Lack of 14-3-3 proteins in *Saccharomyces cerevisiae* results in cell-to-cell heterogeneity in the expression of Pho4-regulated genes *SPL2* and *PHO84*

Janneke H.M. Teunissen, Marjolein E. Crooijmans, Pepijn P.P. Teunisse and G. Paul H. van Heusden *

Abstract

**Background:** Ion homeostasis is an essential property of living organisms. The yeast *Saccharomyces cerevisiae* is an ideal model organism to investigate ion homeostasis at all levels. In this yeast genes involved in high-affinity phosphate uptake (*PHO* genes) are strongly induced during both phosphate and potassium starvation, indicating a link between phosphate and potassium homeostasis. However, the signal transduction processes involved are not completely understood. As 14-3-3 proteins are key regulators of signal transduction processes, we investigated the effect of deletion of the 14-3-3 genes *BMH1* or *BMH2* on gene expression during potassium starvation and focused especially on the expression of genes involved in phosphate uptake.

**Results:** Genome-wide analysis of the effect of disruption of either *BMH1* or *BMH2* revealed that the mRNA levels of the *PHO* genes *PHO84* and *SPL2* are greatly reduced in the mutant strains compared to the levels in wild type strains. This was especially apparent at standard potassium and phosphate concentrations. Furthermore the promoter of these genes is less active after deletion of *BMH1*. Microscopic and flow cytometric analysis of cells with GFP-tagged *SPL2* showed that disruption of *BMH1* resulted in two populations of genetically identical cells, cells expressing the protein and the majority of cells with no detectible expression. Heterogeneity was also observed for the expression of GFP under control of the *PHO84* promoter. Upon deletion of *PHO80* encoding a regulator of the transcription factor Pho4, the effect of the *BMH1* deletion on *SPL2* and *PHO84* promoter was lost, suggesting that the *BMH1* deletion mainly influences processes upstream of the Pho4 transcription factor.

**Conclusion:** Our data indicate that yeast cells can be in either of two states, expressing or not expressing genes required for high-affinity phosphate uptake and that 14-3-3 proteins are involved in the process(es) that establish the activation state of the *PHO* regulon.

**Keywords:** *Saccharomyces cerevisiae*, 14-3-3 proteins, *SPL2*, *PHO84*, *PHO* regulon, Gene expression, Potassium starvation
identified 105 genes of which the RNA levels were significantly \( (P < 0.01) \) up-regulated more than 2.0-fold and 172 genes of which the mRNA levels were significantly down-regulated more than 2.0-fold [7]. It was found that several genes involved in phosphate metabolism were up-regulated during potassium starvation, indicating a link between potassium homeostasis and phosphate metabolism [7, 8].

Intracellular phosphate levels are maintained by an interplay between low- and high-affinity phosphate transporters (for review see: [9, 10]). At high phosphate levels, the low affinity phosphate transporters Pho87 and Pho90 are responsible for phosphate uptake. Under these conditions the Pho4 transcription factor is inactive following phosphorylation by the cyclin – cyclin-dependent kinase complex Pho80 – Pho85 [11, 12]. Upon phosphate shortage the Pho80 – Pho85 complex is inactivated, the Pho4 transcription factor becomes de-phosphorylated and enters the nucleus resulting in expression of a number of genes involved in phosphate uptake (PHO genes) [13]. These genes include among others PHOS, encoding an extracellular phosphatase [14], PHO84, encoding a high affinity phosphate transporter [15] and SPL2, encoding a protein with similarity to cyclin-dependent kinase inhibitors which down-regulates low-affinity phosphate transporters [16, 17]. Evidence has been provided that during potassium starvation PHO genes are activated by a similar mechanism as during phosphate starvation and that the entire PHO signaling pathway is required for regulation of PHO84 expression [8].

14-3-3 proteins are regulatory proteins identified in all eukaryotic organisms often in multiple isoforms capable of binding to hundreds of phosphorylated proteins (for review see: [18–21]). The yeast *S. cerevisiae* has two genes encoding 14-3-3 proteins, *BMH1* and *BMH2* [22–25]. As 14-3-3 proteins participate in many signal transduction processes it is likely that they also have a role in the regulation of the PHO genes. It has been hypothesized that physiological changes in phosphate concentrations can modulate the affinity and specificity of interaction of 14-3-3 with its multiple targets [26]. This may implicate a role of 14-3-3 proteins in phosphate sensing mechanisms and thus in the regulation of the PHO genes. To further understand the control of the expression of SPL2 and PHO84 and the possible involvement of 14-3-3 proteins in a follow-up to our previous study [7] we investigated the effect of deletion of *BMH1* or *BMH2* on the transcriptional response to potassium starvation. These experiments revealed a very low expression of the PHO genes *PHO84* and *SPL2* at standard phosphate and potassium concentrations, indicating that 14-3-3 proteins are indeed involved in the regulation of PHO genes. We further present evidence that deletion of *BMH1* results in heterogeneity in expression of PHO genes in genetically identical yeast cells.

**Methods**

**Strains, plasmids, primers, media and culture conditions**

In this study the yeast strain BY4741 and strains derived from BY4741 were used, as listed in Table 1. Plasmids and primers used in this study are listed in Tables 2 and 3, respectively. For cultivation of yeast at defined potassium concentrations YNB medium containing very low concentrations of alkali metal cations, developed by the Translucent consortium, was used [27]. If required, histidine,

| Strain | Genotype | Source/Reference |
|--------|----------|------------------|
| BY4741 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Euroscarf |
| bmh1Δ (GG3240) | bmh1Δ::loxP in BY4741 | This study |
| bmh2Δ (GG3241) | bmh2Δ::loxP in BY4741 | This study |
| pho80Δ (GG3432) | Δpho80::KAN.MX in BY4741 | This study |
| bmh1Δ pho80Δ (GG3433) | bmh1Δ::loxP Δpho80::KAN.MX in BY4741 | This study |
| BY4741 SPL2-GFP (GG3434) | SPL2-GFP (HIS3) in BY4741 | This study |
| bmh1Δ SPL2-GFP (GG3435) | bmh1Δ::loxP SPL2-GFP (HIS3) in BY4741 | This study |
| bmh2Δ SPL2-GFP (GG3444) | bmh2Δ::loxP SPL2-GFP (HIS3) in BY4741 | This study |
| BY4741 (pRS305) (GG3436) | leu2Δ0::pRS305[LEU2] in BY4741 | This study |
| BY4741 (pPHO84-GFP) (GG3437) | leu2Δ0::pRS305[pPHO84-GFP][LEU2] in BY4741 | This study |
| BY4741 (pCYC1-GFP) (GG3438) | leu2Δ0::pRS305[pCYC1-GFP][LEU2] in BY4741 | This study |
| bmh1Δ (pRS305) (GG3439) | bmh1Δ::loxP leu2Δ0::pRS305[LEU2] in BY4741 | This study |
| bmh1Δ (pPHO84-GFP) (GG3440) | bmh1Δ::loxP leu2Δ0::pRS305[pPHO84-GFP][LEU2] in BY4741 | This study |
| bmh1Δ (pCYC1-GFP) (GG3441) | bmh1Δ::loxP leu2Δ0::pRS305[pCYC1-GFP][LEU2] in BY4741 | This study |
| BY4741 (pRS305[PHO4]) (GG3442) | leu2Δ0::pRS305[PHO4][LEU2] in BY4741 | This study |
| bmh1Δ (pRS305[PHO4]) (GG3443) | bmh1Δ::loxP leu2Δ0::pRS305[PHO4][LEU2] in BY4741 | This study |
leucine, methionine and/or uracil were added to a final concentration of 20 mg/L. For cultivation at defined phosphate concentrations phosphate-free YNB medium (Formedium, UK) was used. If required, potassium phosphate (pH 5.8) was added to a final concentration of 7.2 mM and potassium chloride was added to a final concentration of 50 mM. To study the effects of potassium starvation yeast strains were grown overnight at 30 °C in supplemented Translucent YNB medium containing 50 mM KCl. This culture was used to inoculate two times 50 ml of supplemented YNB medium containing 50 mM KCl yielding A620nm 0.1. These cultures were grown to A620nm 0.5 and cells were isolated by centrifugation. Cells from one culture were washed twice with supplemented YNB medium containing 50 mM KCl and resuspended in 50 ml supplemented YNB medium containing 50 mM KCl. Cells from the other culture were washed twice with supplemented YNB medium lacking KCl and resuspended in 50 ml supplemented YNB medium lacking KCl. Both cultures were incubated at 180 rpm at 30 °C for 60 min. In a similar way the effects of phosphate starvation were studied. Yeast transformations were performed using the lithium acetate method [28].

### Construction of yeast strains

For disruption of BMH1 and BMH2 a DNA fragment was generated by PCR on plasmid pUG6 using the primer combinations Bmh1-kanMX-Fw – Bmh1-kanMX-Rv and Bmh2-kanMX- Fw – Bmh2-pUG6-Rv, respectively. These DNA fragments were used to transform BY4741 and transformants were selected on YPD plates containing 150 μg/ml G418. Correct integration was verified by PCR. The KAN.MX fragment was removed after introduction of pNatCre [29]. For disruption of PHO80 a DNA fragment was generated by PCR on plasmid pUG6 using the primer combinations PHO80-kanMX-Fw – PHO80-kanMX- Rv. This DNA fragment was used to transform BY4741 and bmh1Δ and transformants were selected on YPD plates containing 150 μg/ml G418. Correct integration was verified by PCR.

To tag chromosomal SPL2 at its 3’-end with GFP a PCR fragment was generated using the primer combination SPL2-GFP-TPHO84 - SPL2-GFP-T cyc1 and plasmid pYM28 [30] as template. This DNA fragment was used to transform BY4741, bmh1Δ and bmh2Δ yielding the histidine prototrophic strains BY4741 SPL2-GFP, bmh1Δ SPL2-GFP and bmh2Δ SPL2-GFP, respectively. Correct integration was verified by PCR.
For integration of pRS305 or plasmids derived from pRS305 BY4741 or bmh1Δ were transformed with these plasmids and leucine prototrophic transformants were selected. Yeast strains carrying plasmids were obtained by transforming parental strains with the appropriate plasmids followed by selection for uracil prototrophy.

Construction of plasmids

Reporter plasmids to analyze promoter activity were generated from pRS316. A fragment with the GFP coding sequences and restriction sites for SpeI and BamHI at the ends was generated by PCR using the primer combination GFP-Fw – GFP-Rv and pUG36 as template. This fragment was ligated into pRS316 after digestion with restriction enzymes SacI and SpeI, whereas terminator fragments were ligated after digestion with BamHI and EcoRI, yielding pRS316[P\text{PHO84}+GFP\rightarrow T\text{PHO84}], pRS316[P\text{SPL2}+GFP\rightarrow T\text{SPL2}] and pRS316[P\text{CYC1}+GFP\rightarrow T\text{CYC1}]. The position of the start codon of SPL2 is unclear. The coding region of SPL2 is annotated

| Table 3 Primers | Sequence (5′-3′) |
|-----------------|-----------------|
| Bmh1-kanMX- Fw  | GCAAGTGAGAAGAAAAAGCAAGTTAAAGATAAACTAAAGATAAAACAGCTGAAGCTTCGTACGC |
| Bmh1-kanMX-Rv   | AGATTATACAGAATTACTTTTCTTTGTGCCTCCTCCCGGCGCGCAGCCATAGGCGACCTGCTAGTCG |
| Bmh2-kanMX-Rv   | GAAAAATATCAATACAAACAAAAGAGATCCGCCGTTACACACAAACAAAAGAGATCCGCCGTTACACACAA |
| Bmh2-kanMX-Rv   | GCAAGAAGACTGGAGTGGTAAATCTTCATTTCCCCTTGTATTTCTGCATAGGCCACTAGTGGATCTG |
| PHO84-qPCR-Fw   | ACAACCTGTTGATCCACAA |
| PHO84-qPCR-Rv   | TGCTTCAGTCAGAGTGGAGATG |
| SPL2-qPCR-Fw    | CCGAGGAGATCGCTTCTCTCA |
| SPL2-qPCR-Rv    | ACGTCTGGCTCTACTTGAAT |
| ACT1-qPCR-Fw    | CTGGCCGTATTGACCAAAC |
| ACT1-qPCR-Rv    | CGGTCATTTCCTTTTGCA |
| PHO80-qPCR-Fw   | AAA GAGCTC AATCTGATATTACGACGGTCG |
| PHO80-qPCR-Rv   | AAA ACTAG GATTGGAGATGTTCTG |
| T-pho84-Fw      | AAA GAATTC CTGACGAGGTCG |
| T-pho84-Rv      | AAA ACTAG GATTGGAGATGTTCTG |
| P-cyc1-Fw       | AAA GAGCTC GTTCCACAGTGGCAGG |
| P-cyc1-Rv       | AAA ACTAG GATTGGAGATGTTCTG |
| T-cyc1-Fw       | AAA GAATTC CTGACGAGGTCG |
| T-cyc1-Rv       | AAA ACTAG GATTGGAGATGTTCTG |
| P-spl2-Fw       | AAA GAGCTC TTACACTTGGAGATACAGAGC |
| P-spl2-Rv2      | AAA ACTAG GATTGGAGATGTTCTG |
| T-spl2-Fw       | AAA GAGCTC TTACACTTGGAGATACAGAGC |
| T-spl2-Rv       | AAA GAATTC AAAGGGCGCGCAGATC |
| GFP-Fw          | AAA ACTAG GATTGGAGATGTTCTG |
| GFP-Rv          | AAA GAATTC TTGTCACATCATCACATCCATA |
| PHO4-Fw         | GG GAATTC GTTCCATGAGTGGGTC |
| PHO4-Rv         | GG GAATTC GTTCCATGAGTGGGTC |

For integration of pRS305 or plasmids derived from pRS305 BY4741 or bmh1Δ were transformed with these plasmids and leucine prototrophic transformants were selected. Yeast strains carrying plasmids were obtained by transforming parental strains with the appropriate plasmids followed by selection for uracil prototrophy.

Construction of plasmids

Reporter plasmids to analyze promoter activity were generated from pRS316. A fragment with the GFP coding sequences and restriction sites for SpeI and BamHI at the ends was generated by PCR using the primer combination GFP-Fw – GFP-Rv and pUG36 as template. This fragment was ligated into pRS316 after digestion with SpeI and BamHI. DNA fragments with restriction sites for SacI and SpeI at the ends containing sequences of the promoter of PHO84, SPL2 and CYC1 were obtained by PCR on genomic BY4741 DNA using the primer combinations P-pho84-Fw – P-pho84-Rv, P-spl2-Fw – P-spl2-Rv2 and P-cyc1-Fw – P-spl2-Fw, respectively. DNA fragments with restriction sites for BamHI and EcoRI at the ends containing sequences of the transcription terminator of PHO84, SPL2 and CYC1 were obtained by PCR on genomic BY4741 DNA using the primer combinations T-pho84-Fw – T-pho84-Rv, T-spl2-Fw – T-spl2-Rv2 and T-cyc1-Fw – T-cyc1-Rv, respectively. The promoter fragments were ligated in pRS316 containing GFP using the restriction enzymes SacI and SpeI, whereas terminator fragments were ligated after digestion with BamHI and EcoRI, yielding pRS316[P\text{PHO84}+GFP\rightarrow T\text{PHO84}], pRS316[P\text{SPL2}+GFP\rightarrow T\text{SPL2}] and pRS316[P\text{CYC1}+GFP\rightarrow T\text{CYC1}]. The position of the start codon of SPL2 is unclear. The coding region of SPL2 is annotated
between coordinates 375,100 and 374,654 of chromosome VIII (SGD, www.yeastgenome.org). Ten bp upstream of the annotated start codon an out of frame ATG sequence exists making the annotated start codon less likely to be the genuine start codon. Therefore a more likely start codon is located 85 bp downstream of the annotated start codon. This problem has been mentioned before (supplementary data in: [31]). For our promoter constructs we considered this downstream ATG as the start codon.

pRS305[PHO84-GFP-TPHO84] and pRS305[PCYC1-GFP-TCYC1] were made by transferring the SacI – HindIII fragments containing the promoter, GFP and terminator from pRS316[PHO84-GFP-TPHO84] and pRS316[PCYC1-GFP-TCYC1], respectively, to pRS305. pRS305[PHO4] was prepared by transferring a BamHI – SalI fragment with PHO4 from pRS313[PHO4] to pRS305.

Transcriptome analysis by SAGE-tag sequencing and qRT-PCR
Cultivation of BY4741, bmh1Δ and bmh2Δ, isolation of RNA and SAGE-tag sequencing was done as described previously [7]. At least 1.0 million matching reads per sample were obtained by SAGE-tag sequencing. qRT-PCR was performed as described earlier [7]. To measure transcript levels of PHO84 and SPL2 primer combinations PHO84-qPCR-Fw – PHO84-qPCR-Rv and SPL2-qPCR-Fw – SPL2-qPCR-Rv, respectively, were used. Transcript levels were normalized against expression of ACT1, measured using the primer combination ACT1-qPCR-Fw – ACT1-qPCR-Rv.

Confocal microscopy and flow cytometry
Yeast cells were grown in potassium- or phosphate-free YNB medium supplemented with KCl, potassium phosphate, histidine, methionine, uracil and leucine, when required. For image acquisition a Zeiss LSM 5 Exciter-Axiolmager M1 confocal microscope with a Plan-Apochromat objective (63X/1.4 Oil DIC) and Zeiss ZEN 2009 software were used. GFP was imaged with excitation at 488 nm and emission at 505/530 nm. Adobe Photoshop software was used to increase the visibility of the GFP signals and cells by linear adjustments of intensities. For flow cytometry, a Merck-Millpore Guava EasyCyte 5 Flow Cytometer was used. Fluorescence was determined after excitation at 488 nm and using the standard green 525/30 nm emission filter. For each analysis 5000 cells were used.

Results
Levels of PHO84 and SPL2 RNA are strongly reduced in bmh mutants
Our previous study showed that potassium starvation resulted in a strong induction of genes like PHO84 and SPL2 known to be activated at low phosphate conditions. In order to address the role of 14-3-3 proteins in the expression of PHO genes we analyzed the effect of potassium starvation on the genome-wide transcript profiles of bmh1Δ and bmh2Δ deletion strains. To this end these strains were grown in potassium-free YNB medium supplemented with 50 mM KCl [27]. Exponentially growing cells were transferred to medium containing 50 mM KCl or lacking KCl and grown for 60 min. RNA was isolated for transcriptome analysis by Serial Analysis of Gene Expression (SAGE)-tag sequencing. The complete dataset is given in Additional file 1. Although the variation in expression between the different replicates was larger than usual, the data did show a strong reduction in the level of PHO84 and SPL2 RNA in the bmh1Δ and bmh2Δ deletion strains (Table 4). This effect is most apparent at standard potassium concentrations (50 mM KCl). Other PHO genes like VTC2, GDE1, VTC1, VTC3, PHO89, PHO8 and PHM6 were affected as well by the bmh1 deletion, but these differences were not significant (Additional file 2). Few other genes were found to be affected by the BMH1 or BMH2 deletion. The RNA levels of 10 genes were significantly (P < 0.01) increased or decreased more than 2.0-fold in the bmh1Δ mutant after growth at standard potassium concentration. In the bmh2Δ strain the RNA level of 6 genes were significantly affected (Additional file 3). A different set of genes was found for the bmh1 and bmh2 deletions, but for some genes the effect was apparent in both mutants, although not significant. The effect of the bmh1 deletion on PHO84 and SPL2 could be confirmed by qRT-PCR (Table 5). The effect of the bmh1 deletion can be complemented by introduction of a wild type BMH1 allele on a centromeric plasmid (YCplac33[BMH1]) (Table 5). Introduction of this plasmid in a wild type strain resulted in up-regulation of the SPL2 and PHO84 expression in line with a role of BMH1 in the regulation of these genes (Table 5).

Table 4 Effect of bmh1 and bmh2 deletion on RNA levels of PHO84 and SPL2 (SAGE-tag sequencing)

| Gene | RNA level (reads per million) (± SD) |
|------|----------------------------------|
|      | BY4741 (n = 4) | bmh1Δ (n = 3) | bmh2Δ (n = 3) |
| PHO84 | 50 mM | 0 mM | 50 mM | 0 mM | 50 mM | 0 mM |
| 50 mM | 50 m | 516 ± 214 | | 2 ± 1st | 243 ± 140 | 6 ± 0.5th | 235 ± 16 |
| SPL2 | 31 ± 5 | 224 ± 69 | 3 ± 0th | 95 ± 33 | 7 ± 1st | 163 ± 26 |

aStudent’s t-test indicated a significant difference between bmh1Δ and BY4741 at 50 mM KCl (P = 0.03)
bStudent’s t-test indicated a significant difference between bmh1Δ and BY4741 at 50 mM KCl (P = 0.0002)
cStudent’s t-test indicated a significant difference between bmh2Δ and BY4741 at 50 mM KCl (P = 0.04)
dStudent’s t-test indicated a significant difference between bmh2Δ and BY4741 at 50 mM KCl (P = 0.004)
than that of the BMH2 deletion we mainly focused on the former deletion. To this end, we made reporter constructs in the centromeric pRS316 plasmid by inserting GFP under control of PHO84 or SPL2 promoter sequences or CYC1 promoter sequences as a control. Expression of CYC1, encoding isoform 1 of Cytochrome c, is not significantly affected by potassium starvation [7]. These reporter plasmids were introduced in wild type and bmh1Δ deletion strains and GFP expression was determined after growth at 50 and 0 mM KCl by flow cytometry. As shown in Table 6 the PHO84 promoter has an approx. 2-fold lower activity in the bmh1Δ mutant, whereas the SPL2 promoter is more than 4-fold less active. These results indicate that the lower levels of PHO84 and SPL2 RNA are at least partly caused by a lower transcription. The increase in RNA levels of PHO84 and SPL2 (Tables 4 and 5) upon potassium starvation is also reflected in the activation of the promoters during potassium starvation (Table 6).

PHO84 and SPL2 are regulated by the transcription factor Pho4 [32]. After introduction of our reporter construct for the PHO84 promoter in a pho4Δ deletion strain hardly any GFP fluorescence could be detected (data not shown), confirming the importance of the Pho4 transcription factor for the expression of PHO84. When phosphate is available Pho4 is de-activated by phosphorylation by the Pho80 – Pho85 complex. Deletion of PHO80 results in activation of the PHO genes. To investigate the effect of PHO80 disruption on the activity of the PHO84 and SPL2 promoters we introduced the reporter constructs for these promoters into wild type and bmh1Δ cells with an additional deletion of PHO80 and determined GFP fluorescence after growth in the presence of 50 mM KCl. As shown in Table 7, both the PHO84 and SPL2 promoters are highly active in the pho80Δ mutant. In the bmh1Δ pho80Δ double mutant the negative effect of the bmh1 deletion is lost. These data suggest that Bmh1 affects PHO84 and SPL2 promoter activity upstream of the Pho4 transcription factor.

### Table 5 RNA levels of PHO84 and SPL2 determined by qRT-PCR and complementation by wild type BMH1

| Strain          | RNA level (arbitrary units) (± SD; n = 3) |
|-----------------|------------------------------------------|
|                 | PHO84 | SPL2 | PHO84 | SPL2 |
| BY4741 pRS313   | 0.18 ± 0.03 | 1.10 ± 0.20 | 0.24 ± 0.12 | 1.50 ± 0.34 |
| bmh1Δ pRS313    | 0.03 ± 0.01 | 0.98 ± 0.31 | 0.05 ± 0.02 | 1.44 ± 0.21 |
| BY4741 pRS313[BMH1] | 1.01 ± 0.01 | 1.23 ± 0.40 | 1.05 ± 0.19 | 2.03 ± 0.22 |
| bmh1Δ pRS313[BMH1] | 0.18 ± 0.12 | 0.78 ± 0.23 | 0.33 ± 0.16 | 1.34 ± 0.11 |

*pRS313, empty plasmid control

Student's t-test indicated a significant difference between bmh1Δ and BY4741 at 50 mM KCl (P = 0.01)

### Table 6 Activity of the PHO84, SPL2 and CYC1 promoter determined by flow cytometry using GFP reporters

| Strain          | GFP fluorescence (arbitrary units) (± SD; n = 3) |
|-----------------|------------------------------------------|
|                 | PHO84 | SPL2 | CYC1 |
| Experiments PHO84 | 30 ± 1 | 58 ± 2 | 60 ± 26 | 5.7 ± 0.9 | 249 ± 38 |
| BY4741 P<sub>PHO84</sub>–GFP–<sub>PHO84</sub> | 198 ± 29 | 179 ± 8 | 18 ± 0.8 | 0.5 ± 0.3 | 219 ± 31 |
| bmh1Δ P<sub>PHO84</sub>–GFP–<sub>PHO84</sub> | 13 ± 2<sup>a</sup> | 37 ± 3 | 1128 ± 176 | 118 ± 17 | 155 ± 12 |
| Experiments SPL2 | 204 ± 12 | 146 ± 54 | 1528 ± 279 | 147 ± 23 | 332 ± 117 |
| BY4741 P<sub>SPL2</sub>–GFP–<sub>SPL2</sub> | 3.7 ± 1.6 | 7.1 ± 2.8 | 7.1 ± 2.8 | 7.1 ± 2.8 | 7.1 ± 2.8 |
| bmh1Δ P<sub>SPL2</sub>–GFP–<sub>SPL2</sub> | 204 ± 12 | 219 ± 30 | 1528 ± 279 | 147 ± 23 | 332 ± 117 |

<sup>a</sup>Student's t-test indicated a significant difference between bmh1Δ and BY4741 at 50 mM KCl (P < 0.001)

<sup>b</sup>Student's t-test indicated a significant difference between bmh1Δ and BY4741 at 50 mM KCl (P = 0.03)
Fig. 1 Heterogenic expression of SPL2-GFP in bmh1Δ cells. a left panel, flow cytometry of BY4741 cells (line, no fill), BY4741 SPL2-GFP cells (red) and bmh1Δ SPL2-GFP cells (blue) grown in YNB medium with 50 mM KCl; right panel, flow cytometry of BY4741 cells (line, no fill), bmh2Δ SPL2-GFP cells (red) and bmh1Δ SPL2-GFP cells (blue) grown in YNB medium with 50 mM KCl. b confocal microscopy of BY4741 SPL2-GFP, bmh1Δ SPL2-GFP and bmh2Δ SPL2-GFP cells grown in YNB medium with 50 mM KCl. Scale bar 10 μm. c confocal microscopy of BY4741 SPL2-GFP, bmh1Δ SPL2-GFP and bmh2Δ SPL2-GFP cells grown for 2 h in YNB medium without KCl. d confocal microscopy of BY4741 SPL2-GFP, bmh1Δ SPL2-GFP and bmh2Δ SPL2-GFP cells grown for 2 h in YNB medium without phosphate. e left panel: flow cytometry of BY4741 cells (line, no fill), BY4741 SPL2-GFP cells grown in YNB medium with 50 mM KCl (blue) or without KCl (red). Right panel flow cytometry of BY4741 cells (line, no fill), bmh1Δ SPL2-GFP cells containing YCplac33 (blue) and YCplac33[BMH1] (red) grown in YNB medium with 50 mM KCl.

Teunissen et al. BMC Genomics (2017) 18:701 Page 7 of 12
microscopy (Fig. 1b). The heterogeneity found for bmh1Δ SPL2-GFP cells cannot be explained by a cell cycle-dependent expression of SPL2 as in both populations single cells, small budded cells and large budded cells are present. Most of the wild type cells expresses Spl2-GFP, only in a few cells Spl2-GFP cannot be detected (Fig. 1b). Similar results were obtained when instead of GFP cyano fluorescent protein (CFP) was used to tag Spl2 (in a typical experiment 84% of the wild type cells showed expression of SPL2-CFP and 42% of the bmh1Δ cells showed expression of SPL2-CFP). Heterogenic expression of SPL2 is consistent with the lower levels of SPL2 RNA and lower activity of the SPL2 promoter in the total population of cells. Analysis of dividing cells revealed that almost 100% of the large budded cells has the same expression level in both the mother and the daughter cell after growth at 50 mM KCl (data not shown). This indicates that the expression state is relatively stable.

To address the question whether bmh1Δ cells that do not express Spl2-GFP at standard potassium concentrations are able to express SPL2-GFP in the absence of potassium, we cultivated wild type, bmh1Δ SPL2-GFP and bmh1Δ SPL2-GFP cells at 0 mM KCl. As shown in Fig. 1(c and e) both confocal microscopy and flow cytometry showed that still two populations of cells exist, one population with high expression of Spl2-GFP, the other population with lower expression. However, the latter population has a clearly detectable expression, indicating that this population of cells is still capable of expression of SPL2-GFP. It is of interest that the localization of Spl2-GFP is changed upon potassium starvation both in wild type and mutant cells (Fig. 1c). This change in localization was not observed for free GFP expressed under control of the SPL2 promoter (Additional file 4). Cultivation in the absence of phosphate resulted in an even further increased expression of SPL2-GFP in both wild type and mutant strains (Fig. 1d). Introduction of a wild type copy of BMH1 to bmh1Δ SPL2-GFP cells restored the expression of SPL2-GFP to nearly the same level as in BY4741 SPL2-GFP cells, although there are still cells present without expression, maybe caused by plasmid loss (Fig. 1f).

**Heterogenic expression of PHO84**

To investigate whether also PHO84 has an heterogenic expression we transferred the reporter constructs for PHO84 promoter activity mentioned above into the integrating plasmid pRS305 and integrated these constructs into the genome of wild type and bmh1Δ cells. Subsequently, these cells were grown under standard potassium concentrations (50 mM) and then transferred to a medium containing 50 mM KCl or to medium lacking potassium or phosphate. As shown in Fig. 2, at 50 mM KCl in wild type cells two populations of cells are found, one expressing GFP indicating an active PHO84 promoter, the other population having a low expression of GFP, indicating a less active PHO84 promoter. Transferring these cells to a medium lacking potassium or phosphate resulted in expression in all cells, indicative for activation of the PHO84 promoter. At 50 mM KCl the bmh1Δ cells show two populations of cells, cells expressing GFP and cells with a very low expression of GFP. By using a high laser power during microscopy, in the latter population of cells GFP could be detected, indicating that in all cells the PHO84 promoter has at least some activity. However, the expression was considerably lower in bmh1Δ cells than in wild type cells. Transferring to a medium without potassium or without phosphate resulted in induction of expression in all cells. However, the induction by low phosphate was much stronger than by low potassium. As a control similar experiments were performed to investigate the effect of the bmh1Δ deletion on the CYC1 promoter. As shown in Fig. 2 (c and d) the CYC1 promoter is active in all cells in media with 50 mM KCl, as well as in media lacking potassium or phosphate.

**Effect of an additional copy of PHO4**

Induction of expression of the PHO genes at low phosphate concentrations requires the activation of the transcription factor Pho4. To investigate the effect of an additional copy of the PHO4 gene on the expression of SPL2-GFP we inserted PHO4 in the integrating vector pRS305 and integrated this vector into the genome of wild type and bmh1Δ cells expressing SPL2-GFP. The resulting strains were grown at 50 and 0 mM KCl and the expression of Spl2-GFP was analyzed by confocal microscopy and flow cytometry. As shown in Fig. 3 addition of an extra copy of PHO4 resulted in an increased expression of Spl2-GFP in almost all cells and the absence of heterogeneity in expression. These data suggest that the effect of the bmh1 deletion is not downstream of Pho4. Quantification of expression by flow cytometry showed that with the additional copy of PHO4 the expression of SPL2-GFP in the bmh1Δ cells is still lower than in the BY4741 cells (BY4741 pRS305: 14.5 ± 0.7; BY4741 pRS305[PHO4]: 43 ± 3, bmh1Δ pRS305: 7.0 ± 0.3, bmh1Δ pRS305[PHO4]: 25 ± 2; arbitrary units, ± SD, n = 4).

**Discussion and conclusions**

Upon potassium starvation mRNA levels of the PHO84 and SPL2 genes both related to phosphate metabolism are highly elevated [6–8]. In the present study we showed that deletion of either one of the 14-3-3 genes BMH1 or BMH2 resulted in decreased mRNA levels of these genes, especially apparent at standard potassium concentrations (Tables 4 and 5). These lower mRNA
levels can partly be explained by a lower activity of the \textit{PHO84} and \textit{SPL2} promoter in the \textit{bmh1}Δ mutant (Table 6). Further analysis at the cellular level by confocal microscopy and flow cytometry revealed that at standard phosphate and potassium concentrations in \textit{bmh1}Δ cells the expression of \textit{SPL2} is highly heterogenic with only a small fraction of the cells having a substantial expression of this gene (Fig. 1). This observation can explain the low levels of mRNA and the low activity of the \textit{PHO84} and \textit{SPL2} promoters in the total population of cells (Tables 4, 5 and 6). Upon potassium or phosphate deprivation the expression of these genes is induced in all cells, including those that have little to no expression at standard potassium and phosphate concentrations (Figs. 1 and 2). Analysis of \textit{bmh2}Δ \textit{SPL2-GFP} cells showed that a smaller fraction of the cells lack expression of \textit{SPL2-GFP}. Less effect of the \textit{bmh2}Δ deletion compared to the \textit{bmh1}Δ deletion may be expected as the levels of the Bmh2 protein are 5–10-fold lower than those of the Bmh1 protein [33].
Heterogenic expression of PHO genes has been reported before [31]. These authors identified positive and negative feedback loops, leading to bi-stability in phosphate transporter usage and as a result individual cells expressing predominantly either low- or high-affinity transporters. SPL2 plays a key role in these feedback loops as induction of SPL2 is necessary and sufficient for PHO pathway-dependent down-regulation of low-affinity transporters, causing individual cells to express either low- or high-affinity transporters. The origin of the cell-to-cell variability in gene expression in genetically identical cells is unclear. It may arise from noise in gene expression [31, 34–37].

Research on PHOS has provided evidence that nucleosome positioning plays a role in variations in gene expression at the single cell level [38, 39]. Under phosphate-rich conditions, PHOS gene expression is very low, and the promoter is occupied by nucleosomes. Upon phosphate starvation, there is a shift to a more nucleosome-free state. However, the small fraction of cells that expresses PHOS under phosphate-rich conditions also exhibits this nucleosome-free state [39]. Non-coding RNA may also be involved in the cell-to-cell variation in gene expression [40]. As 14-3-3 proteins have hundreds of binding partners 14-3-3 proteins can potentially influence transcription at different levels. 14-3-3 proteins bind to histone H3 [41] indicating that the effect may be directly at the nucleosome level. It has been shown in mammalian cells that interaction of 14-3-3 proteins with histone H3 leads to transcriptional activation [42]. On the other hand, after deletion of PHO80 the PHO84 and SPL2 promoters are strongly activated, both in the wild type and bmh1A cells and the effect of the bmh1 deletion is lost, suggesting that 14-3-3 proteins do not affect processes downstream of Pho4. It has been shown that Pho4 can induce changes in the nucleosome positioning of the PHO5 gene [43]. Thus, 14-3-3 proteins may influence nucleosome positioning indirectly by affecting the activation state of Pho4. In addition to PHO84 and SPL2, many more genes including PHO5 are activated by Pho4 during phosphate depletion (Additional file 2). Except for affecting the activation state of Pho4, Bmh1 may also affect transcription of the PHO genes at other levels. Recently, it has been shown that in addition to Pho4 the Af2 transcription factor is involved in the regulation of SPL2 [44]. However, regulation of this transcription factor by 14-3-3 proteins still has to be shown.

The observation that the interaction between 14-3-3ζ and phosphorylated human HspB6 is destabilized at physiologically relevant phosphate concentrations (5–15 mM) [26], may be of interest. This observation may indicate that modulation of the interaction between 14-3-3 proteins and their interaction partners by phosphate contributes to the regulation of the expression of PHO genes. One of the relevant 14-3-3 binding partners is the Kcs1 protein [45–48], an inositol hexakisphosphate and inositol heptakisphosphate kinase [49]. The Pho80 – Pho85 complex is inhibited by Pho81 in conjunction with inositol heptakisphosphate (eIP7) [50]. The function of Kcs1 in phosphate regulation is unclear, but Kcs1 may have a negative effect on the production of eIP7 and may play a role in the establishment of feedback loops stabilizing the activation state of the PHO regulon [51]. However, the role of the interaction of Kcs1 with 14-3-3 proteins is unknown.

Additional files

**Additional file 1:** Effect of deletion of BMH1 or BMH2 on the RNA levels after growth in YNB with 50 or 0 mM KCl. (PDF 56 kb)

**Additional file 2:** Effect of BMH1 and BMH2 deletion and potassium starvation on RNA levels of PHO genes. (PDF 56 kb)

**Additional file 3:** Genes of which the RNA level increased or decreased significantly (P < 0.01) more than 2.0-fold upon deletion of BMH1 or BMH2 after growth in YNB with 50 mM KCl. (XLSX 13 kb)

**Additional file 4:** Localization of free GFP expressed under control of the SPL2 promoter after cultivation in the absence of potassium. (PDF 84 kb)
Acknowledgements
We would like to thank Wouter Hendriksen for his help in constructing the bmh1Δ and bmh2Δ strains, Ginny Anemaet for her help in cultivation of yeast strains for transcriptome analysis and in constructing pRS313[PHO84-GFP-TPHO84] and pRS316[P PHO84-GFP-TPHO84] and Dennis van der Wiel for his help in construction of the pho80Δ disruption strains.

Funding
This study was partly funded by the Netherlands Organization for Scientific Research (NWO) - Earth and Life Sciences (ALW) (SYSMO) - grant 826.09.006.

Availability of data and materials
The datasets generated and analyzed during the current study are available in the NCBI’s Gene Expression Omnibus ( GEO) repository, and are accessible through GEO Series accession numbers GSE57093 and GSE85564. Strains and plasmids are available upon request.

Authors’ contributions
JMHt and MEC carried out most of the experiments and contributed to writing the manuscript; PPPT analyzed the effect of an additional copy PHO4; GPHvHt designed the study, some experiments and prepared the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 29 March 2017 Accepted: 31 August 2017
Published online: 06 September 2017

References
1. Vitvitsky VM, Garg SK, Keep RF, Albin RL, Banerjee R. Na+ and K+ ion imbalances in Alzheimer's disease. Biochim Biophys Acta. 1822;2012:1671–81.
2. David Y, Cachaux LP, Ivens S, Lapilover E, Heinemann U, Kaufer D, et al. Astrocytic dysfunction in epileptogenesis: consequence of altered potassium and glutamate homeostasis? J Neurosci. 2009;29:10588–99.
3. Ariño J, Ramos J, Sychrová H. Alkali metal cation transport and homeostasis in yeasts. Microbiol Mol Biol Rev. 2010;74:95–120.
4. Gynt MS, Philpott CC. Regulation of cation balance in Saccharomyces cerevisiae. Genetcs. 2013;193:677–713.
5. Ariño J, Aydar E, Druhle S, Ganser D, Jorini J, Kahn M, et al. Systems Biology of Monovalent Cation Homeostasis in Yeast: The Translucent Contribution. Adv Microb Physiol. 2014;64:1–63.
6. Barrette L, Andress L, Valverde-Saubí D, Casamayor A, Ariño J. The short-term response of yeast to potassium starvation. Environ Microbiol. 2012;14:3026–42.
7. Anemaet IG, van Heusden GPH. Transcriptional response of Saccharomyces cerevisiae to potassium starvation. BMC Genomics. 2014;15:1040.
8. Canadell D, González A, Casado C, Ariño J. Functional interactions between potassium and phosphate homeostasis in Saccharomyces cerevisiae. Mol Microbiol. 2015;95:555–72.
9. Wykoff DD, O’Shea EK. Phosphate transport and sensing in Saccharomyces cerevisiae. Methods Enzymol. 2001;339:194–214.
10. Conrad M, Schothorst J, Kankipati HN, Van Zeebroeck G, Rubio-Texeira M, O’Shea EK. Disruption of the Transcription Factor PHO4 by a Cyclin-CDK Complex, PHO80-PHO85. Science. 1994;263:1153–6.
11. Kaffman A, Herskowitz I, Tijan R, O’Shea EK. Phosphorylation of the Transcription Factor PHO4 by a Cyclin-CDK Complex, PHO80-PHO85. Science. 1994;263:1153–6.
12. Komell A, O’Shea EK. Roles of phosphorylation sites in regulating activity of the transcription factor Pho4. Science. 1999;284:977–80.
13. O’Neill EM, Kaffman A, Jolly ER, O’Shea EK. Regulation of PHO4 nuclear localization by the PHO80-PHO85 cyclin-CDK complex. Science. 1996;271:1209–12.
14. Vogel K, Hinnen A. The yeast phosphate transport system. Mol Microbiol. 1990;4:253–65.
15. Brown CR, Mao C, Falkovskaia E, Jurica MS, Boeger H. Linking stochastic fluctuations in chromatin structure and gene expression. PLoS Biol. 2013;11:e1001621.
39. Small E, Xi L, Wang J. Single-cell nucleosome mapping reveals the molecular basis of gene expression heterogeneity. Proc Natl Acad Sci U S A. 2014;111:E2462–71.
40. Castelnovo M, Stutz F. Role of chromatin, environmental changes and single cell heterogeneity in non-coding transcription and gene regulation. Curr Opin Cell Biol. 2015;34:16–22.
41. Walter W, Clynes D, Tang Y, Marmorstein R, Mellor J, Berger SL. 14-3-3 interaction with histone H3 involves a dual modification pattern of phosphoacetylation. Mol Cell Biol. 2008;28:2840–9.
42. Winter S, Simboeck E, Fischle W, Zupkovitz G, Dohnal I, Mechtler K, et al. 14-3-3 Proteins recognize a histone code at histone H3 and are required for transcriptional activation. EMBO J. 2008;27:88–99.
43. Fascher KD, Schmitz J, Hörz W. Role of trans-activating proteins in the generation of active chromatin at the PHOS promoter in S. cerevisiae. EMBO J. 1990;9:2523–8.
44. Pérez-Sampietro M, Serra-Cardona A, Canadell D, Casas C, Aního J, Herrero E. The yeast AFR2 transcription factor determines selenite toxicity by controlling the low affinity phosphate transport system. Sci Rep. 2015;6:32836.
45. Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR, et al. A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature. 2000;403:623–7.
46. Ho Y, Gruler A, Heilbut A, Bader G, Moore L, Adams S, et al. Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. Nature. 2002;415:180–3.
47. Krogan NJ, Cagney G, Yu H, Zhong G, Guo X, Ignatchenko A, et al. Global landscape of protein complexes in the yeast Saccharomyces cerevisiae. Nature. 2006;440:637–43.
48. Fiedler D, Braberg H, Mehta M, Chechik G, Cagney G, Mukherjee P, et al. Functional organization of the S. cerevisiae phosphorylation network. Cell. 2009;136:63–73.
49. Huang KN, Symington LS. Suppressors of a Saccharomyces cerevisiae plc1 mutation identify alleles of the phosphatase gene PTC1 and of a novel gene encoding a putative basic leucine zipper protein. Genetics. 1995;141:1275–85.
50. Lee Y-S, Mulugu S, York JD, O’Shea EK. Regulation of a cyclin-CDK-CDK inhibitor complex by inositol pyrophosphates. Science. 2007;316:109–12.
51. Nishizawa M, Komai T, Katou Y, Shirahige K, Ito T, Toh-E A. Nutrient-regulated antisense and intragenic RNAs modulate a signal transduction pathway in yeast. PLoS Biol. 2008;6:2817–30.
52. Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics. 1989;122:19–27.
53. Gütten U, Heck S, Fiedler T, Beinhauer J, Hegemann JH. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. 1996;24:2519–24.
54. Gietz RD, Sugino A. New yeast- Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene. 1988;74:257–34.
55. van Heusden GP, Wenzel TJ, Lagendijk EL, de Steenwinkel HY, van den Berg JA. Characterization of the yeast BMH1 gene encoding a putative protein homologous to mammalian protein kinase II activators and protein kinase C inhibitors. FEBS Lett. 1992;302:145–50.