Inorganic polyphosphate abets silencing of a sub-telomeric gene cluster in fission yeast

Ana M. Sanchez¹,²§, Angad Garg¹, Beate Schwer³, Stewart Shuman¹

¹Molecular Biology, Memorial Sloan Kettering Cancer Center, New York, New York, United States
²Gerstner Sloan Kettering Graduate School of Biomedical Sciences, New York, New York, United States
³Microbiology and Immunology, Weill Cornell Medicine, New York, New York, United States
§To whom correspondence should be addressed: sanchea6@mskcc.org

Abstract

Inorganic polyphosphate is a ubiquitous polymer with myriad roles in cell and organismal physiology. Whereas there is evidence for nuclear polyphosphate, its impact on transcriptional regulation in eukaryotes is unknown. Transcriptional profiling of fission yeast cells lacking polyphosphate (via deletion of the catalytic subunit Vtc4 of the Vtc4/Vtc2 polyphosphate polymerase complex) elicited de-repression of four protein-coding genes located within the right sub-telomeric arm of chromosome I that is known to be transcriptionally silenced by the TORC2 complex. These genes were equally de-repressed in vtc2∆ cells and in cells expressing polymerase-dead Vtc4, signifying that polyphosphate synthesis is required for repression of these sub-telomeric genes.

Figure 1. Polyphosphate synthesis by Vtc4 polyP polymerase is required for silencing sub-telomeric genes:
PolyP is especially abundant in yeast cells, e.g., the intracellular concentration of inorganic polyphosphate in budding yeast grown in phosphate-replete medium is 230 mM (with respect to phosphate residues) as compared to 23 mM for orthophosphate (Auesukaree et al. 2004). Yeast polyP is produced by a heterotrimeric membrane-associated VTC complex that synthesizes polyP and simultaneously imports the polyP into the yeast vacuole (Gerasimaite & Mayer 2014, Hothorn et al. 2009, Gerasimaite et al. 2014). Budding yeast has two VTC complexes: the VTC associated with the vacuole consists of Vtc4, Vtc3, and Vtc1 proteins; the VTC associated with the endoplasmic reticulum and nuclear envelope comprises Vtc4, Vtc2, and Vtc1 subunits (Gerasimaite & Mayer 2014, Gerasimaite et al. 2014). Fission yeast has a single heterotrimeric VTC complex that includes a Vtc2 subunit.

Vtc4 is the catalytic subunit of the polyP polymerase; it consists of a cytoplasm-facing N-terminal SPX domain, a central polymerase domain, and a C-terminal membrane anchor domain (Gerasimaite & Mayer 2014). The SPX domain binds and senses the inositol pyrophosphate signaling molecules IP$_7$ and IP$_8$ that stimulate polyP synthesis by VTC (Wild et al. 2016, Gerasimaite et al. 2017, Pascual-Ortiz et al. 2021, Schwer et al. 2022). The Vtc4 polymerase domain, which catalyzes manganese-dependent transfer of an NTP γ-phosphate to an inorganic pyrophosphate or phosphate primer (Hothorn et al. 2009), is a member of the triphosphate tunnel metalloenzyme (TTM) family (Lima et al. 1999, Martinez et al. 2015). Vtc2 and Vtc3 are paralogs homologous to Vtc4, but their TTM domains are catalytically inactive.

In budding yeast, vacuolar polyphosphate comprises ~80% of the total polyP content. There exists a pool of nuclear polyP (dependent on Vtc4) that persists in yeast cells engineered so that the intra-vacuolar pool of polyP is depleted (Azevedo & Saiardi 2014, Azevedo et al. 2020). These findings raise the question of whether polyP plays a role in nuclear transactions, especially in gene expression. To our knowledge, there is scant information on whether physiological levels of polyP impact transcriptional regulation in eukarya. To rectify this knowledge gap, we performed transcriptional profiling of fission yeast vtc4Δ cells that have no detectable intracellular polyP (Pascual-Ortiz et al. 2021, Schwer et al. 2022).

cDNAs obtained from three biological replicates, using poly(A)$^+$ RNA from wild-type and vtc4Δ cells grown to mid-log phase in YES medium at 30°C, were sequenced. Read densities for individual genes were highly reproducible between biological replicates (Pearson coefficients of 0.98 to 0.99). A cutoff of ±2-fold change in normalized transcript read level and an adjusted p-value of ≤0.05 were the criteria applied to derive a list of differentially expressed annotated loci in the vtc4Δ mutant versus the wild-type control. We then focused on differentially expressed genes with average normalized read counts ≥100 in either strain to initially exclude transcripts that were expressed at very low levels in vegetative cells. We thereby identified 36 protein-coding genes that were down-regulated and 6 protein-coding genes that were up-regulated by these criteria in vtc4Δ cells (Table 1). All 36 genes in the former set were down-regulated modestly, between 2-fold and 4-fold vis-à-vis wild-type. Among the vtc4Δ down-regulated genes were those encoding enzymes of glycolysis and sugar metabolism: enolase (Eno102 and Eno101), glyceraldehyde-3-phosphate dehydrogenase Gpd3, glucose dehydrogenase Gcd1, transaldolase Ta1, and phosphoketolase SPBC24C6.09c. Among the up-regulated gene set, 3 coding transcripts were very strongly up-regulated: gdt1(SPAC186.05c) by 111-fold; SPAC186.06 by 52-fold; and SPAC750.01 by 46-fold (Table 1). The 3 other transcripts were increased by only 2-fold in vtc4Δ cells. At this stage, the RNA-seq data suggested that the presence of polyP in wild-type cells...
might strongly repress the expression of a very narrow set of fission yeast transcripts. Therefore, we reset the cut-off criteria at a four-fold change in vtc4Δ versus wild-type and imposed no minimum threshold for read counts. This revealed one additional gene upregulated in vtc4Δ cells: SPAC186.04c by 46-fold (Table 1).

The protein products of the four vtc4Δ up-regulated genes appear unconnected functionally: gdt1 encodes a Golgi calcium and manganese transporter (Colinet et al. 2016); SPAC186.06 is a yeast homolog of phenazine biosynthesis enzyme PhzF (Liger et al. 2005); SPAC750.01 is a putative NADP-dependent aldo/keto reductase; SPAC186.04c is of unknown function. Their unifying property is that they are located physically within a sub-telomeric region of the right arm of chromosome I that is known to be transcriptionally silenced during unstressed vegetative growth (Cohen et al. 2018). The RNA-seq read counts across the cluster of adjacent SPAC186.04c, gdt1, and SPAC186.06 genes in wild-type and vtc4Δ cells show that the up-regulated RNA spans the entire predicted ORF and flanking UTRs, the dimensions of which can be surmised from the vtc4Δ RNA-seq profiles (Fig. 1A).

Weisman and colleagues have established that: (i) the fission yeast TORC2 complex (containing the Tor1 protein kinase) and its downstream effector protein kinase Gad8 are present in the nucleus and bound to chromatin (Cohen et al. 2016, Laribee & Weisman 2020); and (ii) TORC2 and Gad8 function to silence the expression of a set of 7 sub-telomeric genes on chromosomes I and II (Cohen et al. 2018) that, not coincidentally, includes all four of the genes up-regulated by vtc4Δ. These sub-telomeric genes are strongly up-regulated in tor1Δ and gad8Δ cells; they are also de-repressed in ste20A and ryh1Δ cells that respectively lack the TORC2 subunit Ste20 and the Ryh1 GTPase that activates TORC (Cohen et al. 2018). The de-repression of the sub-telomeric loci in tor1Δ and gad8Δ cells correlates with: (i) loss of the repressive H3K9Me2 chromatin mark over the loci; (ii) gain of the H3K4me3 and H4K16Ac activation marks; and (iii) increased locus occupancy by Pol2 (Cohen et al. 2018). Further studies showed that increased expression of the gdt1 (SPAC186.05c), SPAC186.04c, and SPAC186.06 genes in tor1Δ cells depends on Pol2 transcription activators Leo1, Med1, and Gcn5 (Cohen et al. 2022). Our transcriptional profiling implicates the VTC complex as a collaborator in TORC2 silencing of the sub-telomeric four-gene cluster on chromosome I.

A salient question is whether the transcriptional impact of vtc4Δ on sub-telomeric gene silencing is a consequence of the absence of polyP or the interdiction of a hypothetical function of Vtc4 other than polyP synthesis. If the VTC complex is necessary for silencing, then we would expect that deletion of the Vtc2 subunit would phenocopy vtc4Δ. The requirement for catalysis by Vtc4 can be interrogated via a polymerase active site mutation R262A,R264A in Vtc4 that eliminates polyP in vivo (Desfougères et al. 2020). Analogous genetic studies of polyP physiology in mammalian cells are not feasible at present, because the mammalian enzyme(s) responsible for polyP synthesis are not known (Desfougères et al. 2020). A surrogate approach has been to overproduce polyP in mammalian cells via ectopic expression of bacterial polyP kinase and document the effects thereof. When this maneuver was applied to human cells, it was found that: (i) high levels of long-chain polyP accumulated in multiple intracellular compartments; and (ii) 313 genes were down-regulated and 47 genes were upregulated in polyP kinase-expressing cells, by the criterion of a statistically significant 25% difference versus non-expressing cells (Bondy-Chorney et al. 2020). If a ≥2-fold difference cut-off is applied, then 102 genes were down-regulated and 8 genes were upregulated. There are caveats to this approach when it comes to inferences about polyP function. To wit: (i) the levels of polyP achieved are excessive, hence non-physiological; (ii) the polyP may localize to intracellular sites where it is not normally present; and (iii) the polyP that does accumulate is skewed toward very long chains. Indeed, studies in budding yeast indicate that forced accumulation of non-physiological levels of polyphosphate outside the vacuole and membrane compartments, achieved via expression of a bacterial
polyP kinase, is per se cytotoxic (Gerasimaite et al. 2014). Coupling of VTC-mediated polyphosphate polymerase activity to vacuolar or intramembrane import of the polyphosphate product is a means to avoid such toxicity (Gerasimaite et al. 2014).

The present data instate a role for fission yeast polyP in localized gene silencing, presumably as a participant in the TORC2 pathway of sub-telomeric silencing discovered by the Weisman lab. Based on available knowledge, we can speculate on at least two ways in which polyP may accomplish this. First, polyP can exert effects on cell physiology via non-enzymatic lysine polyphosphorylation of target proteins in vivo, including nuclear proteins such as DNA topoisomerase I, Nsr1, and ribosome biogenesis factors (Azevedo et al. 2015, Bentley-DeSousa et al. 2018, Azevedo et al. 2020). Indeed, among the validated targets of in vivo lysine polyphosphorylation in budding yeast are several proteins involved in chromatin biology: histone H2AZ chaperone Chz1, histone acetyltransferase complex subunit Eaf7, nucleosome assembly factor Hpc2 (Bentley-DeSousa et al. 2018). If lysine polyphosphorylation of nuclear proteins that establish or maintain silenced chromatin over the fission yeast chromosome I sub-telomeric cluster is important for their activity, then ablation of polyP synthesis would elicit the observed de-repression. Given that the TORC2 pathway is necessary for silencing the cluster in fission yeast, it is possible TORC2 or pathway components acting downstream are subject to lysine polyphosphorylation. Second, taking a cue from the recent studies in E. coli (Beaufay et al. 2021), polyP might promote the assembly of repressive factors at the affected loci, by providing a scaffold for their recruitment to form a higher order polyP–protein–DNA assembly, with or without accompanying phase separation. Other models or mechanisms for polyP-mediated silencing are in no way off the table. In conclusion, the initial findings here provide an impetus for further interrogation of polyP function in fungal gene expression.

Table 1. List of protein-coding genes that were dysregulated at least two-fold in vtc4∆ cells compared to the wild-type strain. The log2 fold changes are shown.

| Systematic ID | Gene     | Product                                      | log2 fold change |
|--------------|----------|----------------------------------------------|------------------|
|              | upregulated                                                                                       |
| SPAC186.05c  | gdt1     | Golgi calcium and manganese antiporter        | 6.82             |
| SPAC186.06   | PhzF protein family                                                                               | 5.71             |
| SPAC750.01   | NADP-dependent aldo/keto reductase                                                                | 5.52             |
| SPAC186.04c  | –                                                 | 5.55             |
| SPBPB10D8.03 | pseudogene transporter                                                                            | 1.29             |
| SPCC1223.13  | cbf12    | DNA-binding transcription factor               | 1.10             |
| SPBC1711.15c | Schizosaccharomyces pombe specific protein                                                          | 1.00             |
|              | downregulated                                                                                     |
| SPBC16E9.16c | lsd90    | Lsd90 protein                                 | -2.05            |
| SPBC1289.14  | adducin                                          | -1.88            |
| SPCC794.04c  | amino acid transmembrane transporter                                                             | -1.80            |
| SPACUNK4.17  | NAD binding dehydrogenase                                                                         | -1.76            |
| SPBPB21E7.01c| en0102   | enolase                                       | -1.63            |
| Gene Symbol   | Description                                                                 | Log2 Fold Change |
|---------------|------------------------------------------------------------------------------|------------------|
| SPBC336.08    | spc24 NMS complex subunit                                                    | -1.62            |
| SPBC16A3.08c  | oga1 Stm1 homolog                                                           | -1.61            |
| SPAC15E1.02c  | DUF1761 family protein                                                       | -1.59            |
| SPBC354.12    | gpd3 glyceraldehyde 3-phosphate dehydrogenase                              | -1.57            |
| SPAC27D7.03c  | mei2 RNA-binding protein involved in meiosis                                | -1.49            |
| SPBC21C3.19   | rtc3 SBDS family protein                                                     | -1.39            |
| SPAP8A3.04c   | hsp9 heat shock protein                                                      | -1.36            |
| SPCC1235.14   | ght5 high-affinity glucose/fructose:proton symporter                        | -1.32            |
| SPAC637.03    | DUF1774 family                                                              | -1.29            |
| SPBC725.10    | tsp0 mitochondrial outer membrane protein                                   | -1.27            |
| SPBC23G7.13c  | urea transmembrane transporter                                              | -1.27            |
| SPAC13G7.02c  | ssa1 Hsp70 family heat shock protein                                        | -1.18            |
| SPBC660.06    | wwm2 WW domain containing protein                                           | -1.18            |
| SPCC794.09c   | tef101 translation elongation factor EF-1 alpha                             | -1.17            |
| SPAC23H3.15c  | ddr48 DNA damage-responsive protein                                         | -1.17            |
| SPCC794.01c   | gcd1 glucose dehydrogenase                                                  | -1.13            |
| SPBPB8B6.04c  | grt1 DNA-binding transcription factor                                       | -1.09            |
| SPCC737.04    | UPF0300 family protein 6                                                    | -1.08            |
| SPBC839.15c   | tef103 translation elongation factor EF-1 alpha                             | -1.08            |
| SPAC22A12.17c | short chain dehydrogenase                                                   | -1.07            |
| SPAC26H5.09c  | oxidoreductase in NADPH regeneration                                        | -1.07            |
| SPBC24C6.09c  | phosphoketolase family protein                                              | -1.07            |
| SPAC23A1.10   | tef102 translation elongation factor EF-1 alpha                             | -1.07            |
| SPAC1039.09   | isp5 amino acid transmembrane transporter                                   | -1.05            |
Methods

Transcriptome profiling by RNA-seq.
RNA was isolated from *S. pombe* wild-type and *vtc4Δ* cells that were grown in liquid YES medium at 30°C to an A_{600} of 0.5 to 0.6. Cells were harvested by centrifugation and total RNA was extracted via the hot phenol method. The integrity of total RNA was gauged with an Agilent Technologies 2100 Bioanalyzer. The Illumina TruSeq stranded mRNA sample preparation kit was used to purify poly(A)^+ RNA from 500 ng of total RNA and to carry out the subsequent steps of poly(A)^+ RNA fragmentation, strand-specific cDNA synthesis, indexing, and amplification. Indexed libraries were normalized and pooled for paired-end sequencing performed by using an Illumina NovaSeq 6000-S1 flow cell. FASTQ files bearing paired-end reads of length 51 bases (total paired reads of 19.1 million to 27.5 million per biological replicate) were mapped to the *S. pombe* genome (Pombase) using HISAT2-2.1.0 with default parameters (Kim et al. 2015). Mapped reads comprised 93% to 96% of the total reads per replicate. The resulting SAM files were converted to BAM files using Samtools (Li et al. 2009). Count files for individual replicates were generated with HTSeq-0.10.0 (Anders et al. 2015) using exon annotations from Pombase (GFF annotations, genome-version ASM294v2; source 'ensembl'). RPKM analysis and calculations of pairwise correlations (Pearson coefficients of 0.978 to 0.987) were performed as described previously (Schwer et al. 2014). Differential gene expression and fold change analysis was performed in DESeq2 (Love et al. 2014). Cut-off for further evaluation was set for genes that had an adjusted p-value (Benjamini-Hochberg corrected) of ≤0.05 and were up or down by at least two-fold in *vtc4Δ* versus wild-type. Genes were further filtered on the following criteria: (i) ≥2-fold up and the average normalized read count for the mutant strain was ≥100; and (ii) ≥2-fold down and the average normalized read count for the wild-type strain was ≥100. Alternatively, a cut-off of at least a four-fold up or down in *vtc4Δ* versus wild-type was set without regard to the normalized read count values, which flagged *SPAC186.04c* as upregulated in *vtc4Δ* cells.

Reverse transcriptase quantitative PCR analysis.
Total RNA was prepared from exponentially growing cells (three independent cultures for each yeast strain analyzed) via the hot phenol method. The RNAs were treated with DNase I, extracted serially with phenol:chloroform and chloroform, and then precipitated with ethanol. The RNAs were resuspended in 10 mM Tris HCl (pH 6.8) and 1 mM EDTA and adjusted to a concentration of 600 ng/μl. Reverse transcription was performed with 2 μg of this RNA template plus oligo(dT)_{18} and random hexamer primers by using the Maxima First Strand cDNA synthesis kit (Thermo Scientific). After cDNA synthesis for 30 min at 55°C, the reverse transcription reaction mixtures were diluted 10-fold with water. Aliquots (2 μl) were used as templates for gene-specific quantitative PCR (qPCR) reactions directed by the sense and antisense primers listed in Reagents. The qPCR reactions were constituted with the Maxima SYBR Green/ROX master mix (Thermo Scientific) and monitored with an Applied Biosystems QuantStudio 6 Flex Real-Time PCR system. The qPCR reactions were performed in triplicate for each cDNA population. The level of individual cDNAs was calculated relative to that of *act1* cDNA by the comparative Ct method (Schmittgen & Livak 2008). The actin-normalized levels of the four sub-telomeric transcripts in wild-type cells were assigned a value of 1.0 and the mRNA levels in the three *vtc* mutant were then normalized to the wild-type control value.

Data Deposition. The RNA-seq data in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE213524.
Reagents

Oligonucleotide primers used for qPCR analyses

| Gene        | Strand | Sequence               |
|-------------|--------|------------------------|
| act1        | Sense  | 5’–AAGTACCCCATTGAGCACGG |
|             | Antisense | 5’–CAGTCAACAAGCAAGGGTGTC |
| SPAC186.04c | Sense  | 5’–GCGAAGAAAAACCAACAGC |
|             | Antisense | 5’–TCATCGTTTACTCTGATCCGTA |
| gdt1 (SPAC186.05c) | Sense | 5’–AAATTTTCCCGGGCTTTCAT |
|             | Antisense | 5’–TCCGACAATCACCGCTACC |
| SPAC186.06  | Sense  | 5’–GGGAGTGAGGCTGGATCAGT |
|             | Antisense | 5’–CGCCACCAACATGAATATCG |
| SPAC750.01  | Sense  | 5’–TATTGGGAAGACTGGGTGCT |
|             | Antisense | 5’–CCAACCAATTCTCTAGACACCCCA |

Primers for all genes (except for act1) were the same used by Cohen et al. 2018 and 2022.

Fission yeast strains used in this study

| Strain | Genotype                  |
|--------|---------------------------|
| BS78   | h+ vtc2Δ::kanMX           |
| BS128  | h- vtc4Δ::kanMX           |
| BS623  | h+ vtc4-R262A,R264A::kanMX |

All strains are leu1-32 ura4-D18 his3-D1 and either ade6-m216 or ade6-m210.

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