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# Inositol pyrophosphates modulate cell cycle independently of alteration in telomere length

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Abstract

Synthesis of inositol pyrophosphates through activation of Kcs1 plays an important role in the signaling response required for cell cycle progression after mating pheromone arrest. Overexpression of Kcs1 doubled the level of inositol pyrophosphates when compared to wild type cells and 30 minutes following the release from α-factor block further increase in inositol pyrophosphates was observed, which resulted that cells overexpressing Kcs1 reached $G_2$/M phase earlier than wild type cells. Similar effect was observed in *ipk1Δ* cells, which are unable to synthesize $IP_6$-derived inositol pyrophosphates ($IP_7$ and $IP_8$) but will synthesize $IP_5$-derived inositol pyrophosphates (PP-$IP_4$ and $(PP)_2$-$IP_3$). Although *ipk1Δ* cells have shorter telomeres than wild type cells, overexpression of Kcs1 in both strains have similar effect on cell cycle progression. As it is known that PP-$IP_4$ regulates telomere length through Tel1, inositol polyphosphates, cell cycle and telomere length were determined in *tel1Δ* cells. The release of the cells from α-factor block and overexpression of Kcs1 in *tel1Δ* cells produced similar effects on inositol pyrophosphates level and cell cycle progression when compared to wild type cells, although *tel1Δ* cells possesses shorter telomeres than wild type cells. It can be concluded that telomere length does not affect cell cycle progression, since cells with short telomeres (*ipk1Δ* and *tel1Δ*) progress through cell cycle in a similar manner as wild type cells and that overexpression of Kcs1 in cells with either short or normal telomeres will increase S phase progression without affecting telomere length. Furthermore, $IP_5$-derived inositol pyrophosphates can compensate for the loss of $IP_6$-derived inositol pyrophosphates, in modulating S phase progression of the cell cycle.

Keywords: Inositol pyrophosphates; Kcs1; Ipk1; Tel1; Telomere length; Cell cycle

Abbreviations: Plc, phospholipase C; $IP_3$, inositol trisphosphate; $IP_4$, inositol tetrakisphosphate; $IP_5$, inositol pentakisphosphate; $IP_6$, inositol hexakisphosphate; $IP_7$ (also referred as PP-$IP_5$), diphosphoinositol pentakisphosphate; $IP_8$ [also referred as $(PP)_2$-$IP_4$], bisdiphosphoinositol tetrakisphosphate; PP-$IP_4$, diphosphoinositol tetrakisphosphate; $(PP)_2$-$IP_3$, bisdiphosphoinositol trisphosphate; HPLC, high performance liquid chromatography.

## Introduction

In mammalian cells, the activation of several isoforms of phosphoinositide-specific phospholipase C (Plc) has been demonstrated in nuclei during proliferation and synchronous progression through the cell cycle. However, downstream targets of Plc-generated second messengers in nuclei have remained poorly described (reviewed in Visnjic and Banfic, 2007; Follo et al., 2014). Irvine et al. (1993) first observed that there are many similarities between nuclear phospholipid signalling in mammals and phospholipid signalling in the budding yeasts *Saccharomyces cerevisiae* (Irvine, 2003). Budding yeasts have a sole Plc gene, PLC1, that can generate diacylglycerol and inositol 1,4,5-trisphosphate from phosphatidyl-inositol 4,5-bisphosphate, but they do not utilize diacylglycerol to activate protein kinase C and have no I(1,4,5)$P_3$ receptor in their genome to mobilize calcium. Instead, yeast use I(1,4,5)$P_3$ as a precursor for the synthesis of higher inositol phosphates, and these products have been proved to regulate important nuclear events in several genetic and biochemical studies of Plc-mediated signalling (Tsui and York, 2010). Therefore, the focus of interest has progressively shifted toward understanding of pathways responsible for generation of higher phosphates and pyrophosphates and their possible roles in eukaryotic cells.

In *S. cerevisiae*, four different kinases participate in generation of inositol phosphates and pyrophosphates. Ipk2 phosphorylates I(1,4,5)$P_3$ into I(1,4,5,6)$P_4$ ($IP_4$) and I(1,3,4,5,6)$P_5$ ($IP_5$). A major role of Ipk2 and its products, $IP_4$ and $IP_5$, is transcriptional regulation by modulation of the chromatin remodelling in response to nutrients (Odom et al., 2000; Steger et al., 2003). The yeast Ipk1 and its product, $IP_6$, which is generated by phosphorylation of $IP_5$, regulate mRNA export through conjunction with Gle1 which regulates the activity of the DEAD-box protein Dbp5 at the nuclear pore complexes cytoplasmic face (York et al., 1999; Folkman et al., 2014) and nonhomologous end joining (Hanakahi et al., 2000). The synthesis of diphosphoinositol phosphates or pyrophosphates in yeasts is mediated by two kinases: Kcs1 phosphorylates $IP_5$ into 5-PP-$IP_4$, and $IP_6$ into 5-PP-$IP_5$ ($IP_7$); Vip1 phosphorylates $IP_6$ into $IP_7$ isomer 1-PP-$IP_5$. $(PP)_2$-$IP_4$ ($IP_8$) is generated by either Kcs1-mediated phosphorylation of 1-PP-$IP_5$ or Vip1-mediated phosphorylation of 5-PP-$IP_5$ (Tsui and York, 2010). Kcs1 has been reported to modulate telomere length by generation of 5-PP-$IP_4$ (Saiardi et al., 2005; York et al., 2005), and Vip1-generated $IP_7$ binds to the cyclin-CDK-CDK inhibitor complex to regulate transcription of the yeast phosphate (Pi)-responsive (PHO) genes (Lee et al., 2007).

Our recent study demonstrated changes in inositol phosphate levels in α-factor-treated *S. cerevisiae*, which allows cells to progress synchronously through the cell cycle after release from a $G_1$ block. The results of the study showed an increase in the activity of Plc1 early after release from the block with a concomitant increase in the level of $IP_7$ and $IP_8$. The enzymatic activity of Kcs1 in vitro and HPLC analysis of $^3$[H]inositol-labeled *kcs1*Δ cells confirmed that Kcs1 is the principal kinase responsible for generation of pyrophosphates in synchronously progressing cells. Furthermore, disruption of the *DDP1* gene, which encodes a phosphatase that dephosphorylates both $IP_8$ and $IP_7$ down to $IP_6$, increased the level of pyrophosphates following release from α-factor block. While *ddp1*Δ cells reached $G_2$/M phase earlier than wild type cells, FACS analysis of α-factor synchronized *plc1*Δ and *kcs1*Δ yeast mutants revealed a block in S phase, suggesting that progression through the S phase may be regulated by inositol pyrophosphates (Banfic et al., 2013).

In this study we have investigated how overexpression of Kcs1 affects level of inositol pyrophosphates and progression of cells through the cell cycle. Furthermore, as *ipk1Δ* cells are unable to synthesize $IP_6$, $IP_7$ or $IP_8$, but will accumulate alternative inositol pyrophosphates, PP-$IP_4$ and $(PP)_2$-$IP_3$ and have telomeres slightly shorter than wild type (Saiardi et al., 2005; York et al., 2005), therefore herein we have investigated how

overexpression of Kcs1 affects the level of alternative inositol pyrophosphates, telomere length and progression of cells through the cell cycle.

## Materials and methods

### Materials

Reagents were obtained from the following sources: all media and supplements for yeast growth from Formedium; α-factor mating pheromone from Zymo Research; glass beads (diameter 425-600 μm), RNaseA and proteinase K from Sigma; [$^3$H]inositol (30 Ci/mmol) from Perkin Elmer or American Radiolabeled Chemicals and Sytox from Invitrogen. All other chemicals were of analytical grade.

### Yeast strains and growth condition

*S. cerevisiae* strains used in this study are isogenic with W303 MAT**a** (*leu2-3,112*, *his3-11,15*, *ura3-1*, *ade2-1*, *trp1-1*, *rad5-535*, *can1-100*). Deletion strains *ipk1*::*KANMX*, *tel1*::*ura3*, as well as strains in which Kcs1 was overexpressed were generated as described previously (York et al., 2005). For labeling experiments, cells were inoculated in synthetic medium without inositol with addition of 5.7 mg/ml ammonium sulphate, 0.82 mg/ml amino acids, 2% glucose, and 5 μCi/ml $^3$[H]inositol (labeling medium) and grown to achieve isotopic equilibrium (8-9 divisions) in a shaker incubator at 30 $^o$C and 200 r.p.m. (Banfic et al., 2013). At the end of incubation, cells were collected, counted, resuspended in fresh labeling medium containing 2% galactose at the concentration of 2.5 x $10^6$/ml, grown into mid-logarithmic phase, and then incubated in the presence of 5 μM α-factor mating pheromone for 6 h. The $G_1$-arrested cells were washed twice with synthetic medium lacking inositol, released into fresh labeling medium containing 2% raffinose, and allowed to progress synchronously through the cell cycle. At the indicated time points, cells were harvested by centrifugation (2,000 x g, 4 $^o$C, 4 min.) For flow cytometric analysis and telomere length determination, cells were grown as described above, except that cold inositol was added into the medium at the same concentration as $^3$[H]inositol.

### Extraction and analysis of $^3$[H]inositol-labeled inositol polyphosphates

For inositol polyphosphate analysis, the pellet was resuspended in 0.2 ml of extraction buffer (1M $HClO_4$, 3 mM EDTA and 0.1 mg/ml $IP_6$), glass beads were added and cells were disrupted by vortexing, as described above. After centrifugation for 1 min in microcentrifuge, the soluble extract was transferred to a new tube, 0.2 ml of neutralization buffer (1 M $K_2CO_3$ and 3 mM EDTA) was added to achieve pH 6-8, neutralization was allowed overnight at 4 $^o$C, the final volume was adjusted to 0.5 ml, samples were filtered using Spin-X microcentrifuge tubes and stored for HPLC analysis. Separation of all the inositol phosphates was achieved using high resolution 5 μM Partisphere SAX column (Whatman) at a flow rate of 1 ml/min, with a gradient generated by mixing buffers A (1 mM EDTA) and B 1.3 M $(NH_4)_2HPO_4$ X $H_3PO_4$ (pH 3.8) as follows: 0-10 min, 0% buffer B; 10-75 min, 0-100% buffer B; 75-85 min, 100% buffer B using Ultimate 3000 HPLC system (Dionex) (Banfic et al., 2013).

## Flow cytometric analysis

At indicated time points, 1 ml of cell suspension was harvested, fixed with 9 ml of 90% ethanol and stored at -20 $^{o}$C. Cells were washed by 50 mM sodium citrate pH 7.4, sonicated, incubated in 50 mM sodium citrate pH 7.4 containing 0.25 mg/ml of RNase A at 50 $^{o}$C for 1 h, then treated with 1 mg/ml of proteinase K at 50 $^{o}$C for 1 h, washed and resuspended in 50 mM sodium citrate containing 1 μM Sytox. The DNA fluorescence analyses were performed on at least 10,000 cells for each sample with the FACSCalibur system (Becton Dickinson), and the data were analysed using CellQuest and ModFit software (Becton Dickinson) (Banfic et al., 2013).

## Southern analysis of telomere length

Genomic DNA was prepared from 25-ml cultures and digested with PstI before electrophoresis on a 1% Tris borate-EDTA-agarose gel. The fragments were transferred to Hybond N+ nylon membranes (Amersham Biosciences) and hybridized at 65 $^{o}$C to a probe derived from pYT14 containing telomeric DNA and a portion of the Y' subtelomeric repeat. The probe was labeled with of [α-$^{32}$P]dCTP using ready-to-go labeling beads (Amersham Biosciences) as described previously (York et al., 2005).

## Results and discussion

Since our previous study showed that increase in inositol pyrophosphates level generated by increase in the activity of Kcs1 is responsible for increase in progression through S phase of the cell cycle (Banfic et al., 2013) we overexpressed Kcs1 in wild type cells to see how this will affect level of inositol pyrophosphates and cell cycle progression. As shown in Fig. 1, overexpression of Kcs1 for 8 hours, during which cells were also arrested in $G_1$-phase, doubled the level of inositol pyrophosphates ($IP_7$ and $IP_8$) when compared to wild type cells. Following the release from α-factor block further increase in the radioactivity of inositol pyrophosphates was observed, and the amount of radioactivity doubled the amount found in wild type cells 30 minutes after the release from α–factor block. Furthermore, FACS analysis showed that cells overexpressing Kcs1 reached $G_2$/M phase earlier than wild type cells (Fig. 2a).

As shown in Fig. 1, *ipk1Δ* cells are unable to synthesize $IP_6$, $IP_7$ or $IP_8$, but will accumulate alternative inositol pyrophosphates, PP-$IP_4$ and $(PP)_2$-$IP_3$ (Saiardi et al., 2005; York et al., 2005), and 30 min after the release from α-factor arrest increase in PP-$IP_4$ and $(PP)_2$-$IP_3$ was observed, which was similar to the increase in $IP_7$ and $IP_8$ in wild type cells (Fig. 1). When cell cycle analysis of *ipk1Δ* cells was performed, no significant differences were observed in the number of cells that reached $G_2$/M phase in comparison to the wild type cells (Fig. 2b). Overexpression of Kcs1 in *ipk1Δ* cells produced similar effects on the basal level and the levels of PP-$IP_4$ and $(PP)_2$-$IP_3$ 30 min following the release from α–factor block, as did overexpression of the enzyme in wild type cells inositol pyrophosphates (Fig. 1). Also, as in wild type cells, cell cycle analysis showed that *ipk1Δ* cells overexpressing Kcs1 reached $G_2$/M phase earlier than *ipk1Δ* cells (Fig. 2b). Altogether these results suggest that progression through S phase of the cell cycle is modulated by Kcs1-mediated production of inositol pyrophosphates and that $IP_5$-derived inositol pyrophosphates, PP-$IP_4$ and $(PP)_2$-$IP_3$, can compensate for the loss of $IP_6$-derived inositol pyrophosphates, $IP_7$ and $IP_8$.

It is known that *ipk1Δ* cells have shorter telomeres modulated by the level of PP-$IP_4$, and depends on the presence of telomere regulator Tel1, a phosphatidylinositol 3-kinase related kinases family member and yeast homologue of ATM, the protein mutated in human disease ataxia telangiectasia (Saiardi et al., 2005; York et al., 2005). Therefore, we have investigated what will happen with level of inositol pyrophosphates and cell cycle progression in *tel1Δ* cells overexpressing Kcs1. As shown in Fig. 1, inositol pyrophosphate levels prior and after the release from α–factor block in *tel1Δ* cells and *tel1Δ* cells overexpressing Kcs1 were the same as in wild type cells. Moreover, cell cycle analysis showed that cell cycle progression of *tel1Δ* cells is the same as in wild type cells and that *tel1Δ* cells overexpressing Kcs1 will reach $G_2$/M phase earlier than control cells (Fig. 2). Overexpression of Kcs1 did not change telomere length either in wild type, *ipk1Δ* or *tel1Δ* cells (Fig. 3). This observation is somehow different than one showed by York et al., (2005), where elevated levels of PP-$IP_4$ caused by overexpression of Kcs1 in *ipk1Δ* cells produced further shortening of telomeres. As it is known that telomerase acts in S phase of the cell cycle and it takes several cell cycles to adjust telomere length (Blackburn, 2001), simple explanation for this difference is that York et al., (2005) prepared genomic DNA from cells completed several cell divisions prior to analysis, while we overexpressed Kcs1 during the same period when cells were blocked by α-factor, allowing them to complete just one cycle prior telomere length analysis. On the other hand, when results of telomere length determination and cell cycle progression are compared it is obvious that telomere length does not affect cell cycle progression, since cells with short telomeres (*ipk1Δ* and *tel1Δ*) progress through cell cycle in a similar manner as wild type cells and that overexpression of Kcs1 in cells with both short or normal telomeres will increase S phase progression without affecting telomere length.

Present study further corroborates our previous finding (Banfic et al., 2013) that progression through S phase of the cell cycle is modulated by Kcs1-mediated production of inositol pyrophosphates and extends it by showing that $IP_5$-derived inositol pyrophosphates, PP-$IP_4$ and $(PP)_2$-$IP_3$, can compensate for the loss of $IP_6$-derived inositol pyrophosphates, $IP_7$ and $IP_8$. There are numerous ways how inositol pyrophosphates may regulate cell cycle progression, this may include events on vacuole biogenesis and cell wall integrity (Dubois et al., 2002), proper endocytic trafficking (Saiardi et al., 2002), cyclin-CDK-CDK inhibitor complex (Lee et al., 2007), chromatin remodelling (Steger et al., 2003) or RNA polymerase I-mediated rRNA transcription (Thota et al., 2015) and for all of them understanding of structural analysis of diphosphoinositol polyphosphate kinase is crucial (Shears et al., 2013). Furthermore, above all of them, two might be of particular significance. First, is the relationship between inositol pyrophosphates and cellular bioenergetics. Szijgyarto et al. (2011) showed that sustained decrease in $IP_7$ level, promote metabolic adjustments and exhibit elevated cellular ATP level, which was associated with upregulation of glycolytic gene expression and profound reduction in mitochondrial function. It can be speculated that change in inositol pyrophosphates level, by controlling cell ATP content and general synthetic activity, may not only control cell growth as suggested by Szijgyarto et al. (2011) and Irvine and Denton (2011), but may also control cell cycle progression. Second is the possibility that inositol pyrophosphates through protein diphosphorylation may control cell cycle progression. Saiardi et al. (2004), first demonstrated that certain proteins can be phosphorylated, at least *in vitro*, by inositol pyrophosphates. Subsequently it emerged that the β-phosphate of diphosphate groups of inositol pyrophosphates are added to pre-existing Ser-phosphate that is initially provided by a casein kinase II dependent phosphorylation event (Bhandari et al., 2007). A remarkable aspect of phosphate donation by inositol pyrophosphates is that it is non-enzymatic and that all inositol pyrophosphates have similar pyrophosphorylating ability (Bhandari et al., 2007). Two studies have been published investigating how pyrophosphorylation may modulate protein activity in yeast and human cells (Azevedo et al.,

2009, Szijgyrato et al., 2011) and both demonstrated that pyrophosphorylation mediates protein-protein interactions *in vitro*. Although no-one has yet directly demonstrated that inositol pyrophosphates can diphosphorylate proteins *in vivo* (Shears, 2015), perhaps model we used in this study may be useful to clarify this question, because we have demonstrated that different inositol pyrophosphates can compensate for each other and that increase in inositol pyrophosphate level will modulate S phase progression. At the moment we can only speculate that inositol pyrophosphates may diphosphorylate one of proteins, such as Sic1, which promotes S phase entry via binding with Cdc4 and act as a timer of cyclin-dependent kinases waves in the yeast cell cycle (Barberis, 2012).

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Figure legends

Fig. 1. Changes in inositol phosphate levels in wild type, *ipk1Δ* and *tel1Δ* cells, and the same strains overexpressing KCS1. Cells were labelled, synchronized in $G_1$ with α-factor, released into fresh medium, and inositol phosphates were analysed by HPLC as described in Materials and methods. HPLC elution profile of inositol phosphates at time 0 or 30 min after the release from α–factor block.

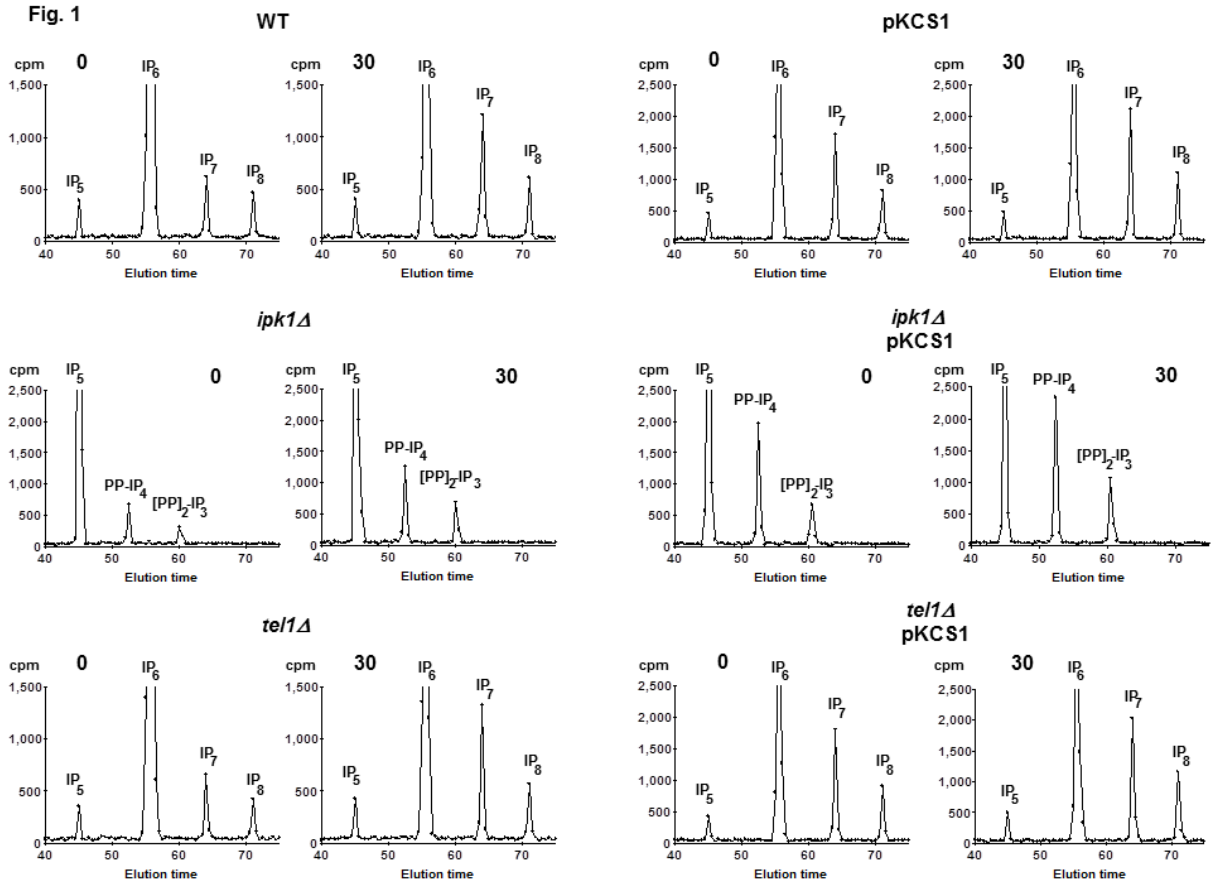

Fig. 2. Progression of -wild type, *ipk1Δ* and *tel1Δ* cells, and the same strains overexpressing KCS1 through the cell cycle following the release from α–factor block. Cells were synchronized in $G_1$ with α-factor, released into fresh medium, harvested at indicated times, stained with Sytox, and assessed for cell cycle distribution by flow cytometric analysis as described under Materials and methods.

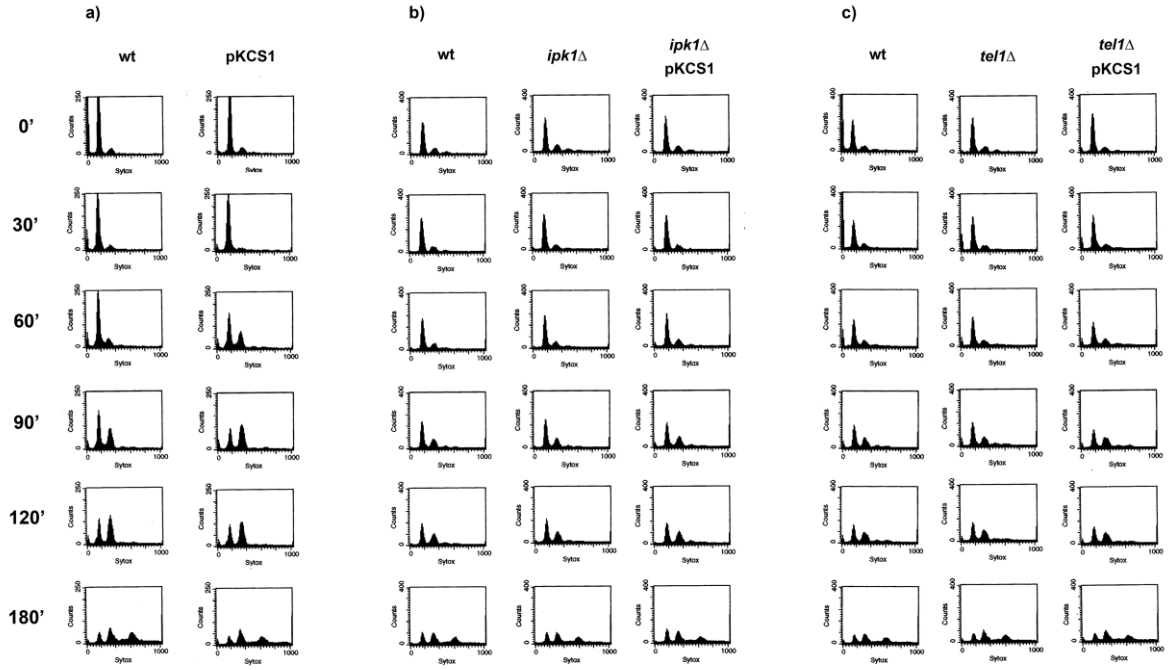

Fig. 3. Telomere analysis of wild type, *ipk1Δ* and *tel1Δ* cells, and the same strains overexpressing KCS1. Cells were synchronized in $G_1$ with α-factor, released into fresh medium, harvested 30 min after the release from α–factor block, and telomere length was determined as described in Materials and methods. Lane 1, wild type; lane 2, wild type overexpressing pKCS1; lane 3, *ipk1Δ*; lane 4, *tel1Δ*; lane 5, *ipk1Δ* overexpressing pKCS1; lane 6, *tel1Δ* overexpressing pKCS1.

**Fig. 3**