Functional Analysis of Phosphorylation on *Saccharomyces cerevisiae* Syntaxin 1 Homologues Sso1p and Sso2p

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**Abstract**

**Background:** The *Saccharomyces cerevisiae* syntaxin 1 homologues Sso1p and Sso2p perform an essential function in membrane fusion in exocytosis. While deletion of either *SSO1* or *SSO2* causes no obvious phenotype in vegetatively grown cells, deletion of both genes is lethal. In sporulating diploid *S. cerevisiae* cells only Sso1p, but not Sso2p, is needed for membrane fusion during prospore membrane formation. Mass spectrometry and *in vivo* labeling data suggest that serines 23, 24, and 79 in Sso1p and serines 31 and 34 in Sso2p can be phosphorylated *in vivo*. Here we set out to assess the contribution of phosphorylation on Sso protein *in vivo* function.

**Principal Findings:** Different mutant versions of *SSO1* and *SSO2* were generated to target the phosphorylation sites in Sso1p and Sso2p. Basal or overexpression of phospho-mimicking or putative non-phosphorylated Sso1p or Sso2p mutants resulted in no obvious growth phenotype. However, S79A and S79E mutations caused a mild defect in the ability of Sso1p to complement the temperature-sensitive growth phenotype of sso2-1 sso1.4 cells. Combination of all mutations did not additionally compromise Sso1p *in vivo* function. When compared to the wild type SSO1 and SSO2, the phosphoamino acid mutants displayed similar genetic interactions with late acting sec mutants. Furthermore, diploid cells expressing only the mutant versions of Sso1p had no detectable sporulation defects. In addition to sporulation, also pseudohyphal and invasive growth modes are regulated by the availability of nutrients. In contrast to sporulating diploid cells, deletion of *SSO1* or *SSO2*, or expression of the phospho-mutant versions of *SSO1* or *SSO2* as the sole copies of SSO genes caused no defects in haploid or diploid pseudohyphal and invasive growth.

**Conclusions:** The identified phosphorylation sites do not significantly contribute to the *in vivo* functionality of Sso1p and Sso2p in *S. cerevisiae*.

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**Introduction**

Eukaryotic cells rely on a highly ordered vesicle transport system to transfer membranes and proteins between different intracellular compartments. A number of proteins have been identified in transport vesicle targeting and fusion with the target membrane. From yeast to man, SNARE family proteins are essential for membrane fusion [1]. SNARE proteins can be divided in distinct subfamilies that all share helical regions with heptad repeats referred to as the SNARE motif [2]. Different SNARE proteins can interact with each other to form a dense helix bundle, the SNARE complex [3,4,5].

The formation of a SNARE complex is typically followed by membrane fusion. Syntaxin family SNARE proteins are integral membrane proteins that belong to Q-SNAREs i.e. they contain a glutamine at the central layer of the SNARE motif bundle [3,5]. In addition to the SNARE motif, syntaxins have an N-terminal domain that is composed of three short helices and a C-terminal transmembrane domain that is followed by a very short hydrophilic tail [5,6]. *S. cerevisiae* expresses two highly homologous syntaxins Sso1p and Sso2p that both mediate membrane fusion during exocytosis at the plasma membrane [7].

The Sso1p the N-terminal domain has been shown to interact with the SNARE motif and regulate the rate of SNARE complex assembly [8]. Together, Sso1p and Sso2p perform an essential function in vegetatively growing haploid and diploid cells [7] where they interact with plasma membrane SNARE proteins Sec9p, Snc1p and Snc2p [9,10]. However, in meiotic diploid cells there is a specific requirement for Sso1p for *de novo* formation of the prospore membrane during meiosis [11,12,13,14].

The functional difference for Sso1p and Sso2p in meiotic cells is not explained by transcriptional regulation, or differences in expression levels. Both proteins are expressed at similar level in meiotic cells, localize to the prospore membrane, and swapping of promoters between *SSO1* and *SSO2* does not render Sso2p functional in prospore membrane formation [11,15,16]. The two...
N-terminal α-helices Ha and Hb of Sso1p are important for its function during meiosis [16]. In addition to the specific requirement of Sso1p, in sporulating cells the Q-SNARE Sec9p is replaced by a homologous protein Spo20 [17,18,19]. Recent results indicate that phosphatidic acid and PI(4,5)P2 are important for membrane fusion during prospore membrane formation [15]. However, the signals that regulate the activity of Sso1p and the initiation of meiotic SNARE complex formation are unknown.

Post-translational modifications are central modifiers of protein activity [20,21]. Mass spectrometry studies have revealed in vivo phosphorylation sites in the amino terminal part of Sso1p and Sso2p [22]. In this study we set out to establish the contribution of these phosphoamino acids on the functional regulation of Sso1 and Sso2 proteins. In addition, we tested, whether, in analogy to meiosis and sporulation, also pseudohyphal and invasive growth, two nutritionally regulated cell differentiation processes display differential requirements for Sso1p and Sso2p.

Figure 1. A schematic diagram illustrating the Sso1p and Sso2p homology and the domain structure of Sso1p. A) Habc, H3 SNARE motif, and transmembrane domain (TMD) are indicated. Serine 23, serine 24, serine 79 in Sso1p (red arrows) and serine 31 and serine 34 in Sso2p (blue arrows) indicate the identified in vivo phosphorylation sites [22,23]. Additional amino acids mutagenized (Serine 59 in Sso1p and Threonine 28 in Sso2p) are indicated by black arrows. B) The three dimensional structure of Sso1p (PDB 1FIO, [8]) with an added random N-terminal peptide for amino acids 1–30. For the phosphoamino acids the side chains are shown. Phosphorylation sites identified by mass spectrometry or in vivo labeling are indicated by red colour. The additional amino acid mutagenized (Serine 59) is indicated by black colour. doi:10.1371/journal.pone.0013323.g001
Results and Discussion

Sso1 and Sso2 Phosphorylation

Sso1p and Sso2p are highly homologous (75% identical, 88% similarity) (Figure 1A). Despite their similarity, only Sso1p, but not Sso2p is functional in prospore membrane formation in meiotic diploid cells [11,16]. This suggests that mechanisms exist that enable cells to discriminate between these two homologous QSNARE proteins for SNARE complex formation in meiotic diploid cells. Recent analysis of S. cerevisiae phosphoproteome has identified serines 23 and 24 in Sso1p and serines 31 and 34 in Sso2p as in vivo phosphorylation sites [22]. In addition, serine 79 was previously reported as an in vivo phosphorylation site in Sso1p [23]. Subsequent analysis showed that S79 phosphorylation reduced participation of Sso1p in haploid cell SNARE complexes [23]. These amino acids (Figure 1A) represent potential regulatory means to modulate Sso protein in vivo function and differentiate between these proteins during sporulation.

The structure of a cytosolic fragment of Sso1p (amino acids 31–225) has been determined [8]. This structure is missing the very amino-terminus that contains several phosphorylation sites in Sso1p and the homologous Sso2p. The amino-terminal peptides of several syntaxins do not refraction well in crystals. This suggests that even when present in the analyzed protein the peptide is unstructured in monomeric syntaxins. In order to better visualize the localization of the indicated in vivo phosphorylation sites a random peptide model was generated for the amino-terminal peptide of Sso1p (Figure 1B). When the putative phosphoamino acids were displayed in this model and the known structure, it is evident that Sso1p S23, S24 and S79 are located either in the Hb helix or at the unstructured amino-terminal peptide. At both locations, they are apparently accessible for cytosolic interactions.

Sso1p and Sso2p Phosphorylation Mutants are Functional In Vivo

In order to assess the functionality of Sso1p and Sso2p phosphorylation sites the putative phosphoamino acids S23, S24 and S79 in Sso1p and S31 and S34 in Sso2p were mutagenized to alanine or glutamic acid to mimic either constitutively non-phosphorylated or phosphorylated forms of these amino acids. These mutant sso1 and sso2 genes were cloned to centromeric low copy and to 2 μ high copy vectors. In both vectors the expression of SSO genes was maintained under the endogenous SSO1 and SSO2 promoters, respectively. In order to test the functionality of these mutant proteins in cells where they were the only Sso proteins expressed, these plasmids were transformed into the GAL1-SSO1 sso1Δ sso2Δ cells (H3664) where the wt SSO1 expression can be shut down by shifting cells from galactose containing medium to glucose containing medium. In glucose medium cells transformed with the empty vector ceased to grow (Table 1 and Table 2). However, no difference in growth, even at high temperatures, was observed for cells expressing either the mutant versions or the wt SSO1 or SSO2 at low or high levels (Table 1 and Table 2).

In order to assess the mutant protein functionality in a different way, the temperature-sensitive sso1Δ sso2-1 (H2177) yeast strain was transformed with plasmids for expression of the mutant Sso1p or Sso2p or the empty vector as a control. The ability of the mutant versions of Sso1p and Sso2p to rescue the temperature-sensitivity of this strain was scored (Figure 2, Table 1 and Table 2). Previously, phosphorylation of S79 was shown to reduce the recruitment of Sso1p to exocytic SNARE complexes in haploid yeast cells [23]. The sso1Δ(S79A) mutant overexpression could efficiently rescue rich medium sensitivity of snc1Δ sec24Δ cells [23]. In that study, the ability of the phosphorylation mimicking S79E/D mutant was not tested.

When compared to the wild type SSO1 (expressed from a centromeric, low copy plasmid) both sso1Δ(S79A) and sso1Δ(S79E) were slightly less efficient in rescuing the temperature-sensitivity of sso2-1 sso1Δ (H2177) cells (Figure 2A). Repeatedly, sso1Δ(S79E) was slightly less efficient than sso1Δ(S79A) in its suppression capacity in sso2-1 sso1Δ cells. However, when all the identified phosphoamino acids in Sso1p or Sso2p were mutagenized separately or simultaneously to alanines or glutamic acids, no additional phenotype over the S79E or S79A was observed (Table 1). Furthermore, when compared to overexpression of wt SSO1 or SSO2, overexpression of the mutant versions of sso1 or sso2 did not result in additional growth phenotypes (Table 2).

Table 1. Complementation capacity of sso and sec mutants.

| Mutants | 24°C | 30°C | 34°C | 37°C | 38°C |
|---------|------|------|------|------|------|
| GAL1-SSO1 sso1Δ sso2Δ | +   | -   | -   | -   | -   |
| sso1Δ S59A | +   | +   | +   | +   | +   |
| sso1Δ S59E | +   | +   | +   | +   | +   |
| sso1Δ S59A S79A | +   | +   | +   | +   | +   |
| sso1Δ S59E S79E | +   | +   | +   | +   | +   |
| sso1Δ S59A S79A | +   | +   | +   | +   | +   |
| sso1Δ S59E S79E | +   | +   | +   | +   | +   |
| sso1Δ wt | +   | +   | +   | +   | +   |
| sso2 T28A S31A S34A | +   | +   | +   | +   | +   |
| sso2 T28E S31E S34E | +   | +   | +   | +   | +   |
| sso2 wt | +   | +   | +   | +   | +   |
| Vector | -   | -   | -   | -   | -   |
| sso1 Δ sso2 -1 | +   | +   | +   | +   | +   |
| sso1Δ S59A | +   | +   | +   | +   | +   |
| sso1Δ S59E | +   | +   | +   | +   | +   |
| sso1Δ S59A S79A | +   | +   | +   | +   | +   |
| sso1Δ S59E S79E | +   | +   | +   | +   | +   |
| sso1Δ S59A S79A | +   | +   | +   | +   | +   |
| sso1Δ S59E S79E | +   | +   | +   | +   | +   |
| sso1Δ wt | +   | +   | +   | +   | +   |
| sso2 T28A S31A S34A | +   | +   | +   | +   | +   |
| sso2 T28E S31E S34E | +   | +   | +   | +   | +   |
| sso2 wt | +   | +   | +   | +   | +   |
| Vector | -   | -   | -   | -   | -   |
| nd, not determined. | | | | | |

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It has been shown that S and T phosphorylation sites cluster [24]. At the same time that cells can use processive phosphorylation of adjacent amino acids to modulate the strength or threshold of the responses [25]. Although, the sensitivity of mass spectrometry is high is possible that not all phosphopeptides are resolved with the current methods. Because the already generated mutations did not significantly affect Sso1p or Sso2p activity, additional mutations were generated in Sso1p (Serine 59) and Sso2p (Threonine 28) that locate adjacent to the identified phosphoamino acids (Figure 1A and B). When tested for complementation or multicopy suppression of the defective Sso1p or Sso2p, the functionality of mutant proteins containing all mutations was comparable to that of the wt proteins in vivo (Figure 2A, Table 1). Similarly, these mutants were able to complement and multicopy suppress the temperature-sensitive phenotype of \textit{ssol-1} \textit{ssol2-1} (H2608) cells (Table 2). All \textit{ssol} and \textit{ssol2} mutants were expressed at similar expression levels irrespective of the growth temperature of the cells (Figure 2B). This indicates that introduction of these mutations does not affect the stability of Sso1p and Sso2p. In addition, this indicates that the observed minor defect in complementation of \textit{ssol2-1 ssol1A} cell temperature-sensitivity is not due to reduced expression levels.

Table 2. Multicopy suppression capacity of \textit{ssol} mutants.

| Mutants | 24°C | 28°C | 30°C | 31°C | 32°C | 33°C | 34°C | 35°C | 36°C | 37°C |
|---------|------|------|------|------|------|------|------|------|------|------|
| \textit{GAL1-SSO1} \textit{ssol1A} \textit{ssol2-1} | nd | nd | nd | nd | + | nd | nd | + |
| \textit{Sso1 S59A} | + | nd | + | nd | nd | nd | + | nd | nd | + |
| \textit{Sso1 S59E} | + | nd | + | nd | nd | nd | + | nd | nd | + |
| \textit{Sso1 S59A S79A} | + | nd | + | nd | nd | nd | + | nd | nd | + |
| \textit{Sso1 S59E S79E} | + | nd | + | nd | nd | nd | + | nd | nd | + |
| \textit{Sso1 S23A S24A} | + | nd | + | nd | nd | nd | + | nd | nd | + |
| \textit{Sso1 S23E S24E} | + | nd | + | nd | nd | nd | + | nd | nd | + |
| \textit{Sso1 S23A S24A S59A} | + | nd | + | nd | nd | nd | + | nd | nd | + |
| \textit{Sso1 S23E S24E S59E S79A} | + | nd | + | nd | nd | nd | + | nd | nd | + |
| \textit{Sso1 wt} | + | nd | + | nd | nd | nd | + | nd | nd | + |
| \textit{sso2 T28A S31A S34A} | + | nd | + | nd | nd | nd | + | nd | nd | + |
| \textit{sso2 T28E S31E S34E} | + | nd | + | nd | nd | nd | + | nd | nd | + |
| \textit{Vector} | – | nd | – | nd | nd | – | nd | nd | – | – |

\textit{SSO1} \textit{ssol1-4 sso2-1} |

| Mutants | 24°C | 28°C | 30°C | 31°C | 32°C | 33°C | 34°C | 35°C | 36°C | 37°C |
|---------|------|------|------|------|------|------|------|------|------|------|
| \textit{Sso1 S79A} | + | + | + | + | + | – | – | – | – | – |
| \textit{Sso1 S79E} | + | + | + | + | + | – | – | – | – | – |
| \textit{Sso1 S23A S24A S59A S79A} | + | + | + | + | + | – | – | – | – | – |
| \textit{Sso1 S23E S24E S59 S79E} | + | + | + | + | + | – | – | – | – | – |
| \textit{Sso1 wt} | + | + | + | + | + | – | – | – | – | – |
| \textit{Sso2 T28A S31A S34A} | + | nd | + | nd | nd | nd | + | + | + | + |
| \textit{Sso2 T28E S31E S34E} | + | nd | + | nd | nd | nd | + | + | + | + |
| \textit{Sso2 wt} | + | nd | + | nd | nd | nd | + | + | + | + |
| \textit{Vector} | + | nd | + | nd | nd | nd | + | + | + | + |

\textit{nd}, not determined.

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Previously, high copy expression of \textit{SSO1} or \textit{SSO2} was shown to suppress \textit{sec1-1}, \textit{sec9-4} and \textit{sec15-1} mutant cell growth defect at the restrictive temperature [7]. The mutants generated here were as efficient as the wild type \textit{SSO1} and \textit{SSO2} to rescue \textit{sec1-1}, \textit{sec9-4} and \textit{sec15-1} growth defect at the restrictive temperature (data not shown). Collectively, our results show that phosphorylation or dephosphorylation of the tested amino acids in \textit{SSO1} or \textit{SSO2} is not essential for vegetative growth of haploid \textit{S. cerevisiae} cells. At the same time, these mutants had no detectable dominant negative effects on cell growth. However, in line with the previous results reporting a role for \textit{Sso1p S79} phosphorylation in \textit{Sso1p} regulation [23], \textit{Sso1pS79E} and \textit{Sso1pS79A} mutants were not fully as effective as the wt \textit{Sso1p} in complementing the temperature-sensitive growth of the \textit{sec2-1 ssol1A} cells.

Phosphorylation Mutants of \textit{Sso1p} Do Not Affect Sporulation

\textit{Sso1}, but not \textit{SSO2} is essential for prospore membrane formation [11]. We used this essential function of \textit{Sso1p} to map possible contribution of the identified phosphoamino acids for \textit{Sso1p} function in this cell differentiation process. For this \textit{ssol1A}/\textit{ssol1A} diploid cells were generated where different mutant versions
of sso1 (expressed from endogenous SSO1 promoter) were integrated at the ura3–52 locus. For each mutant, three independent transformants were induced to sporulate synchronously. The formation of spores was quantified by counting cells that were able to form tetrads (Table 3). The results show that mutations in the tested amino acids in Sso1p do not affect Sso1p functionality in prospore membrane formation. That no sporulation phenotype was observed in these Sso1p mutants was surprising given the fact that S79A alone has been shown to affect SNARE complex assembly [23].

In prospore membrane formation Sso1p forms complexes with a Sec9p homologue Spo20p and Snc2p to drive membrane fusion [17,18]. Our results suggest that in meiotic diploid cells the prospore membrane formation is not critically sensitive to S79 phosphorylation. We can not exclude the possibility that there are additional amino acids that have phospho- or some other post-translational modifications that regulate Sso protein function. The mechanism how S. cerevisiae cells can selectively use Sso1p, instead of the highly homologous Sso2p, for membrane fusion in meiotic diploid cells, remains enigmatic. We have previously identified Mso1p as an essential protein for prospore membrane formation [26]. Mso1p exists in complex with Sec1p, a regulator of SNARE complex assembly [26,27]. Interestingly, Mso1p binds preferentially Sso1p [27]. Interactions with Mso1p may provide additional specificity for the selective activity of Sso1p in prospore membrane formation.

Haploid or Diploid Cell Pseudohyphal or Invasive Growth are Not Differentially Regulated by SSO1 or SSO2

When starved for nitrogen, diploid cells undergo a developmental transition from a single cell yeast form to a filamentous pseudohyphal form [28]. Pseudohyphal filaments are composed of chains of elongated cells that radiate away from the colony and penetrate the agar substratum on which they are grown [28,29]. In this process changes in cell polarity and budding mode take place. Although poorly understood, it is conceivable that changes in cell polarity and budding mode involve regulation of protein and membrane transport to the plasma membrane and thus require the activity of Sso1p and/or Sso2p. Different types of pseudohyphal growth are observed in S. cerevisiae cells. In addition to the originally identified nitrogen starvation triggered diploid cell differentiation process [28], subsequent studies have shown that both haploid and diploid cells can be induced to form short branched pseudohyphae in liquid cultures in response to “fusel” alcohols such as 1-butanol [30].

Table 3. Quantification of Tetrads in sso1 Mutants.

| Mutant        | tetrads | no tetrads | total | tetrad % |
|---------------|---------|------------|-------|----------|
| Sso1p S79A    | 343     | 78         | 421   | 81       |
| Sso1p S79E    | 374     | 78         | 452   | 83       |
| Sso1p S23A S24A S59A S79A | 316     | 72         | 388   | 81       |
| Sso1p S23E S24E S59E S79E | 293     | 70         | 363   | 81       |
| Sso1p wt      | 293     | 66         | 359   | 82       |
| Vector        | 0       | 395        | 395   | 0        |

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In order to assess the possible specific roles of SSO1 or SSO2 in nitrogen starvation or alcohol induced pseudohyphal growth, SSO1 and SSO2 were deleted both in the haploid and diploid cells of \( \Sigma 1278b \) background widely used in studies concerning pseudohyphal growth. In addition, as a negative control haploid and diploid cells of S288c background were tested for pseudohyphal growth. Previously, S288c cells were shown to be defective for pseudohyphal growth due to a mutation in FLO8 [31]. As a positive control for pseudohyphal growth \( \Sigma 1278b \) cells deleted for SEM1 were used. Previously, deletion of SEM1 was shown to enhance pseudohyphal growth [32]. Homozygous diploid cells deleted either for SSO1 or SSO2 were capable of forming pseudohyphae on low nitrogen SLAD plates (Figure 3, upper panel). Similarly, haploid cells deleted either for SSO1 or SSO2 formed extensive hyphae on YPD plates supplemented with 1% 1-butanol (Figure 3, lower panel). In order to test the possible contribution of phosphorylation on Sso1p and Sso2p activity during pseudohyphal growth, haploid and diploid cells (\( \Sigma 1278b \) background) were generated that express as their sole copy of Sso proteins the phospho-mutant versions of Sso1p or Sso2p. Microscopic analysis of these cells revealed that Sso1p(S23S24S59S79) or Sso2p(T28S31S34) alanine or glutamic acid mutations display no obvious defect in pseudohyphal growth (Figure 4).

On solid growth medium (agar) both haploid and diploid cells of \( \Sigma 1278b \) background display invasive growth [30]. To assess whether SSO1 or SSO2 are specifically involved in the regulation of invasive growth, SSO1 or SSO2 deleted cells expressing the phospho-mutant versions of Sso1p or Sso2p as their sole copy of Sso proteins (in \( \Sigma 1278b \) background) were tested for invasive growth. For this, equal amounts of (OD\textsubscript{600} 1) haploid and diploid cells of four independent colonies were spotted on YPD plates. Cells were allowed to grow at 30°C for 3 days followed by incubation at room temperature for two additional days [30]. The plates were rinsed with a gentle stream of deionized water to remove non-invaded cells. As shown in Figure 5A, deletion of SSO1 or SSO2 in haploid MATa (or MATa, data not shown) or diploid cells had no effect on invasive growth. Similarly, the phosphoamino acid mimicking or abolishing mutations in Sso1p or Sso2p had no obvious effect on the ability of cells to invade the agar (Figure 5B). At the same time, the non-invasive control strain S288c (Figure 5, negative ctrl) was unable to invade the agar and the cells were easily washed away.

Collectively, our results suggest that differential participation of Sso1p and Sso2p in membrane fusion during a nutrient triggered cell differentiation process is not a general mode of regulation for cell growth. In addition, our results show that the currently identified
phosphoamino acids are not essential for Sso1p or Sso2p function in vivo. This could be due to differential targeting of downstream factors by different nutrient triggered signaling events in membrane fusion during sporulation and pseudohyphal growth. Alternatively, it is possible that protein phosphorylation is not a decisive event in membrane fusion regulation in these cellular processes or that additional, currently uncharacterized phosphorylation sites or other post-translational modifications exist in Sso1p and Sso2p.

**Materials and Methods**

**Yeast strains**

The yeast strains used are listed in Supplementary Table S1. When not stated otherwise, standard growth media were used [33]. LEU2 and LYS2 were deleted in H1925 and H1926 by transforming the cells with Sal I cut pAD1 orCla I cut pAD2 [34].

**Figure 4.** Haploid and diploid pseudohyphal growth is not affected by mutations in the putative phosphoamino acids in Sso1p or Sso2p. A) Diploid cells expressing as their sole copy of SSO either the wt SSO1 (H3970), SSO1S79A (H3966), SSO1 S79E (H3967), SSO1S23245979A (H3968) or SSO1S23245979E (H3969), or the wild type SSO2 (H3973), SSO2T28S3134A (H3971) or SSO2T28S3134E (H3972). B) Haploid cells expressing as their sole copy of SSO either the wt SSO1 (H3959), SSO1S79A (H3955), SSO1 S79E (H3956), SSO1S23245979A (H3957) or SSO1S23245979E (H3958), or the wild type SSO2 (H3965), SSO2T28S3134A (H3963) or SSO2T28S3134E (H3964). Treatment of cells as described in Figure 3. The negative control was for Haploid (H973) and diploid (H3088) cells of S288c background where a negative regulator of pseudohyphal growth (SEM1) was deleted [32].

**Figure 5.** SSO1 or SSO2 are not required for, and phosphomutations in Sso1p or Sso2p do not affect, diploid or haploid cell invasive growth. Patches of cells from four independent colonies were grown at 30°C for 3 days and incubated at room temperature for an additional 2 days. Non-invasive cells were rinsed away with a gentle stream of deionized water from the agar surface. A) Invasive growth of cells deleted either for SSO1 (diploid H3843), (haploid H3839) or SSO2 (diploid H3845), (haploid H3841). B) Upper panel: Invasive growth of cells expressing as their sole copy of SSO genes the wt SSO1 (diploid H3970), (haploid H3959) the phosphomimicking SSO1S79E (diploid H3967), (haploid H3956), SSO1S23245979E (diploid H3968), (haploid H3958), or the putative non-phosphorylated SSO1S79A (diploid H3966), (haploid H3955), SSO1S23245979A (diploid H3967), (haploid H3956). Lower panel: Invasive growth of cells expressing as their sole copy of SSO genes the wt SSO2 (diploid H3973), (haploid H3965) or the phosphomimicking SSO2T28S3134E (diploid H3972), (haploid H3964) or the putative non-phosphorylated SSO2T28S3134A (diploid H3971), (haploid H3963).

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enable loss of the URA3 marker and flanking vector sequences) and replica-plated to 5-FOA plates. Papillae from the 5-FOA plates were streaked onto YPD and replica-plated either to SC-leu or SC-lys to identify the desired auxotrophic mutants. SSO1 and SSO2 were deleted with kanMX by using the PCR cassette based transformation method [35]. The sequence information of the oligonucleotides used in this study is available upon request. The deletions were verified both by PCR and by Western blotting with Sso1p and Sso2p specific antibodies [11]. Diploid strains were obtained by mating of appropriate haploid cells. In order to test the functionality of different sso1 mutants in the diploid sso1/sso1 strain during sporulation, H3114 was transformed with integrative plasmids linearized by a SsoI cut within the URA3 and selected for growth at 24°C in the absence of uracil. For pseudohyphal growth, plasmids expressing wt or mutant versions of SSO1 or SSO2 were integrated to haploid cells (H3836, H3837, H3839 and H3841) where either the SSO1 or SSO2 had previously been deleted. In the resulting integrants (H3960, H3961, H3962, H3963, H3964 and H3965) either SSO1 or SSO2 was then deleted using either kanMX or hphNT1 containing PCR cassettes [35]. Appropriate haploid cells were then mated to generate homogygous diploids where the mutant versions of SSO1 or SSO2 were the sole copy of SSO genes. The obtained diploid strains were verified for expression of SSO1 or SSO2 by Western blotting with Sso1p and Sso2p specific antibodies [11].

Plasmids

SSO1 genomic fragment (453 bp upstream of ATG and 501 bp downstream of stop) in B1475 was mutated using the QuickChange method (Stratagene) to generated S23, S24, S59, S79 mutations to alanine or glutamic acid. The mutagenized genes were sequenced and cloned as BamHI-EcoRI fragments into pRS406, pRS416 and pRS426. Using B1474 as a template, the genomic SSO2 fragment (435 bp upstream of ATG and 1005 bp downstream of stop) was similarly mutagenized to change T28 and S31 and S34 to alanine or glutamic acid. The mutagenized genes were sequenced and cloned as BamHI-EcoRI fragments into pRS406, pRS416 and pRS426.

Complementation and Suppression Tests for Temperature-sensitive Growth

The complementation or multicopy suppression of the temperature-sensitive growth phenotypes was assayed by transforming plasmids expressing the wild type, mutant versions of SSO1 or SSO2 or the empty vector to sso2Δ GALI-SSO1 strain (H3664) or to sso mutant strains H2177 and H2608. In case of H3664 cells were grown on SC-ura 2% galactose followed by replication to SC-ura 5% galactose. The development of tetrads was monitored by microscopy. The tetrads were counted using hemocytometer (Assistent, Germany).

Supporting Information

Table S1 Yeast strains. Found at: doi:10.1371/journal.pone.0013323.s001 (0.12 MB DOC)

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Author Contributions

Conceived and designed the experiments: JJ. Performed the experiments: QY. Analyzed the data: QY, JJ. Wrote the paper: JJ.

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Yeast Cell Lysates

For evaluation of Sso1p and Sso2p mutant protein expression levels, cells were grown to OD600 1, the cultures were split into two identical halves and grown either at 24°C or 37°C for another 2 h. Cells were broken by vortexing in the presence of 0.45 mm glass beads in 2% SDS supplemented with a protease inhibitor cocktail (Complete, Roche). Lysates were centrifuged for 10 minutes at 20,200 g followed by heating of supernatants for 5 minutes at 95°C. The protein concentration was determined with BCA™ Protein Assay Kit (Thermo Scientific). Equal amount of total protein from each lysate was subjected to 12% SDS-polyacrylamide gel and analyzed by Western blotting using anti-Sso1p and anti-Sso2p specific antibodies [11].

Liquid Sporulation

Cells were grown overnight in YPD (with 5% glucose) diluted to OD600 0.1 in 1% KAc, 2% peptone, 1% yeast extract (pre-sporulation medium) and grown at 30°C overnight. Cells were washed once with water and resuspended to OD600 1 in 1% KAc. The development of tetrads was monitored by microscopy. The tetrads were counted using hemocytometer (Assistent, Germany).

Pseudohyphal and Invasive Growth

Diploid cell pseudohyphal growth was tested on SLAD plates [28] supplemented with appropriate amino acids at 30°C for 1 day followed by examination with Olympus AX 70 Microscope. Haploid cell pseudohyphal growth was induced on YPD plates supplemented with 1% (v/v) 1-butanol at 30°C for 1 day, and then monitored by microscopy [30]. Invasive growth was tested by spotting equal amount of OD600 1 cells on YPD plates. Cells were allowed to grow at 30°C for 3 days followed by incubation at room temperature for two additional days. The plates were rinsed with a gentle stream of deionized water to remove non-invaded cells and photographed.
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