



# A lysine accumulation phenotype of ScIpk2Delta mutant yeast is rescued by *Solanum tuberosum* inositol phosphate multikinase

Samuel E. K. Caddick, Christopher J. Harrison, Ioanna Stavridou, Sue Johnson, Charles A. Brearley

## ► To cite this version:

Samuel E. K. Caddick, Christopher J. Harrison, Ioanna Stavridou, Sue Johnson, Charles A. Brearley. A lysine accumulation phenotype of ScIpk2Delta mutant yeast is rescued by *Solanum tuberosum* inositol phosphate multikinase. *Biochemical Journal*, 2007, 403 (3), pp.381-389. 10.1042/BJ20061772 . hal-00478705

**HAL Id: hal-00478705**

**<https://hal.science/hal-00478705>**

Submitted on 30 Apr 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

A lysine accumulation phenotype of *ScIpk2Δ* mutant yeast is rescued by *Solanum tuberosum* inositol phosphate multikinase.

Samuel E. K. Caddick, Christopher J. Harrison, Ioanna Stavridou, Sue Johnson,  
5 and Charles A. Brearley\*

School of Biological Sciences, University of East Anglia, Norwich, Norfolk, NR4  
7TJ. Tel. +44 (0)1603 592917 Fax. +44 (0)1603 592250 Email: c.brearley@uea.ac.uk.

10 \*to whom correspondence should be addressed

Running title: lysine synthesis in *ScIpk2Δ* mutants is rescued by a potato inositol polyphosphate multikinase

15 Keywords: IPMK, lysine, arginine, *Solanum tuberosum*, *Saccharomyces cerevisiae*

## SYNOPSIS

Inositol phosphates and the enzymes that interconvert them are key regulators of diverse cellular processes including the transcriptional machinery of arginine synthesis [York, J. D. (2006) Regulation of nuclear processes by inositol polyphosphates. *Biochim.Biophys.Acta* **1761**, 552-559]. Despite considerable interest and debate surrounding the role of *Saccharomyces cerevisiae* inositol polyphosphate kinase (*ScIPK2*, *ARG82*, *ARGRIII*) and its inositol polyphosphate products in these processes, there is an absence of data describing how the transcripts of the arginine synthetic pathway, and the amino acid content of *ScIpk2Δ*, are altered under different nutrient regimes. We have cloned an inositol phosphate multikinase from *Solanum tuberosum*, *StIPMK* (GenBank accession, EF362785), that despite considerable sequence divergence from *ScIPK2*, restores the arginine biosynthesis pathway transcripts *ARG8*, acetylornithine aminotransferase, and *ARG3*, ornithine carbamoyltransferase of *ScIpk2Δ* yeast to Wild Type profiles. *StIPMK* also restores the amino acid profiles of mutant yeast to Wild Type, and does so with ornithine or arginine as sole nitrogen sources. Our data reveal a lysine

20  
25  
30

accumulation phenotype in *ScIpk2Δ* yeast that is restored to Wild Type profile by expression of *StIPMK*, including restoration of the transcript profiles of lysine biosynthetic genes. That StIPMK protein shows only 18.6% identity with ScIPK2p likely indicates that the rescue of transcript and diverse amino acid phenotypes is not mediated through direct interaction of StIPMK with the ArgR-Mcm1 transcription factor complex that is a molecular partner of ScIPK2p.

## INTRODUCTION

Much has been reported about members of the inositol phosphate multikinase family from mammals, yeast and plants (for review see [1]). The catalytic flexibility of these enzymes makes them ideal candidates for regulating the levels of inositol phosphates involved in various signalling events, metabolic pathways and cellular processes. Indeed the role of multikinases in inositol hexakisphosphate biosynthesis in plants [2], the regulation of mRNA export from the nucleus in yeast [3,4], and their contribution to arginine biosynthesis in yeast [5,6,7,8,9,10,11], are a few examples of their biological importance.

The yeast inositol phosphate multikinase, *ScIPK2* (*ARG82*, *ARGRIII*), acts in association with the ArgR-Mcm1 transcription factor complex to regulate arginine biosynthesis. The ARG80p and ARG81p components of the complex bind to arginine boxes in the promoters of arginine biosynthetic and catabolic genes, and ScIPK2p stabilizes this complex [5]. Other studies indicate that the kinase activity of ScIPK2p is necessary [12], while more recent work [13] indicates that Ins(1,4,5) $P_3$  6-kinase activity is sufficient to rescue a growth phenotype of *ScIpk2Δ* yeast. Remarkably, and notwithstanding that the different groups have established loss or gain of arginine biosynthetic competence using a range of different growth and phenotypic assays, only the study of Dubois and Messenguy [8] has directly addressed the arginine content of yeast cells, and among recent studies of inositol phosphate multikinases only that of Resnick *et al.* [14] has examined the levels of transcripts, and only then of *ARG8*.

We have taken the approach of looking directly at the amino acids present in a Wild Type and *ScIpk2Δ* mutant strains, and have attempted to complement the *ScIpk2Δ*

mutant strain phenotype by introducing an inositol phosphate multikinase, *StIPMK*, cloned by ourselves from *Solanum tuberosum* (*StIPMK* GenBank accession, EF362785). Moreover, by parallel analysis of transcripts in the yeast samples from which we have analysed amino acids, we have demonstrated that transformation with *StIPMK* restores arginine metabolic gene transcript levels of the *ScIpk2Δ* mutant to Wild Type profiles. In this paper, we report the cloning of *StIPMK*; an inositol polyphosphate kinase from potato (Desirée cv.). *StIPMK* recombinant protein exhibits a range of catalytic abilities. By way of sequence comparison at the amino acid level with other multikinases and by rescue of inositol phosphate synthesis in *ScIpk2Δ* mutant strain, we demonstrate that *StIPMK* is a canonical member of the multikinase family, and that, despite its considerable dissimilarity to *ScIPK2*, it also alleviates a lysine overproduction phenotype of *ScIpk2Δ*.

## MATERIALS AND METHODS

Oligonucleotide primers were obtained from Sigma-Genosys (Haverhill, UK). Chemicals were obtained from Sigma (Sigma-Aldrich, Dorset, UK) or Fisher Scientific (Loughborough, UK). Restriction enzymes were obtained from Invitrogen (Paisley, UK), Roche (Sussex, UK), New England Biolabs (Hitchin, UK) or Promega (Southampton, UK), while bacterial and yeast strains were obtained from Novagen (Nottingham, UK) and EUROSCARF (Frankfurt, Germany) respectively.

### Cloning *StIPMK*

*StIPMK* cDNA sequence was identified by BLAST-searching the Solanum Genomic Network EST database (<http://www.sgn.cornell.edu>) with the sequence of *Arabidopsis* multikinase *AtIPK2β* [15]. *StIPMK* cDNA was isolated from whole potato cDNA using the following primers: forward, 5'-CTTTGTGTGTCGGGGACTTT-3'; reverse, 5'-TATCAGAGGCGGTATGGCTC-3'. The cDNA was cloned into the vector pGEX-4T-2 (Pfizer, Tadworth, UK) which encodes an N-terminal glutathione S-transferase tag. This was achieved using mutagenic primers to replace the ATG start codon of *StIPMK* with an EcoR1 restriction site and the TAA stop codon with a Xho1 restriction site; inserting the *StIPMK* cDNA in-frame into the vector's multiple cloning site. The primers used to

achieve the mutagenesis were as follows: forward, 5'-TCACTTTCTGTGAGAATTCTT-AAGGTTTCCTC-3'; reverse, 5'-ACGTCTCGAGTTATTCAGAGGCGGTATGGCTC-3'.

## 5 HPLC separation of inositol phosphates

Inositol polyphosphates were resolved by HPLC on a 25 cm Partisphere Strong Anion Exchange (SAX) column (Whatman, Maidstone, UK) eluted at a flow rate of 1 mL/min with a gradient derived from buffer reservoirs containing A (water) and B (1.25 M (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, pH 3.8, H<sub>3</sub>PO<sub>4</sub>) mixed as follows: 0 min, 0% B; 5 min 0% B; 65 min, 100% B; 75 min, 100% B. A [<sup>3</sup>H]InsP<sub>6</sub> standard (740 MBq/mmol) obtained from DuPont/NEN (Stevenage, UK) was included during analysis of yeast inositol phosphates. Radioactivity was determined on-line with a Canberra Packard (Pangbourne, Berkshire, UK) A510 Radiochemical Flo-Detector fitted with a 0.5 ml flow-cell, either by Cerenkov counting or for <sup>3</sup>H analysis by admixture of Flo-Scint AP (Canberra Packard) scintillation cocktail.

15 Radioactivity was estimated with an integration interval of 12 seconds.

## Yeast Growth Media

(i) Rich medium (YPD) contained: 1 % yeast extract (Duchefa Biochemie), 2 % Bacto™ peptone (BD Biosciences Clontech, Crawley, UK), 2 % dextrose prepared at pH 6.5. For solid media, micro agar (Duchefa Biochemie) was added at 20g per L of liquid media. (ii) Synthetic defined medium lacking uracil (SD-U) contained: 37 g minimal SD Base with dextrose and 0.77 g uracil drop out supplement (Clontech) adjusted to pH 5.8 in 1 L volume. For solid media, 46.7 g Minimal SD Agar Base were used with 0.77 g uracil drop out supplement. (iii) Minimal medium lacking inositol for labelling experiments was prepared from stock solutions of macronutrients, trace elements, and vitamins. 1 L of a 5 x stock of macronutrients was prepared with 5 g KH<sub>2</sub>PO<sub>4</sub>, 2.5 g MgSO<sub>4</sub>.7H<sub>2</sub>O, 2.5 g NaCl, 1.67 g CaCl<sub>2</sub>.6H<sub>2</sub>O. 1 L of a 100 x stock of trace elements was prepared with 50 mg H<sub>3</sub>BO<sub>3</sub>, 40 mg ZnSO<sub>4</sub>.7H<sub>2</sub>O, 20 mg FeCl<sub>3</sub>.6H<sub>2</sub>O, 20 mg NaMnO<sub>4</sub>.2H<sub>2</sub>O, 10 mg KI, and 4 mg CuSO<sub>4</sub>.5H<sub>2</sub>O. 1 L of a 500 x stock of vitamins was prepared with 1 g calcium pantothenate, 200 mg niacin, 200 mg pyridoxine.HCl, 200 mg thiamine.HCl, 200 mg *p*-aminobenzoic acid, 100 mg riboflavin, 10 mg biotin, 1 mg folic acid. The pH was

adjusted to 7.0. 1 L of minimal media was made combining the following: 4 g galactose, 3.5 g ammonium sulphate, 1.5 g asparagine, 200 mL macronutrient stock, 10 ml trace element stock, and 2 mL of the 500x vitamin stock, at a final pH of 5.7. (iv) Minimal medium containing ornithine/arginine was made as above, but ammonium sulphate and asparagine were omitted. Ornithine or arginine was added to a final concentration of 3 mg/mL, and inositol was added at 1mg/mL.

### ***Saccharomyces cerevisiae* strains and transformation**

The yeast strains YDR173c (accession number: Y03531, genotype: BY4741; Mat a; his3 $\Delta$ 1; leu2 $\Delta$ 0; met15 $\Delta$ 0; ura3 $\Delta$ 0; YDR173c::kanMX4) and Y0000 (Wild Type) were obtained from EUROSCARF (University of Frankfurt, Germany). A 10 mL YPD overnight culture of YDR173c was grown from a single plated (YPD) colony at 30 °C with shaking at 3.8 Hz. A 3 mL aliquot was sub-cultured into 30 mL YPD and grown to a cell density of  $\sim 0.5 - 1 \times 10^7$  cells/ml. Yeast were transformed according to [16], and 100  $\mu$ L aliquots of transformed cells were plated on solid SD/-U media with 50  $\mu$ g/mL G418 antibiotic selection.

### **RNA isolation from *Saccharomyces cerevisiae* and cDNA synthesis**

RNA was extracted using Trizol (Sigma-Aldrich) and acid-washed glass beads (150-212 micron) following the manufacturer's specification. RNA was precipitated with isopropanol, washed with 70% EtOH and pellets were resuspended in 30  $\mu$ L RNase-free water. RNasin Plus (Promega) was used to inhibit RNase. Contaminating DNA was removed using the TURBO DNA-free system (Ambion).

First strand cDNA synthesis from RNA was performed with M-MLV Reverse Transcriptase RNase H Minus, Point Mutant (Promega) using a poly dT (20mer) oligonucleotide primer (Sigma-Genosys).

Primers were designed to anneal to the following genes from *Saccharomyces cerevisiae*: *ARG8*, acetylornithine aminotransferase: Forward, 5'-ATTGTCGAGCCCA-TACAAGG-3', Reverse, 5'-GAACTCAGCACCAAGCATCA-3'; *ARG3*, ornithine carbamoyltransferase: Forward, 5'-TGTATTTTTGCCCCGTGTGAA-3', Reverse, 5'-GT-TTCAGCTTGGCCTGTTTC-3'; *GLD3*, glyceraldehyde-3-phosphate dehydrogenase:

Forward, 5'-CTCTACCGGTGCTGCTAAGG-3', Reverse, 5'-TCAAGTCAAC-  
AACTCTGGCG-3'; *LYS1*, saccharopine dehydrogenase: Forward, 5'-CCACGA-  
ACACATCCAGTTTG-3', Reverse, 5'-GAATTCGTCAAAGGGACCA-3'; *LYS2*,  
5 alpha aminoadipate reductase: Forward, 5'-CCAAACGGTGACTGTGAATG-3',  
Reverse, 5'-TTGGGAGTTGGGAATTGAAG-3'; *LYS5*, phosphopantetheinyl  
transferase: Forward, 5'-CGTCTCAAGCCAGAATCCTC-3', Reverse, 5'-AAAAAGC-  
TGATAGCGCCAAA-3'; *LYS9*, saccharopine dehydrogenase: Forward, 5'-GAAGC-  
CGAAACGGTCATTAG-3', Reverse, 5'-TGGCAACTGGATAACCAACA-3'; *LYS20*,  
homocitrate synthase isozyme: Forward, 5'-CGATACTGGTTGTGCCATTG-3',  
Reverse, 5'-TTGGTCATCCGTCAAGTTCA-3'.

### End Point RT-PCR Analysis

Yeast were grown in liquid SD/-U with 50 µg/mL G418 antibiotic (*ScIpk2Δ/pYES2.1* and  
*ScIpk2Δ/StIPMK*) or liquid YPD (Wild Type) with 50 µg/mL G418 antibiotic (*ScIpk2Δ*)  
to mid-log phase  $\sim 0.5 - 1 \times 10^7$  cells/ml and then transferred to minimal media containing  
2 % galactose and either ornithine or arginine as the sole nitrogen source. Cultures were  
grown at 30 °C with shaking at 3.8 Hz for 16 hours.

PCR reactions were carried out using cDNA from each yeast strain for all growth  
conditions and for all three genes of interest normalized with respect to GLD3 product  
formation by adjusting template concentration, number of cycles and annealing  
temperature. Final reaction conditions were: annealing temperature: 45 °C; extension  
time: 40 seconds; repeated for 20 cycles at 2.5 mM MgCl<sub>2</sub> using Taq DNA Polymerase.  
Control reactions using RNA confirmed the absence of contaminating DNA. The  
products were separated by agarose gel electrophoresis and visualised with ethidium  
bromide and ultraviolet light.

### *myo*-[2-<sup>3</sup>H]inositol labelling of yeast

For inositol polyphosphate analysis, 1 mL of cells from a 10 mL overnight culture were  
sub-cultured into 10 mL YPD medium (Wild Type and *ScIpk2Δ*) with G418 antibiotic  
selection for the mutant, or SD-Uracil with G418 antibiotic selection (for  
*ScIpk2Δ/pYES2.1* and *ScIpk2Δ/StIPMK*). Yeast were grown to a cell density of  $\sim 0.5 - 1 \times$

10<sup>7</sup> cells/ml, and then pelleted at 2,000 g. Pellets were washed three times with minimal medium without galactose or inositol and then resuspended in 10 mL minimal medium containing 2 % galactose and 1.85 Mbq *myo*-[2-<sup>3</sup>H]inositol. Cells were grown overnight at 30 °C with shaking at 3.8 Hz. Inositol phosphates were extracted according to [16].

5 The extracts were analyzed by HPLC.

### Extraction of amino acids from yeast and HPLC analysis

Overnight cultures were pelleted at 5,000 g and resuspended in 0.5 mL dH<sub>2</sub>O. 0.8 g of acid-washed glass beads (150-212 micron) were added and the mixture vortexed for 1  
10 minute, frozen in liquid nitrogen and thawed. This was repeated 5 times followed by centrifugation for 5 minutes at 13,000 g at 4 °C. Samples were diluted 14 x in 1 M potassium borate, pH 10.4 with KOH, and aliquots (50 µL) were reacted for 5 minutes with an equal volume of ortho-phthaldialdehyde (OPA) prepared according to [17]. The fluorescent adducts were resolved by reverse phase chromatography, 50 µL samples were  
15 injected, onto a 250 x 4.6 mm Phenomenex Synergi 4µ Hydro-RP column eluted at 1 mL/min. A gradient was delivered from buffer reservoirs containing A (25 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 2.5 with H<sub>3</sub>PO<sub>4</sub>), and B (acetonitrile, methanol, H<sub>2</sub>O, 40/50/10, v/v/v). the following gradient was delivered: time (min), %B; 0,5; 20,80; 30,95. A Jasco FP-920 fluorescence detector was used to detect the products with excitation at 332 nm and  
20 emission at 445 nm.

## RESULTS

To confirm the homology of *StIPMK* to other multikinases, the predicted amino acid sequence was submitted, with others, to the CLUSTAL W program available from  
25 <http://www.ebi.ac.uk> (Fig. 1).

The alignment reveals that *StIPMK* is most similar to *AtIPK2β* from *Arabidopsis*, with which it shares 54.9% identity. There is a high degree of similarity across kingdoms among the multikinases and certain domains are conserved in all members of the family [1]. Most importantly, the *StIPMK* amino acid sequence contains the conserved motif,  
30 *PxxxDxKxG* (amino acids 96 to 104), identified by others as a putative inositol phosphate binding site [15]. Like the other plant kinases cloned thus far [7,15], *StIPMK* lacks an



obvious calmodulin binding site. Mammalian kinases, in contrast, have an extended and regulatory C-terminal region, a nuclear localization signal [18] and a calmodulin binding site. Though we did not test the ability of recombinant StIPMK protein to bind calmodulin, we have shown that AtIPK2 $\beta$  protein, which also lacks an obvious calmodulin binding site, exhibits no ability to bind the molecule, and is localized to the nucleus [7].

*StIPMK* possesses the putative ATP-binding site (amino acids 245 to 250) identified by Saiardi and co-workers [19] and the *SSLL*-like motif required for the enzymatic activity of inositol 1,2,3,4,5,6-hexakisphosphate (InsP<sub>6</sub>) kinases [20]. Indeed, one proposal [21] is that the more specific inositol 1,4,5-trisphosphate (Ins(1,4,5)P<sub>3</sub>) 3-kinases present in mammals (thought not yeast or plants) may have evolved from multikinases. Given that InsP<sub>6</sub> kinases possess *PxxxDxKxG* and *SSLL*-like motifs, it is possible that they too evolved from multikinases, though it should be noted that no plant multikinase, thus far, has shown the ability to phosphorylate InsP<sub>6</sub> to *PP*-InsP<sub>7</sub>.

#### ***In vitro* catalytic assay of recombinant StIPMK protein**

Recombinant StIPMK protein, purified by batch elution from a Ni-NTA Agarose resin (Qiagen), was catalytically active against a wide range of inositol phosphate substrates (data not shown). Its behaviour is typical of inositol polyphosphate multikinases [1,5,6,7,11,15,18,19,22], and product formation was witnessed with the following inositol phosphates as substrates: Ins(1,4)P<sub>2</sub>, Ins(1,4,5)P<sub>3</sub>, a racemic mixture of Ins([1/3],4,6)P<sub>3</sub>, Ins(1,3,4,6)P<sub>4</sub>, Ins(1,3,4,5)P<sub>4</sub>, Ins(3,4,5,6)P<sub>4</sub>, Ins(1,3,4,5,6)P<sub>5</sub> and Ins(1,2,3,4,5,6)P<sub>6</sub>. StIPMK was inactive against Ins(4,5)P<sub>2</sub>, but generated unidentified InsP<sub>3</sub>, InsP<sub>4</sub> and InsP<sub>5</sub> products from Ins(1,4)P<sub>2</sub>.

#### **Complementation of the inositol phosphate profile of an *ScIpk2A* yeast mutant**

Deletion of *ScIPK2*, the yeast multikinase, causes pleiotropic effects including altered transcriptional control of arginine metabolism [5,8,23,24], altered vacuolar morphology [25], and defects in mRNA export [26] and chromatin remodelling [27]. These phenotypes have variously been attributed to the absence either of the ScIPK2p protein [5] or the inositol phosphate, diphosphoinositol phosphate, and phosphoinositide products

of the enzyme [14]. The *Saccharomyces cerevisiae* yeast strain YDR173c, obtained from EUROSCARF, lacks the *ScIPK2* open reading frame which is replaced by a KANMX4 marker cassette conferring resistance to the antibiotic G418. To investigate the competence of *StIPMK* to restore the inositol polyphosphate profile of the *ScIpk2Δ* strain, we transformed *ScIpk2Δ* mutant yeast with the vector pYES2.1 harbouring the *StIPMK* cDNA. Protein expression was induced with galactose.

The *ScIpk2Δ* strain, lacking multikinase activity, is unable to synthesise  $\text{InsP}_6$  and accumulates  $\text{Ins}(1,4,5)\text{P}_3$  [3,13]. Yeast were labelled overnight in minimal medium containing *myo*-[2-<sup>3</sup>H]inositol. Inositol phosphates were extracted from all three strains and applied to a Partisphere SAX HPLC column (Fig. 2).

It is apparent from Fig. 2 that, while the *ScIpk2Δ* mutant is unable to synthesise  $\text{InsP}_6$  and accumulates  $\text{InsP}_3$ , the Wild Type and *ScIpk2Δ/StIPMK* strains are able to synthesise peaks with the chromatographic properties of  $\text{InsP}_4$ ,  $\text{InsP}_5$  (*ScIpk2Δ/StIPMK* only) and  $\text{InsP}_6$ . *StIPMK* is therefore able to substitute for the catalytic activities of *ScIPK2p* *in vivo* in yeast, generating  $\text{InsP}_6$ , much as *AtIPK2β* does [15].

Though there is low overall homology between *StIPMK* and *ScIPK2p*, the domain organisation within multikinase proteins and a number of regions of high homology are highly conserved, as highlighted by Shears [10] and confirmed by our own bioinformatic analysis (not shown). The conservation of these domains appears to be sufficient to permit members of the family to participate in pathways of  $\text{InsP}_6$  synthesis in heterologous systems. The crystal structure of *ScIPK2p* has recently been solved [28], and is clearly related to that of the human  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase [29]. Although considerable information regarding the substrate specificity of the *ScIPK2p* is revealed, the report does not afford equal assistance in consideration of the interaction of *ScIPK2p* with the arginine biosynthetic machinery of yeast.

### Complementation of the amino acid profile of an *ScIpk2Δ* yeast mutant

Despite the considerable interest in the role of *ScIPK2p* and the mechanism by which it contributes to arginine metabolism in yeast, only one of the host of recent studies [14] has addressed the transcript levels of metabolic genes involved in the arginine synthetic pathway in *S. cerevisiae*, and only that of Dubois and Messenguy [8] has analysed amino

acid content in *ScIpk2Δ* mutant and *ScIPK2*-transformed yeast, though data for the Wild Type was not provided. Thus, the current debate [13,14,30] that addresses ArgR-Mcm1-independent control of arginine biosynthesis assumes that the complicated growth phenotypes of *ScIpk2Δ*, and the various complementations, have explanation in the regulation of arginine biosynthesis, notwithstanding considerable variation in the composition of media on which these phenotyping studies have been undertaken.

Because the *ScIpk2Δ* mutant is limited in its ability to convert ornithine into arginine, evidenced most commonly as weak growth on poor nitrogen sources [5], we determined to assess the effect of the loss of the *ScIPK2* open reading frame in the *ScIpk2Δ* mutant on: 1), the mutant's ability to regulate arginine biosynthetic gene transcripts; and 2), the relative levels of arginine and ornithine, in yeast grown on ornithine or arginine. Thus, we grew yeast in minimal media containing either ornithine or arginine as the sole nitrogen source for either five hours or 16 hours. Transcript levels were assessed by end-point RT-PCR, and yeast amino acid profiles were determined after derivatization with OPA.

Fig. 3 shows the chromatograms for all four strains grown for 5 and 16 hours in media containing either ornithine or arginine. We chose two time points to accommodate variations in amino acid profiles that may accompany transfer of yeast to fresh or new media.

All strains, including the mutant and empty vector strains, were able to generate arginine when grown for five hours on ornithine (Fig. 3A,C), but the Wild Type and *ScIpk2Δ/StIPMK* strains displayed higher arginine:ornithine ratios than the *ScIpk2Δ* and *ScIpk2Δ/pYES2.1* strains. These results suggest that the Wild Type and *ScIpk2Δ/StIPMK* strains are able to synthesise arginine from ornithine more effectively than the mutant. This result is, despite the differences in yeast strains and growth conditions used, in agreement with the study of Dubois and Messenguy [8] (Table 1B, their report), which reported a three-fold increase in arginine on transformation of the *ScIpk2Δ* mutant [strain 02466c (*argRIII::URA3*)] with *ScIPK2*. Our analysis of *ARG8* and *ARG3* transcripts (see Fig. 4 later), revealing enhanced transcript level in Wild Type compared to *ScIpk2Δ* mutant, is consistent with our measurements of amino acids.

The former study [8] noted poor correlation between ornithine carbamoyltransferase (*ARG3*) activity of yeast extracts and arginine content: repression by arginine in the Wild Type, subsequent de-repression in the 02466c mutant strain, and, further to this, repression in *ARGR111*-transformed 02466c strain. In searching for an explanation of the poor correlation between enzyme activity and arginine levels, the authors of the former study assayed the activity of argininosuccinase encoded by *ARG4*, expression of which is not regulated in an arginine-specific manner. The levels of argininosuccinase activity were similar in mutant, Wild Type and *ScIPK2*-transformed strains, implying that the control of arginine accumulation was not manifest most strongly at this step. In retrospect, the poor correlation between enzyme activities and amino acid levels may have an explanation in inositol phosphate- or phosphoinositide-dependent control of arginine-related phenotypes, independent of the interaction of ScIPK2p protein with the ArgR-Mcm1 complex [13,14,30].

Returning to the present study, the most significant phenotype was the overproduction of lysine in the *ScIpk2Δ* mutant (Fig. 3A,C). While all four strains showed this peak, the peak in the mutant strains was almost as large as the arginine peak and also often larger than the ornithine peak. In the Wild Type and *ScIpk2Δ/StIPMK* strains, this lysine peak is smaller than both the arginine and ornithine peaks when grown in ornithine (Fig. 3 A,C). Again, the study of Dubois and Messenguy [8] is relevant. The authors reported variations in lysine levels between the 02466c mutant and *ScIPK2*-transformed 02466c strains, but did not measure in the Wild Type; lysine was elevated 4-fold in the transformed strain, but again without change in the activities of the lysine pathway, namely of NADP-glutamate forming saccharopine dehydrogenase (product of *LYS9*) and NAD-lysine forming saccharopine dehydrogenase (product of *LYS4*). While our study seems at variance with respect to lysine, we are reassured that *StIPMK* reduced lysine levels of the *ScIpk2Δ* mutant to Wild Type levels. Again, it is likely that growth conditions are critical determinants in experiments of this sort.

It is noticeable from a comparison of Fig. 3A (five hours growth) and 3C (16 hours growth) that, on the ornithine nitrogen source (Fig. 3A), the Wild Type and *ScIpk2Δ/StIPMK* strains have higher arginine:ornithine ratios at five hours growth. One explanation of this is that the Wild Type and *ScIpk2Δ/StIPMK* strains show efficient

response to accumulating arginine. Comparing strains grown for five hours, the *ScIpk2Δ* and *ScIpk2Δ/pYES2.1* strains have a reduced capacity to effect the conversion of ornithine to arginine, witnessed by a much lower arginine:ornithine ratio. Nevertheless, the conversion of ornithine to arginine is significant.

Regarding the levels of lysine, we notice that after five hours growth on ornithine the *ScIpk2Δ/pYES2.1* control and *ScIpk2Δ* strain, especially, have a higher lysine:arginine ratio than the Wild Type and *ScIpk2Δ/StIPMK* strains. Perhaps the simplest explanation is that excess ornithine is converted to lysine and that, after five hours more ornithine has been converted into lysine in the *ScIpk2Δ* and *ScIpk2Δ/pYES2.1* strains because they have limited capacity to convert ornithine to arginine, evidenced as lower levels of *ARG3* and *ARG8* transcripts relative to Wild Type (Fig. 4A). It is also possible that *ScIPK2p* or its *InsP* products are involved in transcriptional regulation of lysine synthesis, in which case our studies indicate that *StIPMK* can substitute for *ScIPK2* in regulation of other pathways of amino acid metabolism (see Fig. 5 later).

In arginine media (Fig. 3B), Wild Type and *ScIpk2Δ/StIPMK* strains have slightly lower arginine:ornithine ratios than the same yeast grown in ornithine (Fig. 3A), and slightly higher lysine:arginine ratios. The *ScIpk2Δ* and *ScIpk2Δ/pYES2.1* strains have a lower arginine:ornithine ratio than the Wild Type and *ScIpk2Δ/StIPMK* strains. In the presence of arginine (Fig. 3B), the mutant strains again have higher lysine:arginine ratios than Wild Type and *ScIpk2Δ/StIPMK* strains.

### Arginine metabolic gene transcript levels

To analyse transcript levels of arginine metabolic genes, we split yeast samples between our amino acid analysis and an end-point RT-PCR analysis. The expression of two arginine metabolic genes was investigated. The first was *ARG8* encoding acetylornithine aminotransferase which catalyzes the conversion of N-acetyl-L-glutamyl-5-phosphate to N-acetyl-ornithine, this is the fourth step from glutamate in the biosynthesis of arginine; and the second was *ARG3* encoding ornithine carbamoyltransferase which catalyzes the formation of L-citrulline from L-ornithine, the sixth step in the biosynthesis of arginine. *GLD3*, encoding glyceraldehyde-3-phosphate dehydrogenase 1, was chosen as a ubiquitously expressed control gene (Fig. 4).

It is apparent that with both ornithine (Fig. 4A) and arginine (Fig. 4B) nitrogen sources the Wild Type and *ScIpk2Δ/StIPMK* strains showed similar levels of expression of *ARG3* and *ARG8*, whereas the *ScIpk2Δ/pYES2.1* control strain shows much lower levels of expression of these two genes in both media. Though the expression levels in panels A and B are not directly comparable, it does appear, comparing growth on ornithine (A) with growth on arginine (B), that the Wild Type and *StIPMK* strains show reduced expression of *ARG8*, in comparison to *ARG3* when grown on arginine. While reduced expression of *ARG8* in *ScIpk2Δ* is at variance with the study of Resnick and co-workers [14] which addressed only *ARG8* expression, we have repeated this experiment on 8 occasions with similar results. Using Northern Blotting techniques, the authors of the former study showed that, when grown in complete medium, *ScIpk2Δ* yeast were unable to regulate *ARG8* gene transcript levels, and showed elevated levels of expression compared to the Wild Type. Expression of a functional multikinase in the *ScIpk2Δ* mutant yeast restored *ARG8* transcript levels to Wild Type, while a catalytically inactive version of IPMK did not rescue this phenotype. It is not clear to what the differences between our and the former study may be attributed, though it is likely that the differences in growth conditions are critical parameters, given the plasticity of amino acid metabolism revealed here (Figs. 3 & 4).

Given our findings (Fig. 3) that the *ScIpk2Δ* strain is unable to synthesise arginine to the levels of the Wild Type strain when grown in ornithine, we do not find it surprising that transcript levels of *ARG3* and *ARG8* genes are lower in the *ScIpk2Δ/pYES2.1* strain than in the Wild Type and *ScIpk2Δ/StIPMK* strains. That is to say, we find a positive correlation between transcript levels and the capacity to synthesize arginine. It is likely, given the lower expression of *ARG3* and *ARG8* genes in the *ScIpk2Δ/pYES2.1* strain, that the enhanced capacity of Wild Type and *ScIpk2Δ/StIPMK* strains to effect ornithine to arginine conversion, compared to the mutant, is maintained even on arginine media, though in this environmental condition the dominant metabolic fluxes must be away from arginine. This result again highlights the likelihood of inositol phosphate-dependent and *ScIPK2p/Arg1-Mcm1*-independent control of arginine-related phenotypes in transformed *ScIpk2Δ* strains.

Nevertheless, comparing *ARG3* and *ARG8*, the ability of the yeast to repress arginine synthetic transcripts when grown on arginine (Fig. 4B) is manifest most strongly at the level of *ARG8*. That is, *ARG8* and *ARG3* are expressed as similar levels on ornithine (Fig. 4A), but expression of *ARG8* is lower than that of *ARG3* in Wild Type, *ScIpk2Δ/pYES2.1* and *ScIpk2Δ/StIPMK* yeast strains grown on arginine. In this context, we note that the ARG8 protein acts within the mitochondrion, whereas ARG3 and later enzymes of the pathway reside outside the mitochondrion. Feedback inhibition of N-acetylglutamate synthase (encoded by *ARG2*) and N-acetylglutamate kinase (encoded by *ARG5,6*) by arginine has also been demonstrated in *S. cerevisiae* and *N.crassa* [31].

### Lysine metabolic gene transcript levels

To establish whether the accumulation of lysine in the *ScIpk2Δ* and *ScIpk2Δ/pYES2.1* strains is a metabolic consequence of compromised arginine biosynthesis, or a result of altered transcriptional regulation of lysine biosynthetic genes normally requiring the presence of ScIPK2p or its catalytic products; we looked at the levels of transcription of lysine biosynthetic genes in yeast grown in either ornithine or arginine (Fig. 5).

The synthesis of lysine in *Saccharomyces cerevisiae* proceeds via a pathway involving L- $\alpha$ -aminoadipic acid [32,33]. We have chosen five genes from this pathway as follows: *LYS20*, one of two nuclear homocitrate synthases [34] that catalyze the condensation of acetyl-CoA and  $\alpha$ -ketoglutarate to form homocitrate; *LYS2*,  $\alpha$ -aminoadipate reductase that catalyzes the reduction of  $\alpha$ -aminoadipate to  $\alpha$ -aminoadipate 6-semialdehyde; *LYS9*, (NADP<sup>+</sup>, glutamate-forming) saccharopine dehydrogenase that catalyzes the formation of saccharopine from  $\alpha$ -aminoadipate 6-semialdehyde; *LYS1*, (NAD<sup>+</sup>, lysine forming) saccharopine dehydrogenase that catalyzes the conversion of saccharopine to L-lysine; and *LYS5*, phosphopantetheinyl transferase that converts inactive apo-form of LYS2 into the catalytically active holo-form. *GLD3* was used as a control transcript and the expression of the *LYS* genes analysed with respect to levels of GLD3.

It is evident from growth in both ornithine (Fig. 5A) and arginine (Fig. 5B) nitrogen sources that the Wild Type and *ScIpk2Δ/StIPMK* yeast have consistently higher expression of all transcripts than the *ScIpk2Δ/pYES2.1* yeast, with the possible exception

of *LYS9* in arginine media (Fig. 5B). We find the most likely explanation for this pattern is that the transcriptional regulation of lysine metabolic genes is not compromised in *ScIpk2Δ* mutant yeast and that repression of metabolic transcripts is occurring in response to accumulating lysine which is greatest in the *ScIpk2Δ* mutant (Fig. 3).

5 In the presence of exogenous lysine, *S. cerevisiae* shows an apparent repression of anabolic transcripts. Lysine is a feedback inhibitor of homocitrate synthase (*LYS20*), inhibition of which reduces metabolic flux, lowering the levels of  $\alpha$ -aminoadipate 6-semialdehyde. The latter pathway intermediate is a co-inducer of the *LYS14* regulatory protein [35] that activates expression of pathway transcripts [34]. We did not measure  
10 the levels of  $\alpha$ -aminoadipate 6-semialdehyde, nor transcripts of *LYS14*, but nevertheless our data (Fig. 5) show ‘repression’ of pathway transcripts in *ScIpk2Δ* that is ‘rescued’ in the *ScIpk2Δ/StIPMK* strain. This ‘repression’ of pathway transcripts is manifest most strongly at *LYS1* and *LYS2* i.e. at the end of the pathway, and less strongly at the level of the *LYS9* and *LYS20*, and *LYS5*, which is a transcriptional activator of *LYS3*. Strongest  
15 repression of *LYS20* was evident in arginine media (compare Fig. 5B with Fig. 5A) in which lysine:arginine and lysine:ornithine ratios were highest (compare Fig. 3D with Fig. 3C) suggesting the ‘repression’ is lysine-induced.

Our data, in support of Feller et al. [34], imply that transcriptional control of lysine anabolic transcripts is manifest markedly at the level of *LYS20*, and perhaps  
20 unexpectedly at *LYS1* and *LYS2*. Considerations of flux control inform us that control of flux through metabolic pathways is manifest throughout a pathway. A corollary of this is that the step of predominant control (highest flux control coefficient) will vary according to ambient cellular conditions, reflecting the network of interactions impinging on the pathway. That we observe control of the lysine pathway in *ScIpk2Δ* mutants is an  
25 unanticipated result, and is all the more remarkable for its ‘rescue’ by a plant inositol phosphate multikinase. Perhaps the most parsimonious explanation is that the accumulation of lysine witnessed in *ScIpk2Δ* and *ScIpk2Δ/pYES2.1* strains grown in ornithine or arginine is a direct result of altered metabolic flux towards lysine from ornithine/arginine due to an impaired ability to efficiently convert ornithine to arginine.

30 However, we cannot rule out the possibility that *ScIPK2* and *StIPMK* are involved somehow in regulating the levels of transcription of *LYS* genes resulting in reduced levels



of transcript in the *ScIpk2Δ/pYES2.1* compared to Wild Type. However, in this case, we would find it hard to rationalise how reduced transcript levels would lead to increased lysine production, particularly given that previously characterized lysine-overproducing mutants show loss of repression of homocitrate synthase, encoded by *LYS20* [36].

5

## DISCUSSION

We have shown that when grown on minimal media with a single defined source of nitrogen, ornithine or arginine, not only does *ScIpk2Δ* mutant yeast have reduced levels of transcripts of *ARG3* and *ARG8*, but it shows a concomitant reduction in arginine biosynthesis. Both these phenotypes, transcript and amino acid, are restored by expression of a potato multikinase gene, *StIPMK*. Our data strongly argue that StIPMK or its inositol phosphate products are involved, in this heterologous system, in the formation/stabilization of the ArgR-Mcm1 transcription complex, as evidenced by reduced levels of *ARG3* and *ARG8* transcripts in absence of multikinase protein and their restoration with *StIPMK*. However, we find ourselves at odds with the work of Resnick *et al.* [14], who reported that in the absence of multikinase protein, *ARG8* transcript levels were mis-regulated, i.e. specifically over-expressed. Our results demonstrate that, despite having reduced levels of *ARG8* transcript relative to Wild Type, the *ScIpk2Δ/pYES2.1* mutant still displays a further reduction of the level of this transcript, relative to *ARG3* transcript, when grown with arginine (Fig. 4B) compared to the situation when grown on ornithine (Fig. 4A). We find this scenario entirely plausible, given that it is the complex of Mcm1, ARG80 and ARG81 that binds to DNA, and it is the ARG81 protein that senses levels of arginine. Thus, some transcriptional control is still evident in *ScIpk2Δ/pYES2.1*.

One unanticipated effect that we have observed is an accumulation of lysine in *ScIpk2Δ* mutant yeast, and an alteration of lysine:ornithine and lysine:arginine ratios that may reflect the diversion of amino acids towards lysine in mutant yeast. Once again, these processes are rescued by expression of a functional potato multikinase. Though lysine is metabolically somewhat distant from arginine, and also ornithine, both arginine and lysine ultimately share origins in TCA cycle intermediates; the two branches of amino acid metabolism are more closely linked however at the level of glutamate. Thus

in yeast, L-glutamate participates in the  $\alpha$ -amino adipate aminotransferase and saccharopine dehydrogenase (NADP<sup>+</sup>, glutamate forming, *LYS9*) catalysed steps of lysine synthesis as N donor. Moreover, glutamate entry into the mitochondrion is the 'start' of the arginine synthetic pathway [31].

5           The huge sequence dissimilarity between plant IPMKs and *ScIPK2*, the equivalent of more than 300 point mutations, highlights, in an analysis of temperature sensitive growth phenotype, a transcriptional role for inositol polyphosphates or other products independent of direct interaction of multikinase protein with the ArgR-Mcm1 transcription machinery [13]. Our results highlight the pleiotropic nature of the regulation  
10 of amino acid metabolism in yeast. By analysis of transcripts and amino acid profiles, our experiments extend analysis of *ScIpk2Δ* phenotypes, and highlight the ability of divergent IPMKs to restore control of lysine and arginine biosynthesis in *ScIpk2Δ* yeast.

#### ACKNOWLEDGEMENTS

15       SEKC was supported by a BBSRC Special Committee Studentship (Ref. 01/B1/P/07138). CH was supported by a BBSRC grant (Ref. D14483) awarded to CAB. IS and SJ were supported by a BBSRC grant (Ref. D18101) awarded to CAB. Yeast Strains were provided by EUROSCARF, University of Frankfurt, Germany.

## REFERENCES

1. Shears, S. B. (2004) How versatile are inositol phosphate kinases? *Biochem. J.* **377**, 265-280
- 5     2. Stevenson-Paulik, J., Bastidas, R. J., Chiou, S. T., Frye, R. A., and York, J. D. (2005) Generation of phytate-free seeds in *Arabidopsis* through disruption of inositol polyphosphate kinases. *Proc. Natl. Acad. Sci. U. S. A* **102**, 12612-12617
- 10    3. Saiardi, A., Caffrey, J. J., Snyder, S. H., and Shears, S. B. (2000) Inositol polyphosphate multikinase (ArgRIII) determines nuclear mRNA export in *Saccharomyces cerevisiae*. *FEBS Lett.* **468**, 28-32
4. Miller, A. L., Suntharalingam, M., Johnson, S. L., Audhya, A., Emr, S. D., and Went, S. R. (2004) Cytoplasmic inositol hexakisphosphate production is sufficient for mediating the Gle1-mRNA export pathway. *J. Biol. Chem.* **279**, 51022-51032
- 15    5. Dubois, E., Dewaste, V., Erneux, C., and Messenguy, F. (2000) Inositol polyphosphate kinase activity of Arg82/ArgRIII is not required for the regulation of the arginine metabolism in yeast. *FEBS Lett.* **486**, 300-304
- 20    6. el Alami, M., Messenguy, F., Scherens, B., and Dubois, E. (2003) Arg82p is a bifunctional protein whose inositol polyphosphate kinase activity is essential for nitrogen and PHO gene expression but not for Mcm1p chaperoning in yeast. *Mol. Microbiol.* **49**, 457-468
7. Xia, H., Brearley, C. A., Elge, S., Kaplan, B., Fromm, H., and Mueller-Roeber, B. (2003) *Arabidopsis* Inositol Polyphosphate 6-/3-Kinase Is a Nuclear Protein That Complements a Yeast Mutant Lacking a Functional ArgR-Mcm1 Transcription Complex. *The Plant Cell* **15**, 1-16
- 25    8. Dubois, E. and Messenguy, F. (1994) Pleiotropic function of ArgRIIIp (Arg82p), one of the regulators of arginine metabolism in *Saccharomyces cerevisiae*. Role in expression of cell-type-specific genes. *Mol. Gen. Genet.* **243**, 315-324
- 30    9. Qiu, H. F., Dubois, E., Broen, P., and Messenguy, F. (1990) Functional analysis of ARGRI and ARGRIII regulatory proteins involved in the regulation of arginine metabolism in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **222**, 192-200
10. Shears, S. B. (2000) Transcriptional regulation: a new dominion for inositol phosphate signaling? *Bioessays* **22**, 786-789
- 35    11. Zhang, T., Caffrey, J. J., and Shears, S. B. (2001) The transcriptional regulator, Arg82, is a hybrid kinase with both monophosphoinositol and diphosphoinositol polyphosphate synthase activity. *FEBS Lett.* **494**, 208-212

12. Odom, A. R., Stahlberg, A., Went, S. R., and York, J. D. (2000) A Role for Nuclear Inositol 1,4,5-Trisphosphate Kinase in Transcriptional Control. *Science* **287**, 2026-2029
- 5 13. Seeds, A. M., Bastidas, R. J., and York, J. D. (2005) Molecular definition of a novel inositol polyphosphate metabolic pathway initiated by inositol 1,4,5-trisphosphate 3-kinase activity in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280**, 27654-27661
14. Resnick, A. C., Snowman, A. M., Kang, B. N., Hurt, K. J., Snyder, S. H., and Saiardi, A. (2005) Inositol polyphosphate multikinase is a nuclear PI3-kinase with transcriptional regulatory activity. *Proc. Natl. Acad. Sci. U. S. A* **102**, 12783-12788
- 10 15. Stevenson-Paulik, J., Odom, A. R., and York, J. D. (2002) Molecular and biochemical characterization of two plant inositol polyphosphate 6-/3-/5-kinases. *J. Biol. Chem.* **277**, 42711-42718
- 15 16. Sweetman, D., Johnson, S., Caddick, S. E., Hanke, D. E., and Brearley, C. A. (2006) Characterization of an *Arabidopsis* inositol 1,3,4,5,6-pentakisphosphate 2-kinase (AtIPK1). *Biochem. J.* **394**, 95-103
17. van Eijk, H. M., Rooyakkers, D. R., Soeters, P. B., and Deutz, N. E. (1999) Determination of amino acid isotope enrichment using liquid chromatography-mass spectrometry. *Anal. Biochem.* **271**, 8-17
- 20 18. Nalaskowski, M. M., Deschermeier, C., Fanick, W., and Mayr, G. W. (2002) The human homologue of yeast ArgRIII protein is an inositol phosphate multikinase with predominantly nuclear localization. *Biochem. J.* **366**, 549-556
- 25 19. Saiardi, A., Nagata, E., Luo, H. R., Sawa, A., Luo, X., Snowman, A. M., and Snyder, S. H. (2001) Mammalian inositol polyphosphate multikinase synthesizes inositol 1,4,5-trisphosphate and an inositol pyrophosphate. *Proc. Natl. Acad. Sci. U. S. A* **98**, 2306-2311
20. Saiardi, A., Nagata, E., Luo, H. R., Snowman, A. M., and Snyder, S. H. (2001) Identification and characterization of a novel inositol hexakisphosphate kinase. *J. Biol. Chem.* **276**, 39179-39185
- 30 21. Schell, M. J., Letcher, A. J., Brearley, C. A., Biber, J., Murer, H., and Irvine, R. F. (1999) PiUS (Pi uptake stimulator) is an inositol hexakisphosphate kinase. *FEBS Lett.* **461**, 169-172
22. York, J. D., Guo, S., Odom, A. R., Spiegelberg, B. D., and Stolz, L. E. (2001) An Expanded View Of Inositol Signalling. *Advanced Enzyme Regulation* **41**, 57-71
- 35 23. Bechet, J., Greenson, M., and Wiame, J. M. (1970) Mutations affecting the repressibility of arginine biosynthetic enzymes in *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **12**, 31-39

24. Messenguy, F. and Dubois, E. (1993) Genetic evidence for a role for MCM1 in the regulation of arginine metabolism in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **13**, 2586-2592
- 5 25. Dubois, E., Scherens, B., Vierendeels, F., Ho, M. M. W., Messenguy, F., and Shears, S. B. (2002) In *Saccharomyces cerevisiae*, the Inositol Polyphosphate Kinase Activity of Kcs1p Is Required for Resistance to Salt Stress, Cell Wall Integrity, and Vacuolar Morphogenesis. *J. Biol. Chem.* **277**, 23755-
- 10 26. York, J. D., Odom, A. R., Murphy, R., Ives, E. B., and Went, S. R. (1999) A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. *Science* **285**, 96-100
27. Steger, D. J., Haswell, E. S., Miller, A. L., Went, S. R., and O'Shea, E. K. (2003) Regulation of chromatin remodeling by inositol polyphosphates. *Science* **299**, 114-116
- 15 28. Holmes, W. and Jögl, G. (2006) Crystal structure of inositol phosphate multikinase 2 and implications for substrate specificity. *J. Biol. Chem.*
29. Gonzalez, B., Schell, M. J., Letcher, A. J., Veprintsev, D. B., Irvine, R. F., and Williams, R. L. (2004) Structure of a human inositol 1,4,5-trisphosphate 3-kinase: substrate binding reveals why it is not a phosphoinositide 3-kinase. *Mol. Cell* **15**, 689-701
- 20 30. York, J. D. (2006) Regulation of nuclear processes by inositol polyphosphates. *Biochim. Biophys. Acta* **1761**, 552-559
31. Pauwels, K., Abadjieva, A., Hilven, P., Stankiewicz, A., and Crabeel, M. (2003) The N-acetylglutamate synthase/N-acetylglutamate kinase metabolon of *Saccharomyces cerevisiae* allows co-ordinated feedback regulation of the first two steps in arginine biosynthesis. *Eur. J. Biochem.* **270**, 1014-1024
- 25 32. Zabriskie, T. M. and Jackson, M. D. (2000) Lysine biosynthesis and metabolism in fungi. *Nat. Prod. Rep.* **17**, 85-97
33. Nishida, H. and Nishiyama, M. (2000) What is characteristic of fungal lysine synthesis through the alpha-aminoadipate pathway? *J. Mol. Evol.* **51**, 299-302
- 30 34. Feller, A., Ramos, F., Pierard, A., and Dubois, E. (1999) In *Saccharomyces cerevisiae*, feedback inhibition of homocitrate synthase isoenzymes by lysine modulates the activation of LYS gene expression by Lys14p. *Eur. J. Biochem.* **261**, 163-170
- 35 35. Ramos, F., Dubois, E., and Pierard, A. (1988) Control of enzyme synthesis in the lysine biosynthetic pathway of *Saccharomyces cerevisiae*. Evidence for a regulatory role of gene LYS14. *Eur. J. Biochem.* **171**, 171-176

36. Gasent-Ramirez, J. M. and Benitez, T. (1997) Lysine-overproducing mutants of *Saccharomyces cerevisiae* baker's yeast isolated in continuous culture. *Appl. Environ. Microbiol.* **63**, 4800-4806

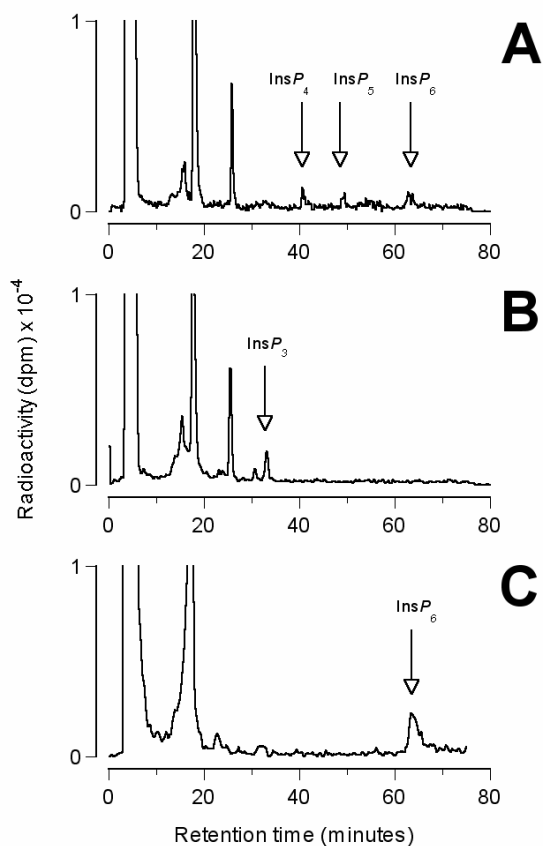
**Fig. 1**

Potato	1	-----MLKVF-----	-----OHQVAGHEAGIGK-----	-----LGPLVDES
Yeast	1	---MDTVNNYRVL---	EHKAAGHDGTLTDGDGLLIFKPAFPQEL	
Arabidopsis a	1	-----MLKVP-----	EHQVAGHTASDGK-----	-----LGPLVDDQG
Arabidopsis b	1	-----MQLKVP-----	EHQVAGHTAKDCK-----	-----PGPLVDDKG
Rat	1	MAAEPPALRLRPPGSTGDSPPVPRLLGGCVPLS	SHQVAGHMYGKDK-----	-----VGTLOHPDG
Human	1	MTLPGGPTGMARPGGARPCSPG-----	LERAPRRSVGEIRLLFEARCAAVAAAAAGEP	
Potato	28	RFYKPLQG-----	DERGANEVAFYSSLSLN-----	-----SGIPEHIQRFFETFY
Yeast	39	EFYKATQVRDVSRRKSSADGDAPLCSWMP	TYLGVNNEGAKIEQSGDAALLKIDERLSDST	
Arabidopsis a	28	RFEKPLQG-----	DSRGEHEAKFYESFTSN-----	-----MKVPDHIHRVFEVYH
Arabidopsis b	29	RFEKPLQG-----	DSRGEIEVKFYESFSSN-----	-----TEVPEHIHRVFEVYH
Rat	55	TVLKQLQP-----	PPRGPRELEFYTMVYAAD-----	-----CADAVLLELRKHLPEKMY
Human	55	RARGAKRRGGQVPNGLPAPPAPVIPQLTVTAE	-----EPDVPPTSPGPPERERDCLPAAG	
Potato	68	GTQLVE-----	ASDGSGLPLPHLVLEDIALCHVNPSIMDIKIGSRTWAPEASEKYIO	
Yeast	99	DNLDSI-----	PVKSEKSKQYLVLENLLYGFSSKPNIDDKDKGTLYDSKASLEKRE	
Arabidopsis a	68	GTQLVE-----	ASDGSGLPLPHLVLDVVS	GYANPSVMDVKIGSRTWYFDVSEYFK
Arabidopsis b	69	GTQAVE-----	GSDG---AAMVLENLLAEYTKPSVMDVKMGSRTWYDPASEEYIO	
Rat	98	GVWSP-----	SAPN---DVLKLEDVTHKFNKPCIMDVKIGRKSYPFASAEKIO	
Human	111	SSHQQPRRLSTSSVSS	GTGSSSLLEDESDLLSDSESRSGNVQLEAG--	EDVGQKNHWQ
Potato	119	KCLKKDRSSSLSLGFRISGLQIYRS	KELGFWKPGKKAQKLSTEEVKLVLRREFVSS---	
Yeast	150	RMKRVSETTSSGSLGFRICGMKIQ--	KNPSVLNQLSLEYEEEDSDYIFINKLYGRS--	
Arabidopsis a	119	KCLKKDRQTTTSLGFRVSGFKIFDHQESSFWRAEKKLVLCYNADGARLALRKFFVSS---		
Arabidopsis b	117	KCLKKDRQTTTSSGFRISGFEVYDHKESSEFWKPERKLLRGLYVDGARLTLRKFFVSS---		
Rat	146	QQVSKYP--LMEEIGLVLGMRVYHLHSDSYETQNHYGRGLTKETLKEGVSKFHFNGFC		
Human	169	KIRTMVNLPVISPFKKRYAWVQLAGHTG	SFKAAGTSGLLILKRCSEPERYCLARLMADALR	
Potato	176	-----	NTLNDLDRPDCAFASST---	VYGGSTG-----
Yeast	206	-----	RTDQNVSDAIELYFNNP---	HLSDARKH-----
Arabidopsis a	176	-----	NSPADSNLTNCAFASE---	VYGGCNG-----
Arabidopsis b	174	-----	NSLSDTGSKPDSAFASS---	VYGGSHG-----
Rat	204	LRKDAVA-----	ASIQKVEKILQWFENQKQLNFEYASSLLFVYEGSSQPATTKSNDRTL	
Human	229	GCVPAFHGVVERDGE SYLQLQDLDLDGDFGFCVLDCKMGVRTYLEEELTKARERPKLRKDM		
Potato	200	----ILSOLLELKAWFEDOTIFHLYS-----	CSILVIFEKELALKGKNPGA-----	
Yeast	231	---QLKKTFLKRLQLFYNTMLEEEVR---	MISSSLLFIYEGDPERWELLNDVDK-LM	
Arabidopsis a	200	---ILAOLLELKDWFEETOTLYHFNS-----	CSILMIYENESILMQGGDAPAPRA	
Arabidopsis b	198	---ILTOLLELKTFENOTLYHFNS-----	CSILMVYENESILKGNDDA---RP	
Rat	257	AGRFLSKGALSADADVLECNNEFHLS	SPANGTSVGKSLSKAYSRHRKLYAKKHQS---	Q
Human	289	YKKMLAVDPEAPTEEHAQRAVTKPRYMQWREGISS	STTLGFRIEGTKKADGSCSTDFKT	
Potato	242	HIKLIDFAHVEGOGVIDHNFLGG-----	-----LCSLIKFISSDILT	
Yeast	281	RDDFDIDDDDDDDDDDDDDDAEGSS-----	EGPKDKKTTGSLS	
Arabidopsis a	247	QVKLVDFAHVLDGNGVIDHNFLGG-----	-----LCSFIKFIKIDILQ	
Arabidopsis b	242	QVKLVDLAHVLDGNGVIDHNFLGG-----	-----LCSFINFIREILQ	
Rat	313	TSLKVEITLEQDNGWKSMSQEHNGNV-----	-----LSQLEKVFYHLPA	
Human	349	TRSREQVLRVFEFEVQGDDEEVLRRYLNRLQQIRD	TLEVSEFFRRHEVIGSSLLFVHDHCH	
Potato	279	AP-----	SDCRIDISAKADQKNLTADNGVVADHKSHTASE-----	
Yeast	320	SM-----	SLID-----FAHSEITPGKCYDENVIEGVETL	LDIFMKF---
Arabidopsis a	284	SV-----	EKH-----DETDTSLLENCR-----	
Arabidopsis b	279	SP-----	DES-----ADS-----	
Rat	352	GR-----	QEIAEAEVRMIDFAHVFPSTVDEGYVYGLKHLIAVLR	SILDS
Human	409	RAGVWLIDFGKTTPLPDGQILDHRRPWEEGNREDGYLLGLDNL	IGILASLAER	

**Fig. 1. Alignment of predicted amino acid sequences of members of the inositol phosphate multikinase family.** Potato: *Solanum tuberosum* (*StIPMK*); Yeast: *Saccharomyces cerevisiae* (*ScIPK2*, protein accession: NP\_010458); Arabidopsis a: *Arabidopsis thaliana* (*AtIPK2α*, protein accession: **CAB96043**); Arabidopsis b: *Arabidopsis thaliana* (*AtIPK2β*, protein accession: **CAC43071**); Rat: *Rattus norvegicus*

(*mIPMK*, protein accession: **NP\_599244**); and Human: *Homo sapiens* (*HsITPKa*, protein accession: **NP\_002211**). The alignment was performed using CLUSTAL W available from <http://www.ebi.ac.uk> and shaded with BOXSHADE available from [http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html). Amino acid residues shared by at least half of the sequences are shaded black, and those that are similar are shaded grey.

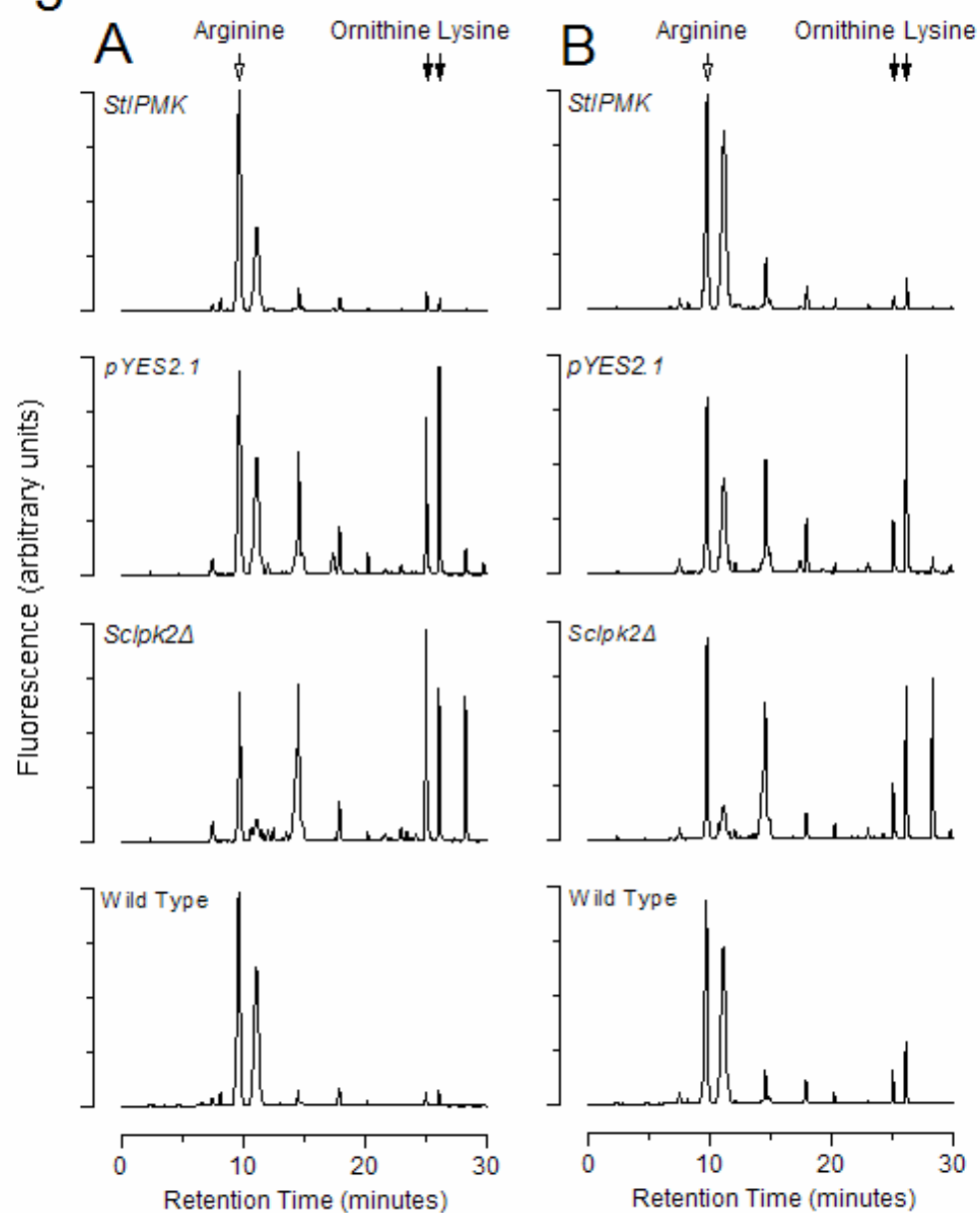
**Fig. 2**

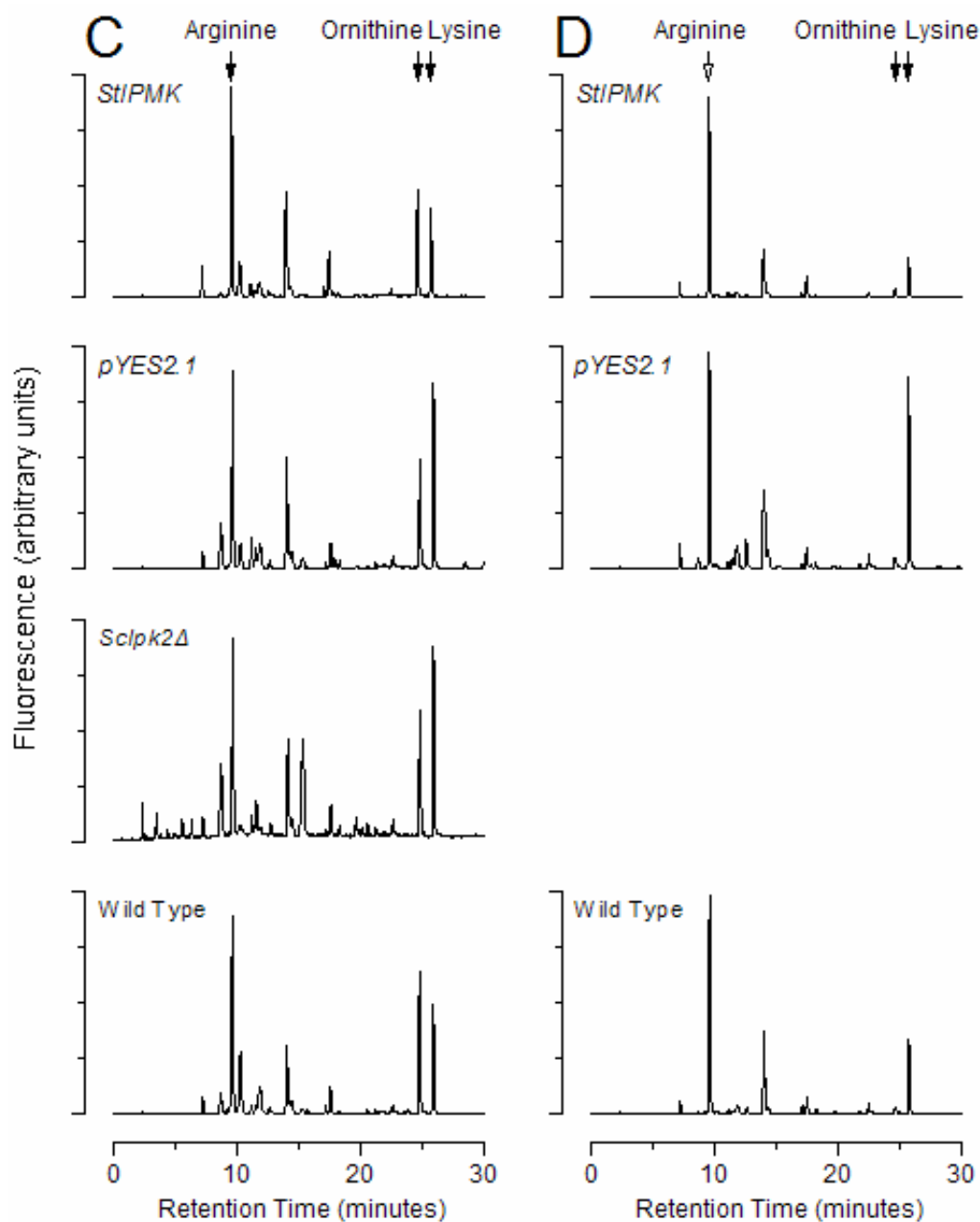


**Fig. 2. HPLC profiles of inositol phosphates present in *Saccharomyces cerevisiae* strains grown in the presence of *myo*-[2-<sup>3</sup>H]-inositol. (A) *ScIpk2Δ/StIPMK* possesses a complement of higher inositol polyphosphates with retention times characteristic of InsP<sub>4</sub>, InsP<sub>5</sub> and InsP<sub>6</sub>. (B) The *ScIpk2Δ* strain accumulates InsP<sub>3</sub> and has no obvious higher inositol polyphosphates. (C) Wild Type accumulates InsP<sub>6</sub>. The data was 3-point smoothed with the flow-detector software.**



Fig. 3



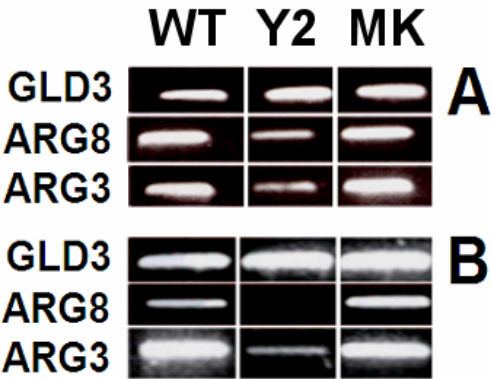


**Fig. 3. Amino acid profiles of yeast grown for 5 or 16 hours in ornithine or arginine media.** Yeast strains were grown to mid-log phase in selective media and then transferred to grow for five hours in minimal media containing either (A) ornithine or (B) arginine, or for 16 hours in either (C) ornithine or (D) arginine. Amino acids were extracted, derivatized with OPA, and the fluorescent adducts resolved on a Phenomenex Synergi 4μ Hydro-RP column. Samples are: Wild Type, *ScIpk2Δ*, *ScIpk2Δ/pYES2.1* (pYES2.1), and

*ScIpk2Δ/StIPMK* (*StIPMK*). The results shown are representative of four experiments (5 hours) and eight experiments (16 hours).

5

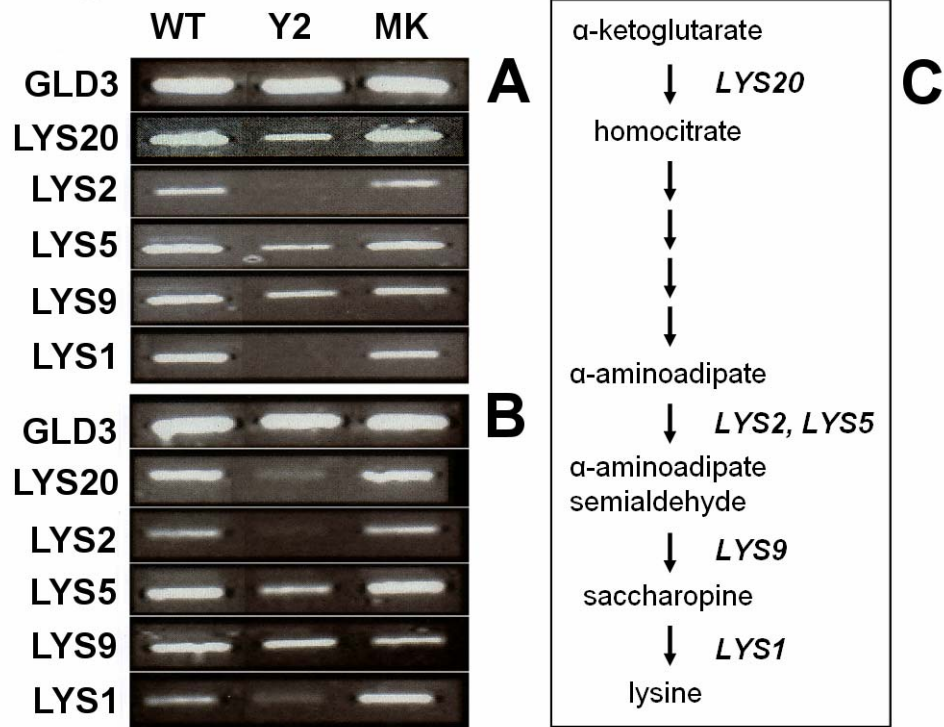
**Fig. 4**



**Fig. 4. End-point RT-PCR of arginine metabolic gene transcripts in *Saccharomyces cerevisiae* in response to ornithine or arginine nitrogen sources.** Yeasts were grown as in Fig. 3C,D. and RNA was prepared from them. Panel (A) shows transcript expression levels after growth in ornithine for 16 hours, while (B) shows expression after growth in arginine for 16 hours. Samples are: (WT) Wild Type, (Y2) *ScIpk2Δ/pYES2.1*, (MK) *ScIpk2Δ/StIPMK*. These data are representative of more than eight experiments.

10

**Fig. 5**



**Fig. 5. End-point RT-PCR of lysine metabolic gene transcripts in *Saccharomyces cerevisiae* in response to ornithine or arginine nitrogen sources.** Yeasts were grown as in Fig. 3C,D. and RNA was prepared from them. Panel (A) shows transcript expression levels after growth in ornithine for 16 hours, while (B) shows expression after growth in arginine for 16 hours. Samples are: (WT) Wild Type, (Y2) *ScIpk2Δ/pYES2.1*, (MK) *ScIpk2Δ/StIPMK*. For details of the gene products see the main text. These data are representative of three experiments. (C) the lysine biosynthetic pathway in yeast showing those steps catalysed by the products of the genes in parts A and B.