influences both the concentration and chain length of polyP in yeast cells.

In this study, we systematically investigated the association of important polyP and phosphate metabolism genes in yeast with intracellular polyP level and chain length in yeast, and constructed a series of engineered strains with the ability to synthase long-chain polyP and with increased intracellular polyP concentration by synthetic biology methods. Based on the established comprehensive genetic framework for polyP metabolism [38,39], a library of potential polyP metabolism-related genes was formed based on previous studies (Table 1). This library includes coding genes for both confirmed polyP synthesizing and degrading enzymes, as well as numerous putative enzymes or proteins that may participate in polyP biosynthesis in yeast. Concurrently, we selected yeast single knockout strains corresponding to the gene library from the commercial Yeast Knockout Collection (YKOC) [40,41] thereby creating a single-gene knockout strain library. By the classical non-destructive intracellular polyP quantification method, DAPI staining [42,43], we screened these single-knockout strains and found that polyP concentrations increased in strains knocking out the *ddp1*, *vip1*, *ppn1*, *ppn2*, *ecm33*, and *ccr4* genes, whereas strains lacking the *vtc1*, *kcs1*, *vma22*, *vma5*, *pho85*, *vtc4*, *vma2*, *vma3*, *ecm14*, and *vph2* genes were almost unable to synthase polyP (Scheme. 1a). PolyP concentrations were also decreased to varying degrees in other strains. Among these, the *Δppn1* strain was chosen as the starting strain for further genetic engineering due to its superior chain length and enhanced cellular polyP level. Using *Δppn1* as a starting strain, we knocked out five genes, *ddp1*, *vip1*, *ppn2*, *ecm33*, and *ccr4*, respectively, and obtained a set of double-knockout strains. Among them, the *Δppn1Δvip1* strain had the highest intracellular polyP concentration and was able to synthase long-chain species. Finally, we used CRISPR/Cas9 to overexpress within the *Δppn1Δvip1* strain one or more of the genes that were found to almost completely inhibit polyP accumulation once deleted through the screening, to obtain 6 engineered strain. The strain which overexpressed *vtc4*, denoted PP2, was tended to accumulate long-chain polyP and had intracellular polyP level of that were 2-fold higher than the wild-type strain. This study systematically sorted out the relationship between key phosphate metabolism genes and yeast polyP content and chain lengths, and provided a basis for future research

Table 1
Library of polyP metabolism-related genes.

Enzyme or protein encoded	Containing genes
VTC complex	*vtc1*, *vtc2*, *vtc3*, *vtc4*, *vtc5*
Endopolyphosphatase	*ppn1*, *ppn2*, *ddp1*
Phosphatase, phosphate transporters and other members of PHO pathway	*pho5*, *pho8*, *pho2*, *pho4*, *pho85*, *pho81*, *pho87*, *pho90*, *pho91*, *pho89*, *pho84*
Vacuolar ATPase subunit	*vph1*, *vph2*, *vma2*, *vma3*, *vma5*, *vma22*, *pkr1*, *vps33*, *vps34*, *rav1*
Inositol polyphosphate kinase	*arg82*, *kcs1*, *vip1*, *plc1*
Other candidates	*opt2*, *cka2*, *pep12*, *pep5*, *rim21*, *trf5*, *tat2*, *ccr4*, *ade1*, *tef4*, *ypt7*, *mek1*, *kre1*, *smi1*, *ecm14*, *ecm33*, *mrp51*, *apm3*, *sir3*, *erg6*, *pfk2*, *pyk2*



Scheme. 1. (a) Screening process of the selected strains by DAPI-staining. (b) Strain construction by synthetic biology methods.

on polyP metabolism in yeast and offered a new option for the design and construction of polyP high-yield yeast strains.

2. Results and discussion

2.1. Library of polyP metabolism-related genes in yeast

To explore the effects of key phosphorus metabolism genes on yeast polyP biosynthesis, we selected 55 phosphorus metabolism - related genes based on literature research [44]. These genes can be divided into 6 categories: (1) VTC complex encoded gene, (2) yeast endopolyphosphatase encoded gene, (3) phosphatase, phosphate transporters and other members of PHO pathway encoded gene, (4) vacuolar ATPase subunit encoded gene, (5) inositol polyphosphate kinase encoded gene, and (6) other candidates (Table 1).

VTC complex is the identified polyphosphate synthetase in yeast [35]. and VTC4 is crucial for polyP synthesis owing to it directly corresponding to polyP synthesis [31]. PPN and DDP are typical endopolyphosphatases in yeast that may affect the cellular polyP chain length [45–47]. In yeast, phosphate metabolism is regulated by PHO pathway [38,39], and genes encoding phosphatase and phosphate transporters may regulate polyP synthesis [48,49]. Vacuoles are the main polyP-storing organelles in yeast, genes regulating the function, permeability or morphology of vacuoles may impact polyP storage, and the proton electromotive force produced in the shape of vacuolar ATPase (V-ATPase) is thought to be necessary for polyP synthesis [44, 50]. Auesukaree et al. confirmed that through genome-wide screening experiments there was also a significant correlation between inositol polyphosphate metabolism and polyP accumulation [37,51]. Since inositol polyphosphate shares the same substrate with polyP, the deletion of inositol polyphosphate kinase encoding genes may show effect on polyP synthesis [37]. The substrate of polyP is ATP, thus the synthesis of polyP will also be affected by other enzymes involved in ATP and phosphate metabolism. Based on Freimoser group's genome-wide study of yeast, we selected enzymes that are involved in physiological processes such as glucose metabolism and cell wall formation as other candidates [44]. The presence or absence of the selected "other candidates" enzymes may influence polyP accumulation, which requires further verification. Detailed information of the 55 genes can be found in Table S1.

2.2. Screening of the responding single-gene knockout strain library

Based on the gene library, we selected the corresponding single-gene knockout mutants on the classic chassis strain *Saccharomyces cerevisiae* BY4741 from YKOC to compose the single-gene knockout strain library. We first studied the growth of these 55 variants and obtained their 24 h growth curves. In synthetic complete (SC) medium, all single-knockout strains grew, with differences in growth rate and biomass, and entered the steady state after 12–17 h (Fig. 1). In steady state, biomass (OD600) of most strains was not significantly differed from the BY4741 wild type strain (WT). The growth of strains knocked out of the VTC complex subunit was almost unchanged, and strains knocked out of endopolyphosphatase DDP1 showed a significant decrease in growth rate, and the organisms were more likely to aggregate and form clumps. There was little change in the overall growth of the pho knockout strains, and the *Δpho85* strain showed a significant slowdown in growth, possibly due to a reduction in nutrient uptake [52]. The growth of the *vma* genes

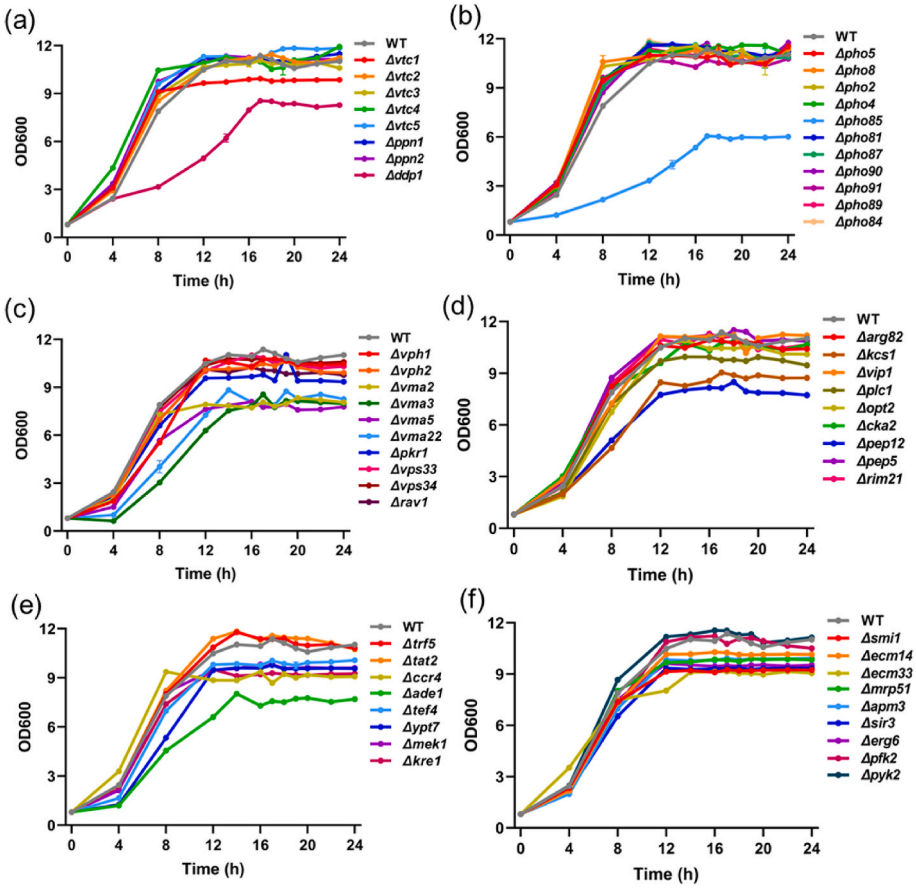


Fig. 1. PolyP assay of single-gene knockout strains. Growth curve of single-gene knocked out strains and double-gene knockout strains. (a) VTC complex and endopolyphosphatase knocked-out strains, (b) phosphatase, phosphate transporters and other members of PHO pathway knocked-out strains, (c) V-ATPase subunits knocked-out strains, (d–f) Inositol polyphosphate kinase and other candidates knocked-out strains.

knockout strains slowed down relative to WT, and their growth was not severely inhibited in the acidic SC medium [53]. We also studied the relationship between polyP chain length and yeast growth time. The Urea-PAGE results showed that the polyP chain length of WT reached the maximum in the steady state. Thus, we set 17 h as the time point for polyP assay.

After screening all single-gene knockout strains by DAPI staining (Scheme. 1a), we found that the cellular polyP level of 6 variants increased compared with WT, while others decreased to different degrees (Fig. 2a–f). The strains with increased polyP were *Δppn1*, *Δppn2*, *Δddp1*, *Δvip1*, *Δccr4* and *Δecm33*. Endopolyphosphatase deletion strains showed an increase on polyP yield which consistent with previous reports [45,46]. *Vip1* and *Kcs1* are both inositol pyrophosphate synthases in yeast [37], but variants without these two genes showed completely opposite results on polyP level. Deletion of *vip1* can increase cellular ATP concentration and influence pH, which may be the reason for the increased polyP production of *Δvip1*. Cellular ATP was measured by commercial kit, compared with WT, the *Δvip1* strain showed a small increase in ATP level (Fig. 3d). Unexpectedly, deletion of *ccr4* and *ecm33* brought a polyP level increase more than 50 %. These two genes encode enzymes that involved in mRNA process and cell wall formation, deletion of them may promote its accumulation [54]. Deletion of the component of VTC and V-ATPase resulted in a significant decrease of polyP level, demonstrating that the presence of these enzymes is necessary for polyP biosynthesis. VTC1 and VTC4 are the receptor and catalytic center of the signaling molecule, respectively, and their deletion leads to a complete block of polyP synthesis [35]. Knockdown of any gene encoding a vesicular membrane ATPase subunit complex with an assembly factor results in a substantial reduction in polyP levels. Activation of vesicular membrane acidity plays an important role in polyP biosynthesis in *Saccharomyces cerevisiae*, and vesicular functions, including protein maturation and activation, as well as metabolite storage and the maintenance of cellular homeostasis, are strongly dependent on the mediation of the V-ATPase complex, so that deletion of the class of subunits markedly inhibits polyP. Cyclin-dependent kinase (CDK) complex plays a key determinant role in the PHO pathway, and CDK activity is primarily regulated by kinase activity primarily by *pho85* [48]. Knocking out *pho85* completely inhibits the PHO pathway, resulting in the inability of polyP synthesis. *Kcs1* is a gene that encodes is an important gene encoding IPMK, which is important for maintaining cell viability, promoting glycolytic metabolism, and inducing mitochondrial damage, and deletion of this gene produces an inhibition of overall cellular metabolism and affects polyP synthesis. *Pfk2* and *pyk2* are important genes that determine cellular glycogen accumulation, and knockdown of the genes facilitates the accumulation of carbohydrates in the strain, a process that is competitive with the strain's synthesis of polyP using ATP, therefore, knockdown of *pfk2* and *pyk2* would curb the accumulation of polyP synthesis to some extent. Other genes like *ade1*, *tef4*, *kre1*, *rim21*, *mrp51*, etc. play key roles in chromosome recombination, DNA and RNA metabolism, ribosome assembly, transcription or protein biosynthesis, and storage of carbohydrates, which play important roles in the maintenance of cellular growth and homeostasis, likewise have an impact on the level of ATP in the cell, will indirectly affect cellular polyP levels.

The polyP chain length of 6 strains with increased polyP concentration was analyzed by urea-PAGE, and the polyP chain length of *Δppn1* increased the most. Here, commercial polyP markers were used for chain length determination. The three polyP standards correspond to the

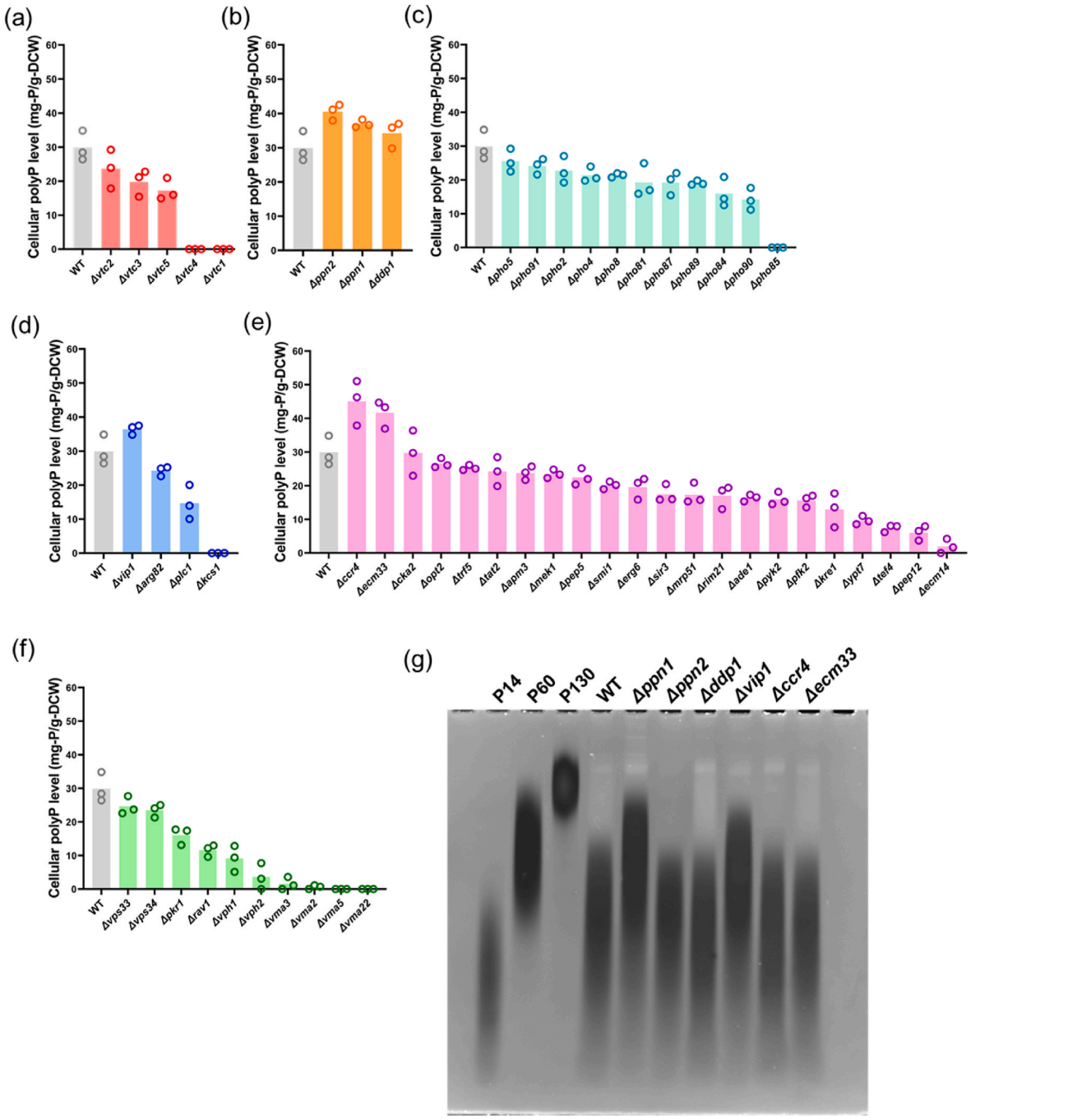


Fig. 2. PolyP assay of single-gene knockout strains. (a–f) DAPI staining quantitative analysis of cellular polyP level of different single-gene knocked down strains. (a) VTC complex, (b)endopolyphosphatase, (c) phosphatase, phosphate transporters and other members of PHO pathway, (d) Inositol polyphosphate kinase, (e) other candidates, (f) V-ATPase subunits. The unit of polyP level means the mass of phosphorus in polyP per gram of dry cell weight (DCW). Each sample repeated 3 times. (g) Urea-PAGE analysis. The commercial polyP markers, P14, P60, and P130, represent that their average chain length is 14, 60, and 130 phosphate groups.

short-chain (P14), medium-chain (P60) and long-chain (P130) components of polyP, respectively. PolyP in WT cells contains almost no long-chain components. Among the six strains with elevated polyP levels, those with deleted *ppn1* and *vip1* genes exhibited a significant increase in polyP chain length compared to the WT. The polyP chain lengths of the four mutants, *Δppn2*, *Δddp1*, *Δccr4* and *Δecm33*, were almost unchanged and even showed a decrease (Fig. 1b). The *Δppn1* and *Δvip1* variants both accumulated long-chain polyP. The BY4741 *Δppn1* variant was chosen as the starting strain for further genetic engineering due to its higher polyP production and longer chain length.

2.3. Construction and polyP analysis of double-gene knockout mutant strains

According to polyP quantitative analysis of single-gene knockout strain library, we found 6 strains showed an increase on polyP level. To improve more on polyP level, the double-gene knockout strains, constructed via homologous recombination (Scheme 1b), based on BY4741 *Δppn1* variant, were prepared for test. We knocked out one of the other 5 genes (*ddp1*, *ppn2*, *ecm33*, *ccr4*, and *vip1*) in the *Δppn1* strain and obtained 5 new double-gene knockout strains (Table 2).

After knocking out the second gene, the strains were slightly less

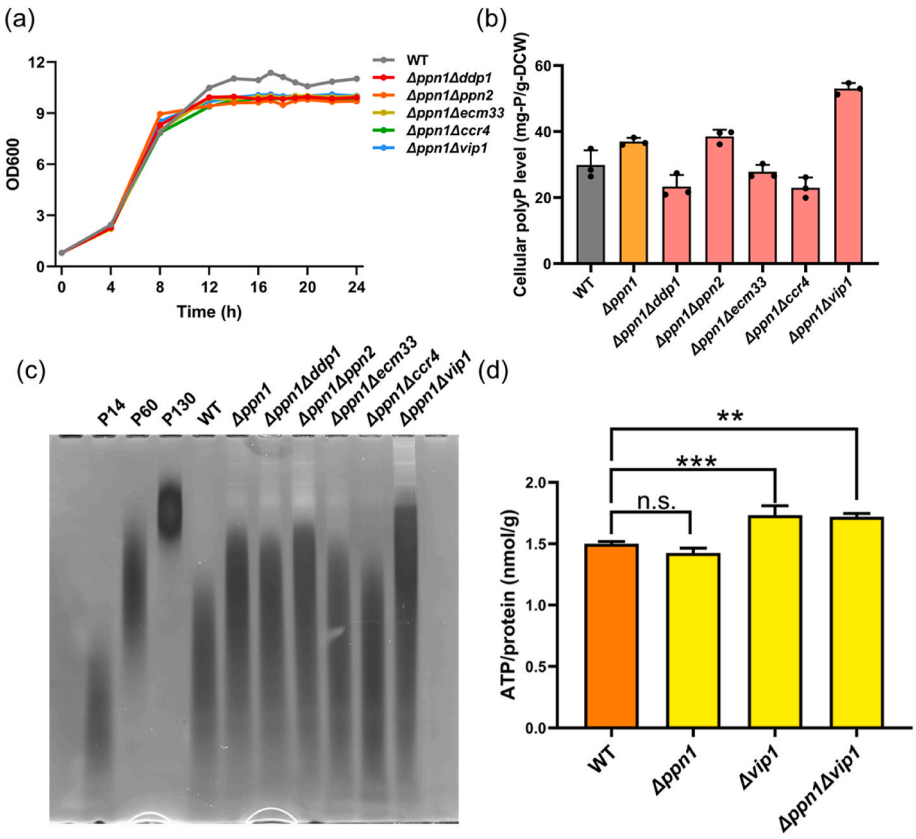


Fig. 3. Analysis of double-gene knockout strains. (a) Growth curve of double-knockout strains. (b) Cellular polyP level. Each sample was repeated 3 times. (c) Urea-PAGE. (d) Cellular ATP level of key strains. Each sample was repeated 3 times. The significances were calculated via one-way ANOVA method (***, $p < 0.001$; **, $p < 0.01$, n.s., no significance).

Table 2
Double-gene knockout mutant strains.

Starting strain	Gene knocked out	Double-gene knockout strain
BY4741 *Δppn1*	*ddp1*	*Δppn1Δddp1*
	ppn2	*Δppn1Δppn2*
	ecm33	*Δppn1Δecm33*
	ccr4	*Δppn1Δccr4*
	vip1	*Δppn1Δvip1*

active than the original strain, but their biomass in the steady state was basically the same (Fig. 3a). Fortunately, the *Δppn1Δvip1* strain showed outstanding properties on polyP level and chain length (Fig. 3b and c). Its polyP chain length was significantly improved, and it accumulated more long-chain polyP than other double-gene knockout strains. The cellular polyP level of *Δppn1Δvip1* strain reached 53.01 mg-P/g-DCW. Due to the absence of *vip1*, cellular ATP level increased and provided more substrate for polyP. ATP assay showed that *Δvip1* and the double-gene knocked out strain owned significantly higher cellular ATP concentration than WT and *Δppn1* (Fig. 3d). On the other hand, the absence of *ppn1* slowed down the degradation of polyP. Knocking out both *ppn1* and *vip1* had a cumulative effect on polyP accumulation without inhibiting yeast growth.

2.4. Further modification of the yeast via CRISPR/Cas9 method

From the single-gene deletion library study, we learnt that deletion of some key genes almost inhibited intracellular polyP accumulation. We hypothesized that overexpressing these genes in the double-gene deletion mutant *Δppn1Δvip1* might further increase polyP concentration. We envisaged that it would lead an additional increase when enzymes like VTC and V-ATPase were overexpressed in double-gene deletion mutant *Δppn1Δvip1*. We employed the CRISPR/Cas9 method for strain engineering (Scheme. 1b) [55]. We designed 6 different parts to attempt to overexpress one or more genes belonging to VTC, V-ATPase and PHO in *Δppn1Δvip1* strain (Fig. 3a). Expression of these fragments requires induction by galactose. *Vtc4* and *vtc1* are the two most critical subunits of VTC, increasing the expression levels of these two subunits could theoretically further increase cellular polyP level. In the scanning of single-gene knockout strains, deletion of the V-ATPase coding gene and *pho85* strongly inhibited polyP synthesis, thus overexpression of these genes may increase their polyP concentration. We inserted the designed parts into the 607b site of the yeast genome, which is known for high expression. Finally, 6 new engineered yeast strains, denoted PP1 ~ PP6, were constructed for testing (Table 3).

In SC medium, The PP1~PP6 strains grew similarly to WT, and the polyP chain lengths did not differ from those of the *Δppn1Δvip1* strain. With exception of PP2, these strains showed a decrease in polyP levels (Fig. 4b). In gal-SC medium, the genes that were integrated began to be expressed, the strains' own metabolic burden increased, and there was a sharp decline in biomass. (Fig. 4c). The cellular polyP level and chain length of PP1 and PP4 performed a decrease. The other four strains

Table 3
Engineered yeast strains for long-chain polyP biosynthesis.

Strain name	Genotype
PP1	*Δppn1Δvip1 607b::vtc1*
PP2	*Δppn1Δvip1 607b::vtc4*
PP3	*Δppn1Δvip1 607b::vma2*
PP4	*Δppn1Δvip1 607b::vtc4-vtc1*
PP5	*Δppn1Δvip1 607b::vtc4-vtc1-vma2*
PP6	*Δppn1Δvip1 607b::vtc4-vtc1-vma2-pho85*

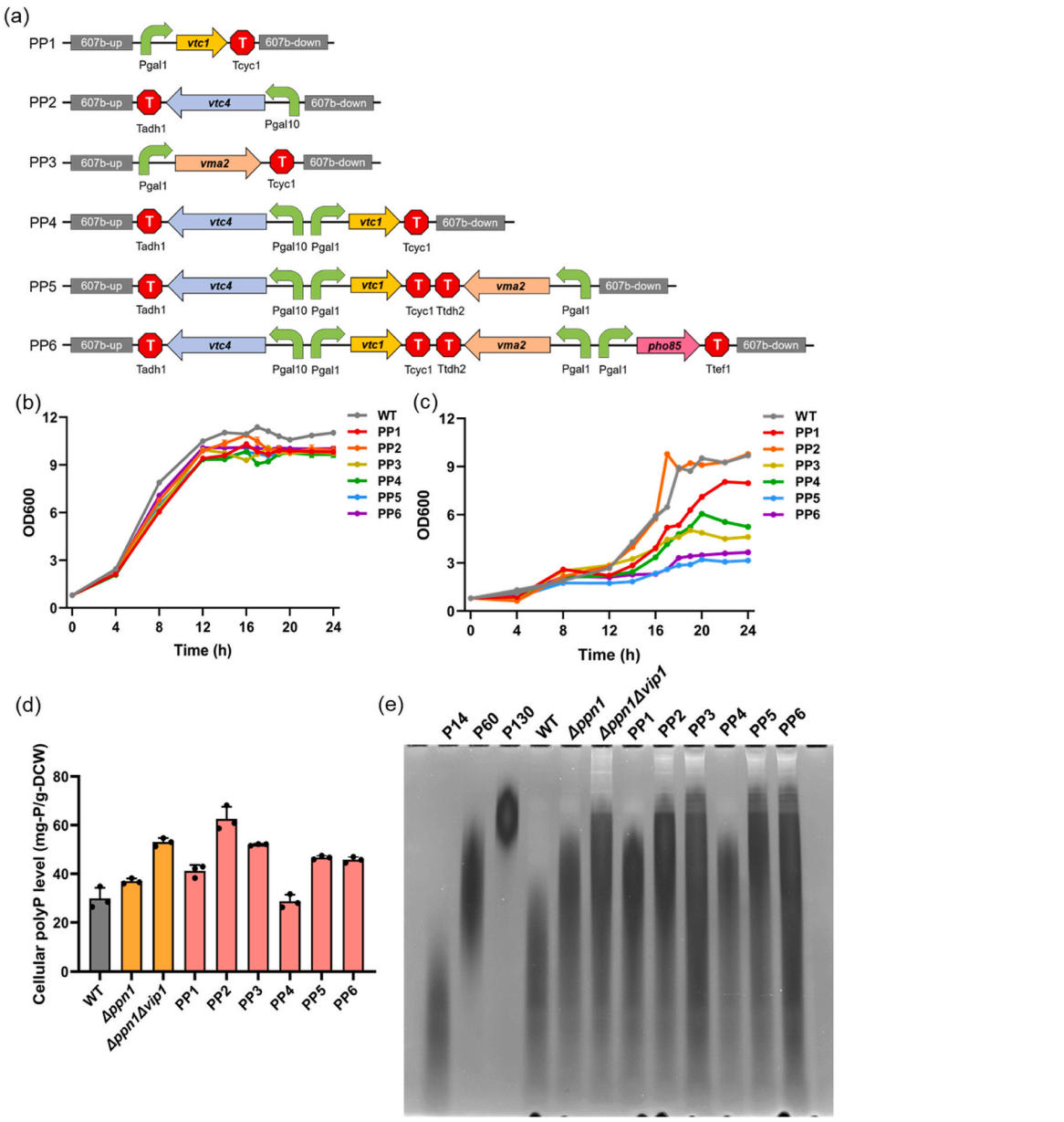


Fig. 4. Analysis of engineered strains cultivated in gal-SC medium. (a) Designed parts for genes overexpression (b) Growth curve of engineered strains in SC medium (glucose). (c) Growth curve of engineered strains in gal-SC medium, in which inserted genes were overexpressed (d) Cellular polyP level. Each sample was repeated 3 times. (e) Urea-PAGE analysis.

showed some increase in polyP levels. Although the upper front of the electrophoretic bands of these four strains did not produce significant changes compared to *Δppn1Δvip1*, it is noteworthy that the chain length distribution of the major components of polyP in the PP2 strain fell almost within the interval corresponding to P60 and P130, implying an elevation of the average chain length of polyP in PP2 cells. At the same time, the cellular polyP level of PP2 gained a significant elevation to 62.6 mg-P/mg-DCW, which was twice that of the wild-type strain. To analyze polyP synthesized by strain PP2 in more detail, we extracted polyP from WT and PP2 strains using the phenol-chloroform method used in a previous study [56]. Extracted polyP was degraded in hydrochloric acid, and the degradation products were analyzed using ammonium molybdate spectrophotometry [43]. After conversion, it was determined that 15.1 mg-P/g-DCW of polyP could be extracted from the WT strain, while 37.2 mg-P/g-DCW could be extracted from the PP2 strain. The phenol-chloroform method resulted in significant yield losses compared to the non-destructive DAPI staining method. However, the amount of polyP extracted from PP2 strain was still twice that of WT, indicating the reliability of DAPI quantification results.

2.5. Exploration of the mechanism of polyP level increase in PP2

The PP2 strain had better performance in terms of polyP concentration and chain length. We explored its polyP accumulation mechanism by qRT-PCR analysis of VTC4, the catalytic center of PolyP synthase, and three polyP-degrading enzymes, PPN2, PPX1, and DDP1, in WT, *Δppn1*, *Δppn1Δvip1*, and PP2 strains. As a VTC4 overexpressing strain, PP2 expressed 46-fold more VTC4 than WT, far more than *Δppn1* and *Δppn1Δvip1*. At the same time, the expression of endopolyphosphatase in both PP2 strains was only half that of WT. It was only under the combined effect of these factors that PP2 achieved a simultaneous increase in polyP concentration and chain length (Fig. 5). We noted that the PP2 strain grew differently in Gal-SC medium and in Glu-SC medium. Growth in Gal-SC medium might cause a decrease in

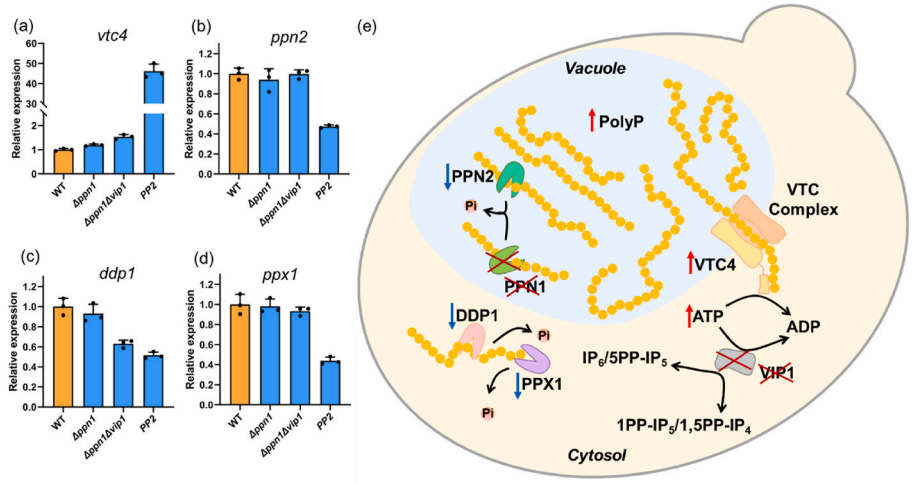


Fig. 5. Mechanism elucidation of polyP accumulation in engineered strain PP2. (a–d) Results of qRT-PCR for wild type, *Δppn1*, *Δppn1Δvip1* and PP2 strains. Relative expression of (a) *vtc4*, (b) *ppn2*, (c) *ddp1* and (d) *ppx1*. (e) Schematics of the polyP accumulation in PP2. The enzymes that knocked out are marked with red crosses. Blue down arrows indicate down regulation of enzymes and red up arrows showed up regulation of molecules.

endopolyphosphatase expression. To assess the effect of different growth conditions caused by endopolyphosphatase, we employed qRT-PCR analysis on yeast wild type grown in Gal-SC (Fig. S7). Compared with the wild-type strain grown in Glu-SC medium, the expression of DDP1 in WT in Gal-SC was almost unchanged, and the expression of PPN2 and PPX1 was slightly decreased, but the decrease was not as significant as in the PP2 strain. Thus, the decrease in endopolyphosphatase expression in PP2 strains is more due to the own properties of PP2 strains than to different growth conditions. We noted that the expression of PPN2, PPX1, and DDP1, all three enzymes, was down-regulated in *Δppn1* and *Δppn1Δvip1*. The *Δppn1Δvip1* also showed a significant decrease in the relative expression of DDP1 compared to ppn1, whereas the relative expression of VTC4 increased, and thus it is the polyP concentration and chain length of *Δppn1Δvip1* that are higher than that of *Δppn1*.

3. Conclusion

This study systematically mapped the genetic landscape of polyP biosynthesis in *S. cerevisiae*, revealing key regulators of polyP levels and chain length. Through screening a library of 55 knockout strains, we identified *ddp1*, *vip1*, and *ppn1* as critical targets for enhancing polyP accumulation, while VTC and V-ATPase subunits emerged as essential for synthesis. The *Δppn1Δvip1* double-knockout strain, coupled with *vtc4* overexpression (PP2), achieved a 2-fold increase in polyP level and preferential synthesis of long-chain species, demonstrating synergistic effects of blocking degradation pathways and boosting synthetic capacity. These findings underscore the importance of balancing polyP synthesis and hydrolysis through targeted genetic interventions. The engineered PP2 strain represents a significant advancement in microbial polyP production, with implications for sustainable manufacturing of high-value polyP for medical, agricultural, and industrial applications. Future work will focus on scaling production and exploring the mechanistic interplay between inositol pyrophosphate signaling and polyP chain elongation, further solidifying yeast as a versatile chassis for polyP biotechnology. It lays the foundation for an in-depth study of polyP synthesis in yeast and provides an optional new idea to utilize yeast as a cellular factory for long-chain polyP.

4. Materials and methods

4.1. Chemicals and reagents

4′,6-diamidino-2-phenylindole (DAPI) and sodium phosphate glass type 45 (P45) were purchased from Sigma-Aldrich. Antibiotics were purchased from Biosharp. Yeast culture medium and electrophoresis related reagents were purchased from Sangong. The qRT-PCR reagents were purchased from Vazyme. All other chemicals and buffers were purchased from Macklin. The ATP Assay Kit was purchased from Beyotime. PolyP markers (P14, P60, P130, EXP-SML Set) were purchased from RegeneTiss.

4.2. Yeast culture

In this study, all *S. cerevisiae* strains were grown on YPD plates to gain single colony. A single colony was picked into 20 mL synthetic complete (SC) medium with full supplementary nutrients and incubated at 30 °C for 12 h to pre-cultivate. Then appropriate volume of yeast culture was transferred to new SC medium to set initial OD600 value at 0.8 to start the cultivation. After enough culture time, the yeast culture was used to subsequent analysis [56]. For single-gene knockout strains, the YPD plates and pre-cultivating medium contained 200 μg/mL G418. For PP1∼PP6, the pre-cultivating medium was SC-medium with glucose. After that, galactose is then used as a carbon source for the culture medium (Gal-SC), allowing the inserted gene to be expressed.

4.3. Construction of engineered yeast variants

Homologous recombination was employed in double-gene knockout variant. For *ppn1* deletion, a pair of primers with homologous sequences to the URA3 operon, Ppn1 upstream and Ppn1 downstream were designed and amplified the URA3 operon from pESC-URA plasmid following DpnI digestion for 1 h [57]. The resulting recombination fragment was then transformed into yeast cells to replace *ppn1* with *ura* and subsequently screened on uracil-deficiency plate. Colonies were further confirmed using specific primers designed by NCBI Primer-BLAST tool. After the conformation of *ppn1* replacement, *ppn1*-hoF/R primers were employed for self-amplified via PCR for URA3 marker-recycling. Colonies were screened on SC containing 1 g/L 5-FOA plate [58] and further confirmed with the specific primers. The *Δppn1* variant was then operated with another similar homologous recombination to replace the second gene.

Starting from the double-gene knockout strain, a reported CRISPR/Cas9 toolkit was applied for gene parts insertion [55]. In brief, the genes of interest were attained by PCR from yeast genome and then assembled to *607b* up- and down-stream homologous arm. The pCUT-*607b-His* plasmid, which carries the Cas9 expression gene and the gRNA

expression box targeting the *607b* site, and each designed part were transformed into *Δppn1Δvip1* strain simultaneously to gain the PP1∼PP6 engineered strains. All prime sequences and electrophoresis maps were listed in Supporting Information (Figs. S4–S6 and Table S2).

4.4. Quantitative analysis of yeast polyP by DAPI staining

For polyphosphate researchers, the cation fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) should be the most powerful tool in polyP analysis. On account of its high sensitivity and unique fluorescent feature of DAPI-polyP complex, DAPI staining is the gold-standard for polyP quantitative analysis. According to Kulakova et al.'s study [42], DAPI-polyP complex would be excited by a 415 nm laser and generating fluorescence-emission at 550 nm which differs from nucleic acid. To estimate the quantity of polyP synthesized by yeast cell, a standard curve reflecting fluorescence intensity-polyP concentration relationship is indispensable. This standard curve was done by using sodium phosphate glass type 45 (P45) as reference substance and shown in Supporting Information (Fig. S1). For yeast polyP estimating, 1.5 mL yeast culture was transferred into a 2 mL Eppendorf tube (EP tube) then centrifuged at 10000×*g*. Optical density should be determined before. After centrifugation, the precipitate was resuspended by 1 mL HEPES solution (20 mM, pH 7.4) then stocked at −20 °C before test. The sample then should be transferred to a 15 mL Corning tube and add HEPES solution to be diluted. The final volume (mL) equals to the determined OD value in number. Mixing the diluted yeast sample, HEPES solution and DAPI solution (100 μM) at a ratio of 2:9:1 as a testing sample, then incubating 5 min. A background sample, by adding pure water instead of DAPI solution, should be prepared at the same time. Acquiring fluorescence intensity of the two samples then making a subtract and substituting the result into standard curve, polyP concentration was finally attained [43]. The concentration unit is μM for phosphate residue of polyphosphate chain, marked as μM-P. Each group was repeated 3 times parallelly. The concentration was calculated against dry cell weight (DCW) by weighing lyophilized yeast. The conversion relationship between yeast OD600 and DCW is shown in the following formula:

1.0 OD600 = 0.34 g/L DCW.

The unit of polyP level is mg-P/g-DCW for phosphorus element mass of polyphosphate per gram dry cell weight. The concentration of polyP is represented by the concentration of phosphorus in polyP.

4.5. Fluorescence imaging

For cellular polyP imaging, steady state yeast cells were washed with distilled water then incubated with 100 μM DAPI solution at 37 °C for 1 h. Then DAPI was removed by centrifugation and the cells were resuspended with distilled water. Transferring 10 μL cell suspension to a clean glass slide and covering the drop with a clean coverslip. Next, the DIC and fluorescence images were captured by Axio Observer Z1 microscope (Zeiss). The imaging parameters were calibrated by undyed yeast cells.

4.6. Urea-PAGE analysis of yeast polyP

Urea-PAGE is the universal method for polyP chain length analysis. With DAPI negative staining, polyP chain length distribution can be clearly shown on the polyacrylamide gel [59]. In brief, yeast culture was transferred into a 1.5 mL EP tube. The volume (mL) of yeast culture was calculated by current optical density in 1/OD600. The medium was removed by centrifugation and the yeast was washed by distilled water. Next, the yeast was resuspended with 60 μL 50 mM Tris buffer (pH 7.2) and boiled at 100 °C for 10 min. After that, the sample was centrifugated at 13000×*g* to remove precipitate. Commercial polyP markers, P14, P60 and P130 were used for chain length estimation. The supernatant was mixed with 2× TBE loading buffer and run on 15 % gel at 150 V for 60 min. The gel was stained by 5 μM DAPI for 3 h then exposed to UV for negative staining. Images were collected by ChemiDoc™ Imaging System.

4.7. qRT-PCR analysis

The strains were harvested at log phase and washed by DEPC-treated water. The total RNA was extracted by FastPure Universal Plant Total RNA Isolation Kit and then applied in reverse transcription reaction with HisyGo RT Red SuperMix. Next, 1 μg cDNA was used in qPCR with primers and ChamQ Blue Universal SYBR qPCR Master Mix. In this study, the *act1* gene was chosen as a loading control to normalize the gene expression. Transcript levels of relevant genes were calculated by the $2^{-\Delta\Delta Ct}$ method [60]. All primers used were shown in Supporting Information (Table S3).

4.8. ATP analysis

A suitable volume of each yeast culture was transferred, centrifuged, and washed. A commercial ATP assay kit (Beyotime) was used to lyse the yeast pellets and assay ATP by chemiluminiscence. Data were collected by a microplate reader, and the protein concentration was measured by a NanoDrop System.

CRediT authorship contribution statement

Zipeng Chen: Writing – original draft, Software, Methodology, Investigation, Data curation. **Yanling Wu:** Methodology, Investigation, Data curation. **Lingfeng Qin:** Methodology, Data curation. **Chen Wang:** Software, Methodology, Data curation. **Zhixin Li:** Investigation, Data curation. **Xiaozhou Luo:** Project administration, Investigation. **Wei Wei:** Writing – review & editing, Validation, Supervision. **Jing Zhao:** Supervision, Project administration, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2025.04.004.

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