Phosphatidylinositol 4,5-bisphosphate is required for invasive growth in *Saccharomyces cerevisiae*

Isabelle Guillas[1,2,3,*], Aurélia Vernay[1,2,3], Jean-Jacques Vitagliano[1,2,3,‡] and Robert A. Arkowitz[1,2,3,§]

[1]Université Nice - Sophia Antipolis, Institute of Biology Valrose, 06108 Nice Cedex 2, France
[2]Centre National de la Recherche Scientifique UMR7277, Institute of Biology Valrose, 06108 Nice Cedex 2, France
[3]Institut National de la Santé et de la Recherche Médicale U1091, 06108 Nice Cedex 2, France

*Present address: Université Pierre et Marie Curie Paris 06, CNRS, EAC 7180, Paris, France
‡Present address: University of Lausanne, Department of Pharmacology and Toxicology, Lausanne, Switzerland
§Author for correspondence (arkowitz@unice.fr)

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Summary

Phosphatidylinositol phosphates are important regulators of processes such as the cytoskeleton organization, membrane trafficking and gene transcription, which are all crucial for polarized cell growth. In particular, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] has essential roles in polarized growth as well as in cellular responses to stress. In the yeast *Saccharomyces cerevisiae*, the sole phosphatidylinositol-4-phosphate 5-kinase (PI4P5K) Mss4p is essential for generating plasma membrane PtdIns(4,5)P_2. Here, we show that Mss4p is required for yeast invasive growth in low-nutrient conditions. We isolated specific *mss4* mutants that were defective in cell elongation, induction of the Flo11p flocculin, adhesion and cell wall integrity. We show that *mss4-f12* cells have reduced plasma membrane PtdIns(4,5)P_2 levels as well as a defect in its polarized distribution, yet Mss4-f12p is catalytically active *in vitro*. In addition, the Mss4-f12 protein was defective in localizing to the plasma membrane. Furthermore, addition of cAMP, but not an activated MAPKKK allele, partially restored the invasive growth defect of *mss4-f12* cells. Taken together, our results indicate that plasma membrane PtdIns(4,5)P_2 is crucial for yeast invasive growth and suggest that this phospholipid functions upstream of the cAMP-dependent protein kinase A signaling pathway.

Key words: Yeast, Invasive growth, Phospholipids, Polarized growth, PtdIns(4,5)P_2, PI4P5K

Introduction

Polarized growth is critical for a range of cellular processes and responses to different environments. Phosphatidylinositol phosphates (PIPs) are important for actin cytoskeleton organization, vesicle trafficking (exo- and endocytosis) and transcription – all of which are required for polarized cell growth (Di Paolo and De Camilli, 2006; Strahl and Thorner, 2007; Vicinanza et al., 2008; Kwiatkowska, 2010; Saarikangas et al., 2010; Shewan et al., 2011). The phosphatidylinositol PtdIns(4,5)P_2 is a major PIP species, generated by phosphorylation of PtdIns(4)P at position 5 by type I phosphatidylinositol-4-phosphate 5-kinases (PI4P5K). In the budding yeast *Saccharomyces cerevisiae*, PtdIns(4,5)P_2 is generated by the conserved PI4P5K Mss4p which localizes to cortical patches PIK patches at the plasma membrane (Desrivières et al., 1998; Homma et al., 1998; Audhya and Emr, 2002; Ling et al., 2012), where the majority of this lipid is found. This sole yeast PI4P5K – a member of the type I subfamily – is essential for cell viability and actin cytoskeleton organization (Desrivières et al., 1998; Homma et al., 1998). In response to different signals, transient site-specific changes in PtdIns(4,5)P_2 levels result in membrane recruitment and activation of signaling proteins which contain particular domains that bind this phospholipid (Di Paolo and De Camilli, 2006; Lemmon, 2008; Vicinanza et al., 2008). For example, pleckstrin homology (PH), basic rich (BR) and AP180 N-terminal homology (ANTH) domains, which specifically bind PtdIns(4,5)P_2, are found in a range of proteins including those implicated in actin organization, exocytosis, endocytosis, membrane trafficking and cell division (e.g. septins) (Audhya and Emr, 2002; Sun et al., 2005; He et al., 2007; Takahashi and Pryciak, 2007; Orlando et al., 2008; Zhang et al., 2008; Bertin et al., 2010). Mss4p is critical for plasma membrane targeting of components of the yeast pheromone response (i.e. Ste5p) and the yeast cell wall integrity MAP-kinase (MAPK) pathways (i.e. Rom2p) and hence PtdIns(4,5)P_2 is important for responses to cell wall perturbations and pheromone (Audhya and Emr, 2002; Garrenton et al., 2006; Garrenton et al., 2010).

Tor kinases play an important role in nutrient sensing (Barbet et al., 1996; Rohde et al., 2008) and pseudohyphal growth, particularly in response to nitrogen deprivation (Cutler et al., 2001; Orlova et al., 2006). Furthermore, genetic and physical interactions between either Mss4p or PtdIns(4,5)P_2-binding proteins and Tor-kinase-containing complexes (TORCs) have been observed (Audhya et al., 2004; Fadri et al., 2005; Berchtold et al., 2012). For example, Slm1p and Slm2p, both PH domain containing PtdIns(4,5)P_2-binding proteins, interact with TORCs (Audhya et al., 2004; Fadri et al., 2005; Berchtold et al., 2012). Mss4p mediated PtdIns(4,5)P_2 production, Slm1p/Slm2p and Tor kinases are also important for sphingolipid signaling (Audhya et al., 2004; Kobayashi et al., 2005; Bultynck et al., 2006; Daquinag et al., 2007; Roelants et al., 2011; Berchtold et al., 2012; Liu et al., 2012). Starved yeast cells respond to the addition of glucose or ergosterol, the major sterol of *S. cerevisiae*, by rapid changes in PIPs (Dahl and Dahl, 1985; Kaibuchi et al., 1986; Frascotti et al., 1990). With respect to glucose, these changes are characterized by rapid

increases in the levels of PtdIns(4,5)P_2 metabolites including phosphatidic acid, PI and PIP (Kaibuchi et al., 1986; Kato et al., 1989; Frascotti et al., 1990) and also PtdIns(4,5)P_2 levels. Ras2p, which is required for filamentous growth, has been implicated in these PIP increases (Kaibuchi et al., 1986; Kato et al., 1989; Frascotti et al., 1990) which require phospholipase C1 (Plc1p) (Coccetti et al., 1998).

PtdIns(4,5)P_2 is likely to play a role in responses to cell wall perturbation (Audhya and Emr, 2002; Morales-Johansson et al., 2004) and is important for responses to mating pheromone (Garrenton et al., 2006; Garrenton et al., 2010). A unique dedicated MAP kinase pathway is critical for each of these processes, i.e. the cell wall integrity and the mating pheromone-dependent MAPK cascades (Chen and Thorner, 2007). In response to limiting nutrients *S. cerevisiae* undergo filamentous growth, which is regulated by both MAPK and Ras2/cAMP-dependent protein kinase A (PKA) pathways (Brückner and Mösch, 2012; Cullen and Sprague, 2012). In haploid and diploid cells this nutrient-regulated growth state is frequently referred to as invasive growth and pseudohyphal growth, respectively. These signaling pathways regulate filamentous growth by inducing the transcription of a range of genes, one of the most important being the adhesion flocculin *FLO11*. Filamentous growth is characterized by three visible changes: cell elongation, increased cell–cell and cell–substrate adhesion due to cell surface changes and alteration in budding pattern to a unipolar-distal budding pattern (Mösch and Fink, 1997; Madhani, 2000).

Given the roles of PtdIns(4,5)P_2 in response to varied stresses including nutrient deprivation and cell wall integrity we examined whether Mss4p and hence this lipid were required for *S. cerevisiae* filamentous growth. We have carried out a genetic screen for *mss4* mutants specifically defective in haploid invasive growth and show that Mss4p is required for this process, in particular cell elongation, induction of the Flo11p flocculin and adhesion to a solid substrate. Our results indicate that PtdIns(4,5)P_2 is critical for haploid invasive growth and suggest that a polarized distribution of this lipid is important for polarized growth in response to nutrient deprivation.

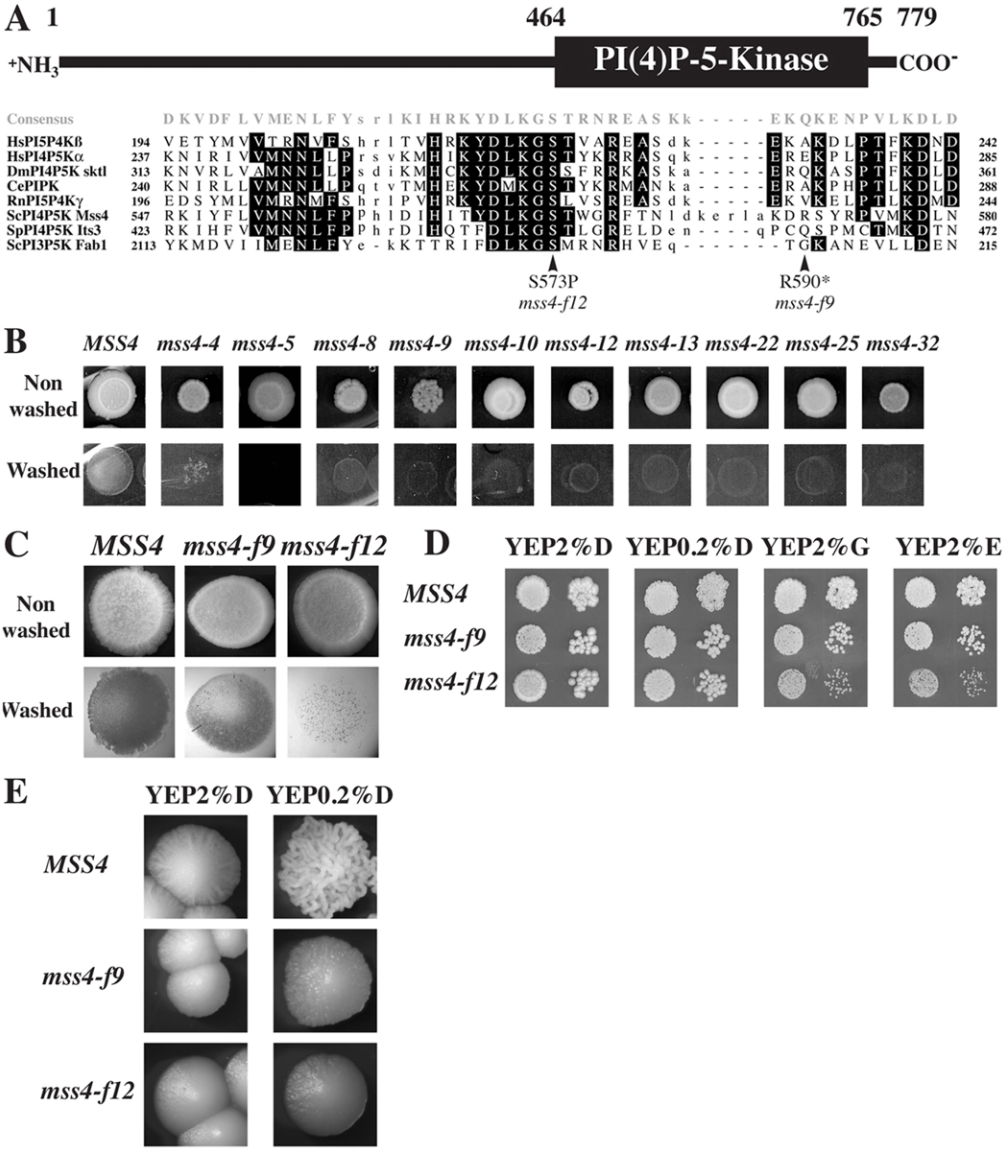

Fig. 1. Mss4p is specifically required for yeast haploid invasive growth. (**A**) Upper panel: schematic representation of Mss4p with the catalytic kinase domain indicated. Lower panel: sequence alignment of PIPK catalytic kinase domains from *H. sapiens*, *D. melanogaster*, *C. elegans*, *R. norvegicus*, *S. cerevisiae* and *S. pombe* using the BLAST algorithm (Altschul et al., 1990). Conserved residues are indicated in black squares. (**B**) Invasive growth defects of *mss4* mutants. The indicated strains were spotted on YEPD and, after 10 days incubation, surface cells were washed off revealing agar invasion. (**C**) Recreated *mss4-f9* and *mss4-f12* mutants are defective for invasive growth. Indicated strains (*mss4-f9*; R590* and *mss4-f12*; S573P) were analyzed as described in Fig. 1B. (**D**) Serial diluitons of the indicated strains were spotted on medium containing glucose (D), glycerol (G) and ethanol (E) and grown for 5 days. (**E**) Enlarged images of colonies grown on the indicated medium for 5 days. Similar results were observed in three independent experiments and representative images are shown.

Table 1. *mss4* mutations responsible for invasive growth defect

Amino acid change	Base mutation	Invasive growth
Ile566Asn	T1697A	++
Ser573Pro	T1717C	−
Thr574Ile	C1721T	++
Arg590Stop	A1768T	+
Lys597Arg	A1790G	++
Lys597Asn	A1791C	++

The changes found in *mss4-f9* (Arg590Stop, Lys597Arg) and *mss4-f22* (Ile566Asn, Thr574Ile, Arg590Stop, Lys597Asn) alleles are shown; ++, invasive growth similar to wild-type strains; +, reduced invasive growth; −, little to no invasive growth.

Results

Isolation of *mss4* invasive growth mutants

Yeast invasive, filamentous growth requires major rearrangements of the actin cytoskeleton together with the activation of two different signal transduction pathways, a MAPK and cAMP-dependent PKA pathway (Brückner and Mösch, 2012; Cullen and Sprague, 2012). We postulated that, similar to the role of PIPs in mammalian cells and the yeast pheromone response, phosphatidylinositol phosphates might be important for polarized growth in response to limiting nutrient conditions. We set out to determine if the PI4P5K Mss4p is required for yeast haploid invasive growth. A randomly mutagenized library of *mss4* mutants was screened for alleles specifically defective in invasive growth. We focused on the Mss4p catalytic kinase domain and a portion of this domain (amino acid 467–601) was mutagenized by error prone PCR (Fig. 1A). A *mss4Δ* strain, which was kept alive with a plasmid copy of *MSS4* driven by the *GAL1* promoter, was used to isolate *mss4* alleles defective for haploid invasive growth. Over 12,000 yeast colonies were screened for their ability to undergo invasive growth on YEPD medium and ten mutants were identified (Fig. 1B) which exhibited various degrees of invasive growth defects. Four mutants, *mss4-f9*, *-f12*, *-f13* and *-f22*, whose defects were reproducibly observed upon plasmid rescue and retransformation, were further studied. Sequencing revealed *mss4-f9* and *-f22* had a mutation, which resulted in the replacement amino acid arginine 590 by a stop codon. In addition mutations, which changed lysine 597 to arginine in *mss4-f9* and isoleucine 566 to asparagine, threonine 574 to isoleucine and lysine 597 to asparagine in *mss4-f22* were identified. Mutants *mss4-f12* and *-f13* both had the same unique mutation, which resulted in the amino acid residue serine 573 changed to a proline. Each individual amino acid change was recreated by mutagenesis and the changes S573P (*mss4-f12*) and R590* (*mss4-f9*) are responsible for the invasive growth defects (Table 1; Fig. 1C) and will be hereafter referred to as *mss4-f12* and *mss4-f9*, respectively (Fig. 1A). To rule out the possibility that yeast cells were able to read through the *mss4-f9* mutation (R590*) and thereby generate full-length kinase, the portion of *MSS4* 3′ to the newly introduced stop codon was also removed. Both of these recreated mutants were functional for viability as cells grew on 5-FOA-containing medium. Fig. 1C shows that both *mss4-f9* and *mss4-f12* mutants are defective in invasive growth, with little to no cells of the latter mutant invading the agar surface. The *mss4-f9* mutant had an intermediate defect with less invasion compared to wild-type cells yet increased invasion compared to *mss4-f12* cells. These mutants were also defective in invasive growth on low ammonia medium (supplementary material Fig. S1); however, due to poor invasive growth in this condition, subsequent analyses were carried out with low glucose medium. We examined the location of the amino acid changes in *mss4-f9* and *mss4-f12* mutants in an alignment of PIP-kinases from a range of organisms and the arginine 590 amino acid residue altered in *mss4-f9* is not conserved in other PIP-kinases (Fig. 1A). The stop codon in the *mss4-f9* mutant results in a truncation of the conserved catalytic domain removing regions of this domain that are likely to be involved in substrate binding, catalysis and plasma membrane targeting (Kunz et al., 2000; Arioka et al., 2004). Strikingly, the serine residue 573, which is altered in *mss4-f12* is highly conserved in all PIP-kinases examined. This residue is in proximity to other residues implicated in binding to PIP lipids. Together, these results demonstrate that the PI4P5K Mss4p is necessary for yeast invasive growth.

Budding and mating are not substantially affected in *mss4-f9* and *mss4-f12* mutants

MSS4 is essential for viability and necessary for organization of the actin cytoskeleton (Desrivières et al., 1998; Homma et al., 1998). The mutations in both *mss4-f9* and *mss4-f12* are localized to the catalytic kinase domain and therefore we examined the budding growth of these mutants in different solid medium (Fig. 1D). These two mutants grew similar to wild-type strains on rich medium (YEP) containing 2% glucose. When grown on medium containing reduced glucose levels (YEP0.2%D) or non-fermentable carbon sources, such as ethanol or glycerol, the *mss4-f12* mutant grew less well than wild-type cells while the growth of *mss4-f9* cells was less affected. YEP medium containing 0.2% glucose, 2% ethanol, or 2% glycerol induces invasive growth in wild-type cells more efficiently than YEP medium with 2% glucose (Cullen and Sprague, 2000). Microscopic examination of the yeast colonies on YEP0.2%D revealed that both *mss4* mutants do not form crenelated colonies in contrast to wild-type cells but rather form colonies with a smooth surface similar to growth on YEP2%D (Fig. 1E) (Halme et al., 2004; Casalone et al., 2005). Similar differences in colony morphology were observed in ethanol and glycerol containing medium (supplementary material Fig. S2). These alterations in colony morphology are correlated with invasive growth

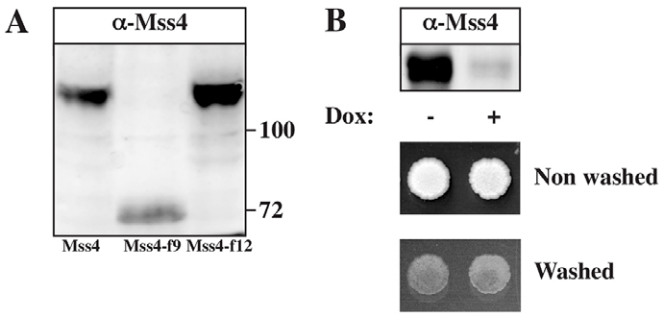

Fig. 2. Increased levels of Mss4p do not perturb invasive growth. (**A**) Cell extracts from indicated strains were analyzed by SDS-PAGE followed by immunoblotting. Immunoblots were probed with anti-Mss4p antisera. (**B**) Upper panel: levels of Mss4p and invasive growth of a wild-type strain carrying a Tet-*MSS4* plasmid. Extracts from cells grown in the presence or absence of doxycycline (20 μM), analyzed as in Fig. 2A. Lower panels: invasive growth was analyzed as in Fig. 1C. Similar results were observed in three independent experiments and representative images are shown.

(Reynolds and Fink, 2001; Casalone et al., 2005). As *MSS4* is important for mating pheromone response (Garrenton et al., 2010), we examined whether the *mss4-f12* cells could respond to mating pheromone and mate efficiently. Both *MSS4* and *mss4-f12* cells formed shmoos in response to mating pheromone (data not shown) and halo assays revealed identical pheromone-dependent growth arrest (supplementary material Fig. S3A). Furthermore, pheromone-dependent gene induction using the *FUS1-lacZ* reporter revealed little to no difference with the wild-type (supplementary material Fig. S3B). In quantitative matings the *mss4-f12* strain exhibited a minor mating defect (mating efficiency 12.0±1.2% versus 23.5±2.4% for *MSS4* strain), which was not substantially affected with an enfeebled mating partner (2.2% for *mss4-f12* strain versus 7.3% for *MSS4* strain). Together, our results indicate that these *mss4* mutants are specifically defective in invasive growth.

Alteration of Mss4p levels does not affect invasive growth

We next examined the expression levels of Mss4p in wild-type and mutant strains to determine if the invasive growth defect could be due to altered Mss4p levels. Fig. 2A shows an immunoblot of cells expressing either Mss4p mutant, Mss4-f9p, or Mss4-f12p, as the sole PI4P5K using an anti-Mss4p polyclonal serum directed against the Mss4p N-terminus. As expected, Mss4-f9p migrated faster than wild-type Mss4p due to the stop codon at position 590. Furthermore, this mutant was expressed at a lower level compared to Mss4p, whereas Mss4-f12p is expressed at similar or greater levels compared to Mss4p. As the *mss4-f12* mutant exhibited a strong invasive growth defect, we examined whether this defect could be due to an altered level of Mss4p. However, two results argued against this explanation. Firstly, the invasive growth defect of each mutant was complemented by a wild-type copy of *MSS4* (supplementary material Fig. S4), indicating that these mutants are recessive. Secondly, we overexpressed Mss4p using the Tet promoter and examined if this resulted in an invasive growth defect. In the absence of the repressor doxycycline, Mss4p is highly overexpressed yet of invasive growth was not affected (Fig. 2B). These results indicate that the invasive growth defects of *mss4-f9* and *mss4-f12* mutants are unlikely to be due to alterations in the level of this PI4P5K.

Cell polarity, cytoskeleton and cell wall integrity in *mss4* mutants

In addition to the changes in cell–cell adhesion that result in crenelated colonies during filamentous growth, cells elongate and undergo a reorganization of cell polarity. Mss4p is critical for cell morphology and actin cytoskeleton organization (Desrivières et al., 1998; Homma et al., 1998). First we examined the cell morphology of *MSS4*, *mss4-f9* and *mss4-f12* mutants. Fig. 3A shows that in rich medium with excess glucose, these mutants exhibit similar morphologies with the *mss4-f12* mutant cells slightly larger than wild-type and *mss4-f9* cells (average cell long axis *MSS4* 5.7±0.8 μm, n=296; *mss4-f9* 5.7±0.8 μm, n=318; *mss4-f12* 6.0±0.8 μm, n=207). We also examined if these *mss4* mutants had an altered bud site selection pattern and found that they budded axially, similar to wild-type cells (wild-type 88%, *mss4-f9* 85% and *mss4-f12* 86% axial budding; n=150–200 cells). Upon glucose depletion, the wild-type formed elongated clumps of cells, whereas the *mss4-f12* mutant was substantially defective in cell elongation and appeared not to form cell clumps (Fig. 3B). The *mss4-f9* mutant exhibited an intermediate phenotype. As unipolar-distal budding is necessary for invasive growth (Mösch and Fink, 1997; Madhani, 2000) we examined the

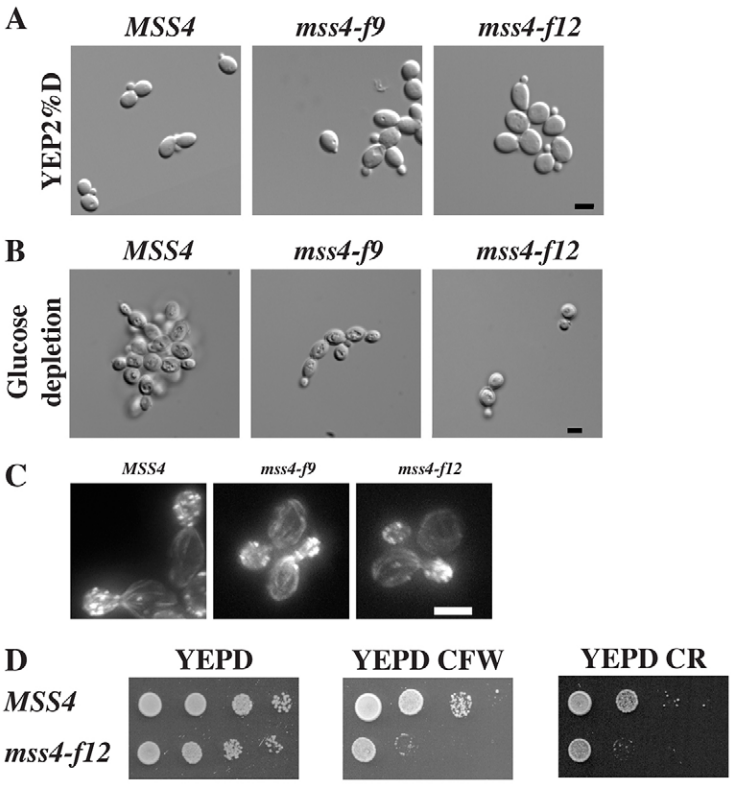

Fig. 3. Morphology and actin cytoskeleton of *mss4* mutant cells are not substantially affected. (**A**,**B**) Images of indicated yeast strains grown in rich medium with 2% glucose or incubated for 6 hr in medium lacking glucose. (**C**) Images of the actin cytoskeleton of indicated strains grown in rich medium with 2% glucose. Actin was visualized with Alexa-Fluor-488–phalloidin. Maximum projections of 30×0.15 μm deconvoluted *z*-sections are shown. Similar results were observed in two experiments and representative images are shown. (**D**) Serial dilutions of indicated strains were spotted on YEPD medium containing 25 μg/ml Calcofluor White (CFW) or 100 μg/ml Congo Red (CR) and incubated for 2 days. Similar results were observed in three independent experiments.

bud site selection of these *mss4* mutants in glucose depletion conditions. Both mutants budded in a unipolar pattern with 88% and 90% unipolar budding for *mss4-f9* and *mss4-f12* cells, respectively, compared to 85% for wild-type cells ($n=200$ cells).

Actin has multiple functions in filamentous growth and reorganization of the actin cytoskeleton is critical during invasive growth (Cali et al., 1998). Hence we examined the distribution of actin cytoskeleton. Fig. 3C shows that actin patches are highly polarized and found predominantly in the buds of *mss4-f9* and *mss4-f12* cells similar to wild-type controls. In addition, the actin cable distribution appeared to be unaffected in these *mss4* mutants, suggesting that the invasive growth defect is not due to a general defect in actin cytoskeleton organization nor in budding pattern. As Mss4p/PtdIns(4,5)P_2 are important for polarized growth (Yakir-Tamang and Gerst, 2009a; Yakir-Tamang and Gerst, 2009b), we investigated the distribution of cell polarity proteins in the *mss4-f12* mutant. The Cdc42p effector Gic2p, the exocyst subunit Sec3p and septins are all involved in cell polarity and bind PtdIns(4,5)P_2 (Takahashi and Pryciak, 2007; Orlando et al., 2008; Zhang et al., 2008; Bertin et al., 2010). A reporter for active Cdc42p ($Gic2_{PBD}$RFP), the polarisome component Spa2p, Sec3p and the septin subunit Cdc3p localize similarly in *MSS4*, *mss4-f12* and *mss4-f9* cells (supplementary material Fig. S5; data not shown). There were somewhat lower Spa2GFP signals in *mss4-f12* cells; however, as the signal was polarized we attribute this difference to expression levels.

The cell wall integrity pathway contributes to invasive growth (Birkaya et al., 2009). Hence we examined whether the *mss4* mutant with the stronger invasive growth defect was sensitive to the cell wall perturbants Congo red (CR) and Calcofluor white (CFW). Fig. 3D shows that *mss4-f12* cells are more sensitive to CR and CFW, although growth of this mutant was still observed. Together our results indicate that while there are no major defects in cell polarity in the *mss4* mutants, cell wall integrity is affected.

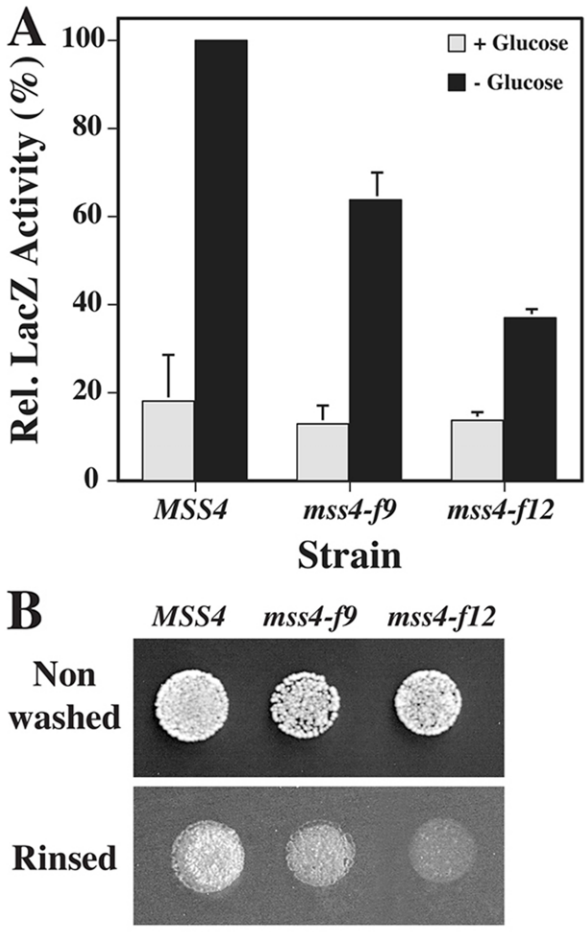

Fig. 4. Induction of the flocculin *FLO11* and cell adhesion is reduced in *mss4* mutants. (**A**) Flo11–LacZ levels in cells grown in the presence or absence of glucose for 7 hr. Values are normalized to the wild-type in the absence of glucose [54.4 β-galactosidase units (μg protein)$^{-1}$ min^{-1}] and are the mean±s.d. of two experiments each with two clones and duplicate determinations. (**B**) Adhesion of *mss4* strains on a solid surface. Indicated strains were spotted onto SC-Trp medium containing 10 mM 3-AT and after 3 days were washed off the surface with a gentle stream of H_2O. Similar results were observed in two independent experiments and representative images are shown.

Induction of the Flo11p cell adhesion flocculin is reduced in *mss4* mutants

The cell adhesion flocculin, Flo11p is critical for haploid invasive growth (Lambrechts et al., 1996; Lo and Dranginis, 1998; Rupp et al., 1999; Guo et al., 2000). Upon glucose depletion, this flocculin, which is downstream of both MAP kinase and cAMP PKA pathways, is upregulated (Rupp et al., 1999; Cullen and Sprague, 2000; Kuchin et al., 2002). We used the *FLO11* promoter fused to the LacZ to investigate whether the *mss4* mutants were able to induce Flo11p expression after glucose depletion. The levels of Flo11–LacZ were determined after 7 hr of growth in medium lacking glucose and Fig. 4A shows that in these conditions wild-type cells induce Flo11p by ~5-fold. The *mss4-f9* mutant induced Flo11p to a lower level, ~60% of control cells, whereas the *mss4-f12* mutant exhibited a further reduction in Flo11p upregulation (~30%). In amino acid starvation conditions, Flo11p is also required for adhesive growth to solid agar. Hence we examined whether the *mss4* mutants could adhere to a solid surface upon His starvation (Roberts and Fink, 1994; Braus et al., 2003; Guldal and Broach, 2006). Fig. 4B shows that in comparison to wild-type cells, *mss4-f9* cells were somewhat reduced in adhesion, whereas the *mss4-f12* mutant was substantially reduced in adhesion to this solid surface. These results suggest that Mss4p is required for Flo11p upregulation and ultimately adhesion.

Cell polarity proteins localize to sites of growth in the *mss4-f12* mutant in low glucose

We next examined the distribution of active Cdc42p, Spa2p, Sec3p and Cdc3p in wild-type and *mss4-f12* cells grown in low glucose (0.2%). In these conditions cells begin to elongate after 6–8 hr and there was still sufficient signals of the reporters. These cell polarity proteins localize similarly in wild-type, *mss4-f12* and *mss4-f9* cells (supplementary material Fig. S6; data not shown), although a reduction in the signals of Spa2–GFP and Cdc3–GFP was observed in the *mss4-f12* mutant. These results indicate that, in glucose depletion conditions, cell polarity is not substantially perturbed in the *mss4-f12* mutant.

The *mss4-f12* mutant has reduced levels of PtdIns(4,5)P_2 *in vivo* but is catalytically active *in vitro*

As both of the identified *mss4* mutants have alterations in the catalytic core domain we examined whether these mutants had

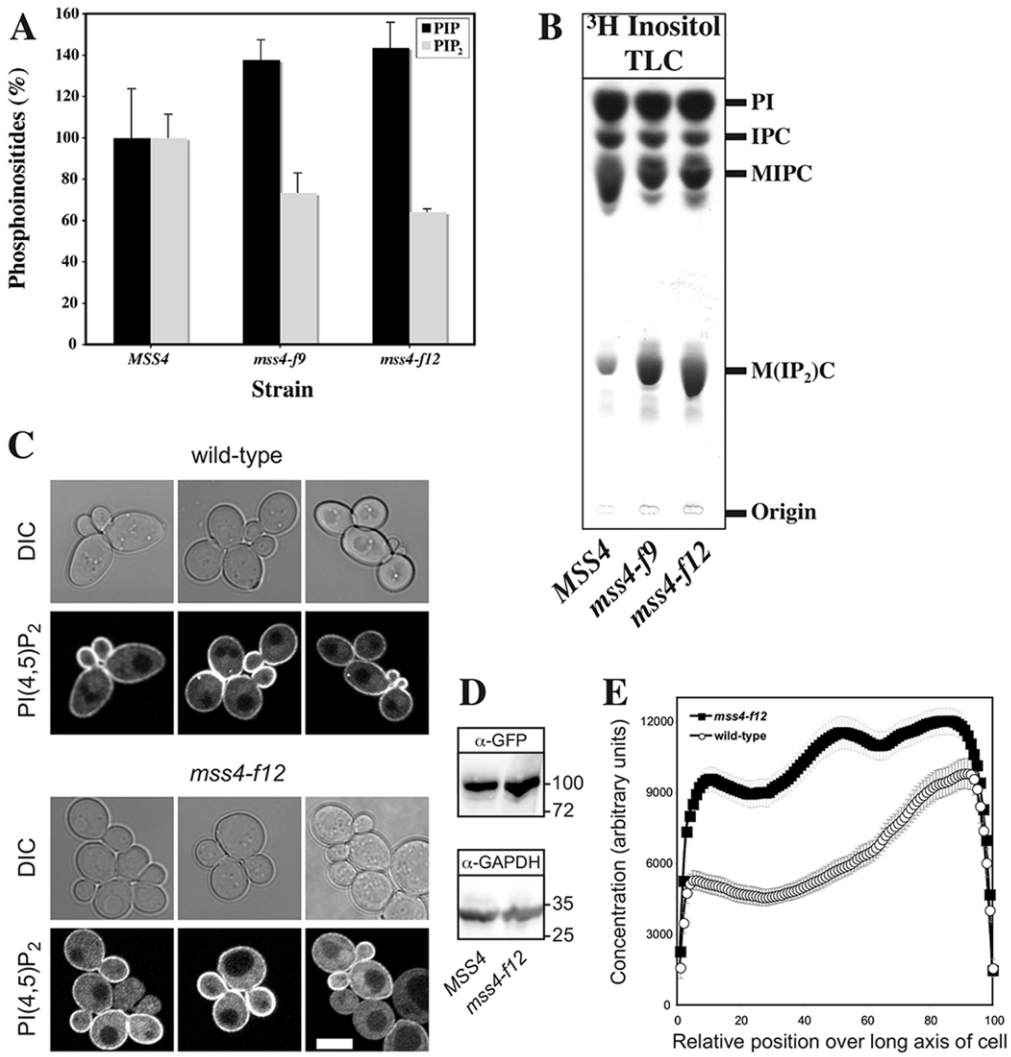

Fig. 5. ***mss4*** **mutants have reduced levels of PtdIns(4,5)P_2, an altered distribution of plasma membrane PtdIns(4,5)P_2 and accumulate mannosyl-di-inositolphosphate-ceramide** ***in vivo*****.** (**A**) Quantification of PtdIns(4)*P* (PIP) and PtdIns(4,5)P_2 (PIP$_2$) levels in indicated strains. Cells were labeled with [^{33}P]P_i and extracted lipids were analyzed by TLC. Levels of PtdIns(4)*P* and PtdIns(4,5)P_2 were normalized to that in wild-type cells (5.2% and 6.1% of total labeled lipids for PtdIns(4,5)P_2 and PtdIns(4)*P*, respectively) and are the means±s.d. of three determinations. (**B**) Cells were labeled with [^{3}H]inositol and extracted complex sphingolipids were analyzed by TLC with the positions of standards indicated. Similar results were observed in five experiments. (**C**) Confocal fluorescence and DIC images of indicated yeast strains expressing PtdIns(4,5)P_2 reporter. Central *z*-sections of representative cells are shown. (**D**) Cell extracts of the indicated strains were analyzed by SDS-PAGE and immunoblots were probed with anti-GFP or anti-GAPDH sera. (**E**) Quantification of PtdIns(4,5)P_2 concentration over long axis of budding cells. Mean±s.e.m. signal concentration over the cell long axis (in relative units, set to 100, determined by the BP program as described in the Materials and Methods) starting from the mother cell. The concentrations were determined from sum projections of $n=28$ and $n=79$, wild-type and *mss4-f12* mutant cells, respectively from two or three independent experiments.

reduced PtdIns(4,5)P_2 levels *in vivo*. Phosphatidylinositol phosphates were labeled *in vivo* with [^{33}P]P_i, lipids were then extracted and analyzed by TLC. Both mutants exhibited ~40% increase in PtdIns(4)*P* levels and a 30–40% decrease in PtdIns(4,5)P_2 levels (Fig. 5A). [^{3}H]inositol labeling was also carried out to determine if complex sphingolipid synthesis was perturbed, as previous studies have suggested that phosphatidylinositol phosphate levels are linked to complex sphingolipid levels (Audhya et al., 2004; Kobayashi et al., 2005; Bultynck et al., 2006; Daquinag et al., 2007; Berchtold et al., 2012). Using inositol labeling, similar results were observed with respect to increased PtdIns(4)*P* levels and reduced PtdIns(4,5)P_2 levels in the *mss4* mutants. However analyses of complex sphingolipids by TLC revealed a substantial increase in the levels of mannosyl-di-inositolphosphate-ceramide [M(IP)$_2$C] (Fig. 5B). Together these results indicate that the *mss4* mutants accumulated PtdIns(4)*P* and M(IP)$_2$C and had decreased levels of PtdIns(4,5)P_2 *in vivo*. We then focused our attention on the strongest mutant, *mss4-f12*.

While the above biochemical analyses revealed that the *mss4-f12* mutant had reduced levels of PtdIns(4,5)P_2, the labeling studies did not provide information on the distribution and levels of PtdIns(4,5)P_2 at the plasma membrane. We used the PtdIns(4,5)P_2 reporter that we had optimized for use in *C. albicans* (Vernay et al., 2012) to visualize plasma membrane PtdIns(4,5)P_2 in *S. cerevisiae*. This GFP–PH$^{Plc\delta}$–PH$^{Plc\delta}$–GFP

reporter, driven by the *GAL1* promoter to minimize toxicity associated with long-term expression was integrated at the *URA3* locus. Confocal *z*-sections revealed that this reporter localized predominantly to the plasma membrane and was enriched in small/medium buds (Fig. 5C). In contrast, *mss4-f12* cells exhibited an increase in cytoplasmic signal as well as an apparent redistribution of signal in the mother cell. By immunoblot, we observed somewhat higher levels of this

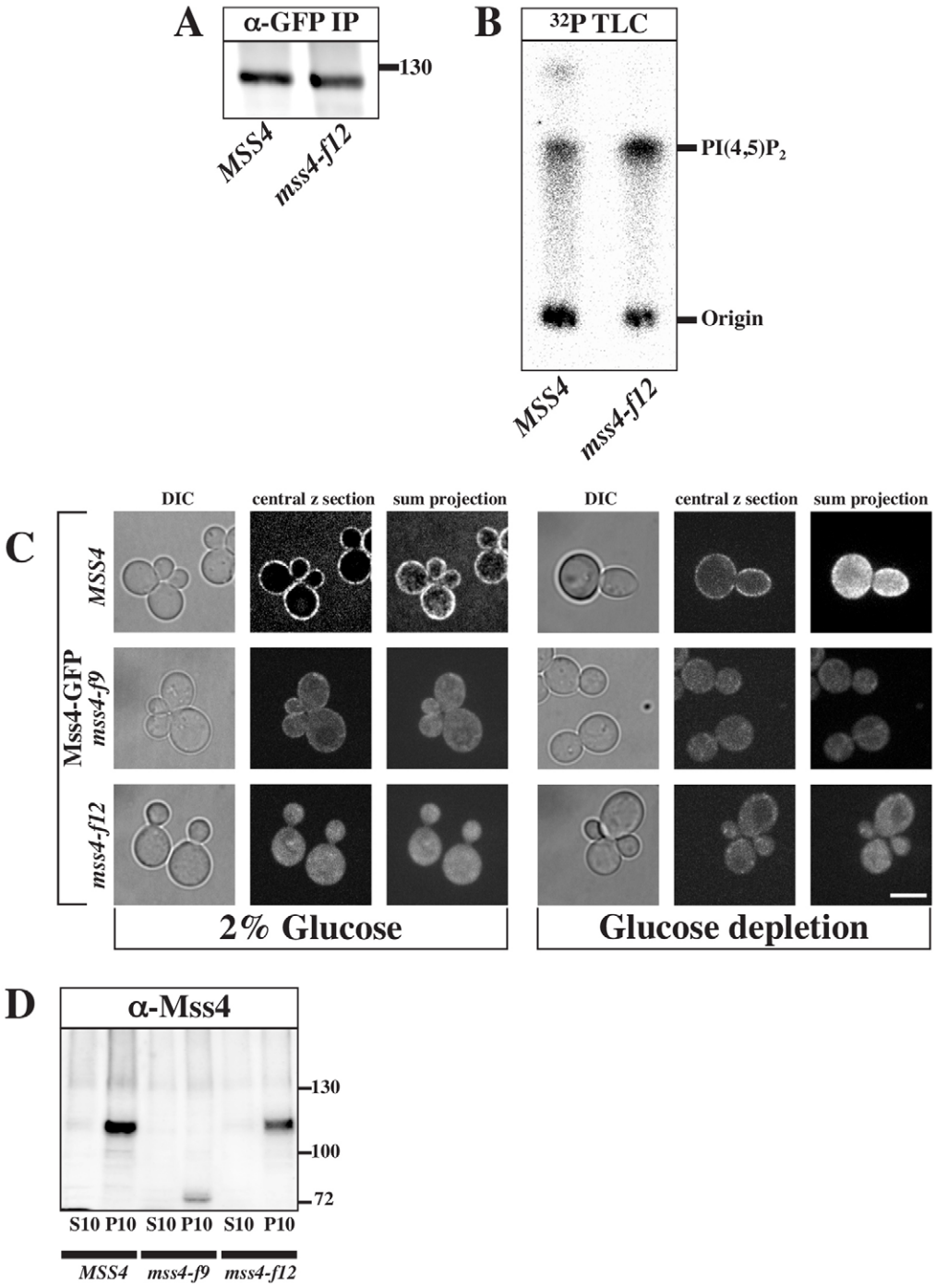

Fig. 6. Mss4-f12p is catalytically active *in vitro* and *in vivo* is defective in localization to the plasma membrane. (**A**) Extracts from cells expressing a sole copy of *MSS4* (either Mss4-GFP or mss4-f12-GFP) incubated with anti-GFP resin. An aliquot (10% of total) was analyzed as in Fig. 2A. (**B**) Mss4p or Mss4-f12p bound to anti-GFP resin was assayed for its ability to phosphorylate PtdIns(4)*P* using [γ-^{32}P]ATP. After a 60-min incubation at 30℃, samples were analyzed by TLC. Similar results were observed in five experiments. (**C**) Fluorescence images of yeast expressing as sole copy either Mss4-GFP, mss4-f9-GFP or mss4-f12-GFP grown in selective medium with 2% (left) or 0.2% glucose for 6–7 hr (right). DIC images and fluorescence image central *z*-sections and sum projections (10–12 *z*-sections) of representative cells are shown. Similar results were observed in three independent experiments. (**D**) Supernatants (10,000 ***g***) and pellets (10,000 ***g***) from yeast expressing the indicated Mss4p form as a sole copy were analyzed as in Fig. 2A. Similar results were observed in two experiments.

PtdIns(4,5)P_2 reporter in the *mss4-f12* mutant compared to wild-type *MSS4* cells (Fig. 5D). We used a tailor-made MATLAB program to analyze the signal concentration over long axis of the cell (Vernay et al., 2012) and Fig. 5E shows that in the wild-type strain there was an average 2-fold increase in PtdIns(4,5)P_2 concentration going from the back of the mother cell to the tip of the bud. In contrast, the *mss4-f12* mutant exhibited little polarized PtdIns(4,5)P_2 distribution with only an average ~25% increase in PtdIns(4,5)P_2 concentration over the cell long axis. From these sum projections, we also observed an increase in the level of the reporter in the *mss4-f12* cells. We determined the ratio of plasma membrane to cytoplasmic signal (from the central *z*-section) and wild-type cells had an average ratio of 1.98±0.57 (n=65 cells) compared to that of the mutant 1.46±0.48 (n=77 cells). Quantitation of the total signal (plasma membrane and intracellular) from central *z*-sections also revealed a slight increase in the reporter level in the *mss4-f12* mutant (average relative value for wild-type *MSS4* 137±37, n=65 cells; *mss4-f12* 156±43, n=77 cells). These results demonstrate that there is a redistribution of plasma membrane PtdIns(4,5)P_2 levels in *mss4-f12* cells resulting in reduced PtdIns(4,5)P_2 polarity.

Mss4p is essential for cell viability and cells expressing kinase defective mutants are not viable (Kobayashi et al., 2005; Ling et al., 2012). Cells expressing the *mss4-f12* mutant as the sole *MSS4* copy were viable, suggesting that this mutant retained catalytic activity. We used GFP fusions to Mss4p and Mss4-f12p to facilitate immuno-purification for *in vitro* kinase assays. C-terminal GFP fusions were expressed in a *mss4Δ* strain and the Mss4-f12–GFP strain exhibited a similar invasive growth defect as the *mss4-f12* mutants (supplementary material Fig. S4). Mss4–GFP fusions were purified from these strains and Fig. 6A shows that similar amounts of Mss4p and Mss4-f12p were immunoprecipitated. These agarose immobilized PI4P5Ks were then assayed for their ability to phosphorylate PtdIns(4)*P* using [γ-^{32}P]ATP. Fig. 6B shows that Mss4-f12p is catalytically active and phosphorylated PtdIns(4)*P* to a similar extent as Mss4p. We had difficulties reproducibly immunoprecipitating Mss4-f9–GFP, yet were able to detect low kinase activity, suggesting that this truncated Mss4p has some activity. These *in vitro* kinase activity assays indicate that mutation of the conserved serine residue at position 573 does not dramatically alter the catalytic activity of this kinase, yet nonetheless perturbs PtdIns(4,5)P_2 levels *in vivo*.

Localization of Mss4p mutants

Mss4p localizes to the plasma membrane and this localization is critical for *in vivo* PI4P5K activity (Homma et al., 1998; Audhya and Emr, 2003; Ling et al., 2012). To examine Mss4-f9p and Mss4-f12p localization, we used the functional GFP fusions (supplementary material Fig. S7) described above which were expressed from their endogenous promoter. We observed that Mss4–GFP localized to the plasma membrane in both rich and glucose depletion medium (Fig. 6C). Strikingly, we observed a reduction of Mss4-f9–GFP and Mss4-f12–GFP at the plasma membrane (Fig. 6C). In contrast to Mss4-f12–GFP, some Mss4-f9–GFP was observed at the plasma membrane. We carried out crude fractionation to determine whether the Mss4-f9 and Mss4-f12 proteins were soluble. Fig. 6D shows that the majority of the wild-type and mutant Mss4 proteins were found in a 10,000 ***g*** pellet with little protein found in the soluble 10,000 ***g*** supernatant. Localization and fractionation of Mss4-f9p indicate that there is less of this protein. Our results indicate that the Mss4-f12p mutant is not targeted or maintained at the plasma membrane, despite being associated with cellular membranes.

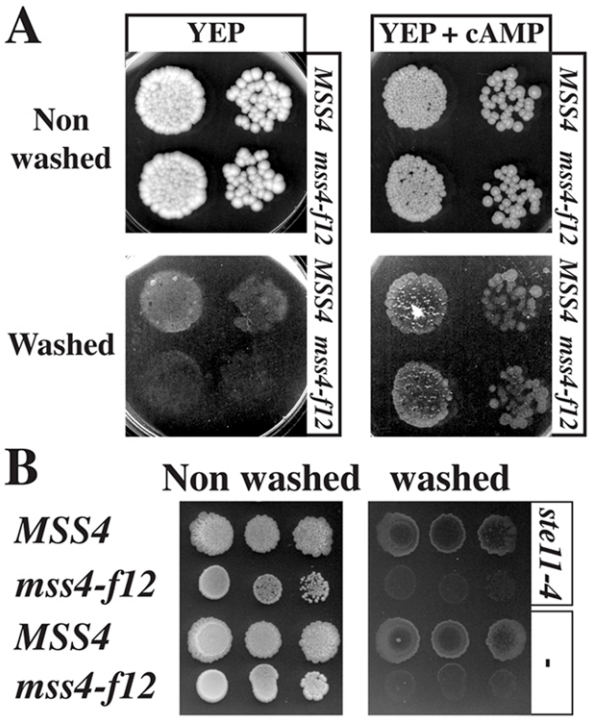

Fig. 7. Exogenously added cAMP restores invasive growth in *mss4-f12* mutant whereas expression of the activated MAPKKK Ste11p does not. (**A**) Serial dilutions of the indicated strains were spotted on YEP medium in the presence or absence of cAMP (5 mM) and, after 5 days, cells on the surface of the agar were washed off as in Fig. 1B. Similar results were observed in three independent experiments and representative images are shown. (**B**) The indicated strains expressing either the activated *ste11-4* allele or empty vector were grown in selective medium, serial dilutions were spotted on medium containing 0.2% glucose and after 5 days growth, surface cells were washed off as in Fig. 1B. Similar results were observed with two transformants in three independent experiments.

Exogenous cAMP addition, but not activated MAPKKK Ste11p, restores the invasive growth in the *mss4-f12* mutant

The induction of Flo11p is dependent on two signaling pathways, the Kss1p MAPK pathway and cAMP-dependent PKA pathway (Rupp et al., 1999; Brückner and Mösch, 2012; Cullen and Sprague, 2012). To determine if Mss4p functioned upstream of the cAMP dependent pathway we examined whether the addition of exogenous cAMP was sufficient to restore the invasive growth defect of *mss4-f12* cells. Fig. 7A shows that medium containing 5 mM cAMP, was sufficient to restore invasive growth, similar to that previously observed for a *gpa2* mutant (Kübler et al., 1997). We next examined whether expression of the activated MAPKKK allele, *ste11-4*, could also restore invasive growth of the *mss4-f12* mutant. Fig. 7B shows that *mss4-f12* cells expressing *ste11-4* were still defective in agar invasion. Quantitation of the morphology of *MSS4* and *mss4-f12* cells expressing *ste11-4*, revealed that these cells were larger, displayed aberrant morphologies (long axis of *MSS4* cells 5.7±0.8 μm, n=137; *mss4-f12* cells 6.2±0.8 μm, n=84; *MSS4*

ste4-11 cells 8.1±1.7 μm, $n=109$; *mss4-f12 ste4-11* cells 7.2±1.0 μm, $n=103$) and grew less well. Together these results suggest that Mss4p functions upstream of the cAMP-dependent PKA pathway but not the Kss1p MAPK pathway.

Discussion

We have isolated a specific *mss4* mutant that is defective for invasive growth in low nutrient conditions. The *mss4-f12* mutant exhibits cell wall integrity defects, yet distribution of active Cdc42p, the polarisome component Spa2p, the exocyst subunit Sec3p and the septin subunit Cdc3p are largely unaffected. Upon glucose depletion, we show that *mss4* mutant cells do not elongate and are substantially defective in *FLO11* induction as well as adhesion. This *mss4-f12* allele has a highly conserved serine residue in the kinase domain changed to a proline residue. We show that Mss4-f12p is catalytically active *in vitro*; however, in *mss4-f12* cells we observed reduced levels of PtdIns(4,5)P_2 and an accumulation of the complex sphingolipid, M(IP)$_2$C. Using a reporter to visualize PtdIns(4,5)P_2 *in vivo*, we demonstrate that *mss4-f12* cells have reduced levels of plasma membrane PtdIns(4,5)P_2 as well as a defect in its polarized distribution. In contrast to the distribution of Mss4p, we did not observe the Mss4-f12 protein at the plasma membrane, which nonetheless appeared to be associated with a membrane fraction. Together our results indicate that plasma membrane PtdIns(4,5)P_2 is critical for yeast invasive growth and suggest that this phospholipid functions upstream of the cAMP-dependent PKA signaling pathway required for invasive growth.

From over 12,000 mutagenized copies of *mss4*, we isolated 10 mutants and confirmed the invasive growth defect in four. Two mutants had an arginine at position 590 replaced by a stop codon and two mutants had a serine residue at position 573 changed to a proline residue. The serine 573 in Mss4p is conserved in all phosphatidylinositol phosphate kinases. Based on the crystal structure of the human type IIβ phosphatidylinositol phosphate kinase, the equivalent residue is part of the kinase catalytic core and is less than 8 Å from residues involved in binding to ATP and phosphatidylinositol phosphate as well as a critically conserved aspartic acid residue that functions as a weak base in kinase catalysis (Rao et al., 1998). However our results indicate that the *in vitro* kinase activity of Mss4-f12p is similar to that of the wild-type. The positively charged surface of PI4P5Ks together with the substrate-binding region has been proposed to function as a coincidence detector that targets phosphatidylinositol phosphate kinases to the plasma membrane (Fairn et al., 2009). Interestingly, the Mss4-f12p mutant did not localize to the plasma membrane, although serine 573 is not in proximity to residues that have been proposed to be important for membrane binding (Rao et al., 1998; Arioka et al., 2004). We propose that the three positively charged residues that form a patch on the surface of the structure of the human type IIβ phosphatidylinositol phosphate kinase adjacent to the equivalent of the *S. cerevisiae* Mss4p Ser 573, i.e. *Hs* Arg 134, Lys 218 and Arg 224 (equivalent to Lys 480, Lys 571 and Arg 577 in *S. cerevisiae* Mss4p) are involved in plasma membrane binding or stabilization at this membrane.

Our results suggest that sufficient plasma membrane PtdIns(4,5)P_2 is critical for haploid invasive growth in *S. cerevisiae*. The phosphatidylinositol-specific phospholipase C (Plc1p), which hydrolyzes PtdIns(4,5)P_2 to IP$_3$ and DAG, is important for pseudohyphal filamentous growth (Ansari et al., 1999). Plc1p binds both the sugar-sensing G-protein-coupled receptor Gpr1p and Gα subunit Gpa2p (Ansari et al., 1999). The pseudohyphal filamentous growth defect, in nitrogen depletion conditions, of the *plc1* deletion mutant is rescued either by activating the MAPK or the cAMP-dependent PKA pathway. Plc1p is also required for induction of the transcriptional reporter *FG(TyA)::lacZ* expression during nitrogen depletion. Perhaps sufficient plasma membrane PtdIns(4,5)P_2 is required during filamentous growth to generate IP$_3$ and DAG that may be required for signaling. The *mss4-f12* mutant grew poorly on non-fermentable carbon sources, which is consistent with the growth defect of the *plc1Δ* mutant (Flick and Thorner, 1993). However, this *mss4* mutant grows at 37℃, in contrast to the *plc1Δ* mutant (Flick and Thorner, 1993), making unlikely a complete loss of Plc1p activity. Alternatively it is possible that plasma membrane PtdIns(4,5)P_2 is important for targeting Plc1p to this membrane; however, we have been unable to detect Plc1p at the plasma membrane (unpublished observations). Addition of exogenous cAMP, but not expression of an activated MAPKKK allele *ste11-4*, restored invasive growth in the *mss4-f12* mutant, similar to what was observed with a *gpa2* mutant (Kübler et al., 1997). These results are consistent with Mss4p functioning upstream of the cAMP-dependent PKA pathway.

Phosphatidylinositol (PI) is a precursor for PIPs and sphingolipids. Growth defects due to inhibition of sphingolipid biosynthesis can be suppressed by Mss4p overexpression, yet levels of complex sphingolipids are unaffected (Kobayashi et al., 2005); however, they are reduced in $stt4^{ts}$ and $mss4^{ts}$ strains (Tabuchi et al., 2006). Furthermore, the calcium sensitive phenotype of the *csg2* complex sphingolipid biosynthesis mutant was suppressed by a $mss4^{ts}$ mutation (Dunn et al., 1998). In contrast, inhibition of the plasma membrane PI4-kinase Stt4p by wortmannin resulted in an increase in complex sphingolipids (Brice et al., 2009). Similarly in the *mss4-f12* mutant we observed reduced levels of PtdIns(4,5)P_2 and an accumulation of the complex sphingolipid, M(IP)$_2$C. Furthermore, inhibition of complex sphingolipid biosynthesis results in an increase in plasma membrane PtdIns(4)*P* and PtdIns(4,5)P_2 (Jesch et al., 2010), suggesting that the increased PI levels observed in the *mss4-f12* mutant are likely to contribute to increased complex sphingolipid levels. This common phospholipid is likely to underlie some of the connections between these two lipid biosynthesis pathways (Henry et al., 2012).

Sustained growth in one direction is required for cells to elongate, such as those observed in filamentous yeast and plant pollen tubes. The PI4P5K Mss4p is critical for filamentous growth in *Candida albicans* (Vernay et al., 2012) and *Neurospora crassa* (Seiler and Plamann, 2003; Mähs et al., 2012). In the pathogenic fungi *C. albicans*, Mss4p is critical for invasive filamentous growth and, in particular, the switch between the yeast and filamentous forms (Vernay et al., 2012). In *N. crassa*, *mss4* mutants were identified in a screen for hyphal morphogenesis defects and exhibited general polarity defects (Seiler and Plamann, 2003). Two ts mutants from this screen both have the conserved Tyr at the equivalent of position 744 in *S. cerevisiae* Mss4p substituted with an Asn, resulting in a complete loss of activity of this *Neurospora* PI4P5K (Mähs et al., 2012). In tobacco and *Arabidopsis*, PI4P5Ks are critical for pollen tube growth and polarity (Ischebeck et al., 2008; Sousa et al., 2008; Ischebeck et al., 2010; Ischebeck et al., 2011; Stenzel et al., 2011). Perturbation of this class of lipid kinase results in altered morphologies and reduced pollen tubes elongation (Ischebeck et al., 2008; Sousa

et al., 2008; Ischebeck et al., 2010; Ischebeck et al., 2011; Stenzel et al., 2011). Together with our findings that Mss4p is required for invasive growth in *S. cerevisiae*, suggest that PtdIns(4,5)P_2 plays an important role in sustained unidirectional growth, likely via extensive membrane traffic. Indeed, our studies in *C. albicans* reveal that a dramatic gradient of PtdIns(4,5)P_2 emanates from the tip of the hyphal filament in wild-type cells.

Our results indicate that the *mss4-f12* mutant has reduced levels of plasma membrane PtdIns(4,5)P_2. However, we did not observe Mss4-f12p on the plasma membrane raising the possibility that PtdIns(4,5)P_2 is generated in an intracellular compartment, some of which ends up at the plasma membrane. In addition the polarized distribution of this phospholipid in budding *mss4-f12* cells is perturbed, yet we did not observe a substantial alteration in the distribution of four cell polarity proteins. Previous studies using either of the PtdIns(4,5)P_2 reporters, GST–GFP–PH$^{Plc\delta}$ or GFP–PH$^{Plc\delta}$–PH$^{Plc\delta}$, did not detect a substantial enrichment of PtdIns(4,5)P_2 in small buds (Stefan et al., 2002; Garrenton et al., 2010); however, an accumulation of this lipid was observed at the bud neck (Garrenton et al., 2010). Using the GFP–PH$^{Plc\delta}$–PH$^{Plc\delta}$–GFP reporter we observed enrichment of PtdIns(4,5)P_2 throughout the plasma membrane of small buds with 2–3-fold the concentration of PtdIns(4,5)P_2 in the bud compared to the back of the mother cells. Previous studies have shown using the inositolpolyphosphate 5-phosphatase synaptojanin Sjl2p and the ANTH domain from Sla2p to visualize PtdIns(4,5)P_2 that this phospholipid is localized to endocytosis sites which are concentrated in small buds (Sun et al., 2007; Brach et al., 2011). The loss of PtdIns(4,5)P_2 polarity in the *mss4-f12* cells is consistent with the suggestion that membrane traffic via endocytosis and exocytosis is important for invasive growth.

In summary, our results suggest that PtdIns(4,5)P_2 is important for invasive growth in *S. cerevisiae* and that this phospholipid is a critical regulator for cell elongation in highly polarized cells.

Materials and Methods

Growth conditions, strains and plasmids

Standard techniques and medium were used for yeast growth and genetic manipulation (Rose et al., 1991). Media was prepared as described (Gimeno et al., 1992; Casalone et al., 2005). Strains, primers and plasmids used are described in supplementary material Tables S1–S3.

The *MSS4* ORF was amplified from genomic DNA using Mss4pBamHI/Mss4mNotI. The resulting 2340 bp product was cloned into pRS416 under the control of *GAL1-10* promoter (resulting in p416GALpMSS4) and pCM188 (Garí et al., 1997) 3′ of the tetracycline regulatable promoter (resulting in pCM188TetpMSS4). The *MSS4* ORF with 704 bp 5′ of the ATG codon was amplified using Mss4pSalI/Mss4mNotI and the resulting 3044 bp product was cloned into pRS414 and pRS415 yielding p414MSS4pMSS4 and p415MSS4pMSS4.

A C-terminal Mss4–GFP fusion was constructed by inserting a unique AatII restriction site in place of the stop codon (using site directed mutagenesis with Mss4pAatIInostop/Mss4mAatIInostop, resulting in p414MSS4pMSS4-AII) and subsequently cloning (Gly-Ala)$_5$–yeGFP-ADH1$_{term}$ (PCR amplified from p416GalCdc24HAGFP with AatII and NotI sites at the 5′ and 3′ ends using AatIIpGAyeGFP/ADHtNotI) into p414MSS4pMSS4-AII, yielding p414MSS4pMSS4GFP. p414MSS4pmss4f9GFP was created by inserting a unique AatII replacing the two codons which coded for R590/S591 by site-directed mutagenesis in p414MSS4pMSS4GFP using primers Mss4pAatII1767/Mss4mAatII1767 resulting in p414MSS4pMSS4-590AII-GFP. This *MSS4* plasmid, which contained two AatII sites, was digested with AatII, removing the 3′ region after R590 and subsequently religated, resulting in p414MSS4pmss4f9GFP. p414MSS4pmss4f12GFP was created by site-directed mutagenesis using the plasmid p414MSS4pMSS4GFP with Mss4C12pT1717C/Mss4C12pT1717C.

To visualize plasma membrane PtdIns(4,5)P_2 a XhoI/NotI GFP–PH$^{Plc\delta}$–PH$^{Plc\delta}$–GFP fragment optimized for expression in *C. albicans* (Vernay et al., 2012) from pExpARG-pADH1GFP-PH$^{Plc\delta}$-PH$^{Plc\delta}$-GFP was cloned into pRS406 with a *GAL1-10* promoter inserted at the XhoI site. This resulting plasmid, p406GALpGFP-PH$^{Plc\delta}$-PH$^{Plc\delta}$-GFP was linearized with StuI and transformed into wild-type (RAY1990) and *mss4-f12* strains (RAY1991). Two independent transformants of each strain (RAY2001/2003 and RAY1993/1999) with this reporter integrated at the *URA3* locus were analyzed for invasive growth and GFP expression by immunoblot and fluorescence microscopy.

The *mss4* deletion mutants were constructed by PCR-based gene disruption as described using Mss4pKO/Mss4mKO and the pBSLoxPHis5SpLoxP plasmid (Arkowitz and Lowe, 1997; Nern and Arkowitz, 1998) and transformed into the 10560-6B strain carrying p416GALpMSS4. Four unique, silent restriction sites were introduced by site-directed mutagenesis in p414MSS4pMSS4, NheI (bp −3; Mss4pNheI-3/Mss4mNheI-3), BglII (bp 607; Mss4pBglII607/Mss4mBglII607), AatII (bp 1401; Mss4pAatII1401/Mss4mAatII1401), XhoI (bp 1807; Mss4pXhoI1807/Mss4mXhoI180) resulting in p414MSS4pMSS4*.

To generate random *mss4* mutants, the region between the unique AatII and XhoI sites (bp 1401–1807) was mutagenized by error prone PCR using p414MSS4pMSS4* as a template and an unbalanced dNTP mixture (5.8 mM dATP, 5 dCTP, 10.5 mM dGTP and 72.5 mM dTTP) (Barale et al., 2006). The AatII/XhoI fragment was then cloned into p414MSS4pMSS4*. Subsequently this library was transformed into RAY1885 and transformants were selected on SC-Trp glucose plates, which repressed *MSS4* expression. Colonies were then cultured overnight in YEPD in 96 well plates and subsequently spotted onto solid YEPD and YEP, incubated for 5 to 10 days. Invasive growth was assessed by washing the plates with a gloved hand, as described (Roberts and Fink, 1994; Guldal and Broach, 2006), and was compared to a control strain. Colonies were screened for invasive growth defects. In mutants that exhibited reduced invasive growth the p416GALpMSS4 plasmid was removed by 5-FOA counter-selection. The invasive growth phenotypes of the mutants were subsequently confirmed on YEPD, YEP and SLAD medium. The *mss4-f9* and *mss4-f12* mutant alleles were recreated by site-directed mutagenesis using the plasmid p414MSS4pMSS4 and primers mss4C9pA1768T/mss4C9mA1768T and Mss4C12pT1717C/Mss4C12mT1717C, resulting in p414MSS4pmss4f9 and p414MSS4pmss4f12, respectively. The region 3′ of the stop codon in the *mss4-f9* mutant was removed by insertion of two AatII sites (one replacing codons encoding for R590/S591 and a second 5′ of the stop codon) by site-directed mutagenesis, digestion with AatII to remove the 3′ region after R590 and subsequent religation resulting in p414MSS4pmss4f9Δ. Individual *mss4* point mutants were made by site-directed mutagenesis. All PCR-amplified products and mutants were confirmed by sequencing.

RAY1885 was transformed with p414MSS4pMSS4, p414MSS4pMSS4GFP, p414MSS4pmss4f9, p414MSS4pmss4f9Δ, p414MSS4pmss4f12, p414MSS4pmss4f9GFP and p414MSS4pmss4f12GFP plasmids, the rescuing plasmids were removed with 5-FOA and the invasiveness defects were examined on YEPD, YEP and SLAD medium. The invasive growth defect of these strains was complemented by the p415MSS4pMSS4 plasmid.

Diploid strains (RAY1945 and RAY1949) were generated using Gal inducible HO endonuclease to switch the mating type of a 10560-6B *mss4-Δ1::HIS5Sp* strain carrying p415MSS4pMSS4. After verification of a *MAT**a*** isolate by response to pheromone and PCR (Huxley et al., 1990) followed by loss of the pGal-HO plasmid, diploids were generated by mating with RAY1940 and subsequent loss of p414MSS4pMSS4. This diploid was transformed with p414MSS4pMSS4 or p414MSS4pmss4f12 resulting in RAY1945 and RAY1949, respectively. These diploids were sporulated, spores enriched and *MAT**a*** isolates identified (RAY2012-2015).

Antibodies and immunoblotting

The *MSS4* ORF was PCR amplified using p416GALMSS4 as a template with Mss4pSalI/Mss4mNotI. The 1922 bp PCR product was digested with SpeI/NotI and the 421 bp fragment cloned into the pGEX-6P plasmid, resulting in pGEX-6PMSS4. The GST fusion protein was expressed in *E. coli* BL21 cells grown at 37°C and induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside for 3 hr. Cells were harvested, resuspended in PBS, lysed by sonication (5×30 sec) and centrifuged 15 min at 10,000 rpm at 4°C. The supernatant was then added to GSH–Sepharose 4B resin (Amersham Biosciences), which was subsequently washed with PBS containing 1 mM PMSF and protease inhibitor mixture (Roche Applied Science). GST–Mss4p was eluted with 50 mM Tris-HCl, pH 8, 10 mM GSH, purity confirmed by SDS-PAGE and rabbits were immunized. For immunoblots, anti-Mss4p (polyclonal; 1:1000), anti-GFP (polyclonal; 1:1000) (Nern and Arkowitz, 2000b) and anti-GAPDH (monoclonal; 1:10,000) were used. Following incubation with peroxidase-conjugated secondary antibodies (Bio-Rad), bands were visualized by enhanced chemiluminescence (luminol-coumaric acid) on an imaging system (Las3000; Fujifilm).

Invasive growth, adhesive growth, cell wall integrity and pheromone-response assays

Exponentially growing cell cultures were diluted to 0.1 or 0.5 OD_{600}/ml and spotted on YEPD, YEP or SLAD (Gimeno et al., 1992) solid medium either in the presence or absence cAMP (5 mM) for invasive growth assays or spotted on SC-Trp solid medium either in the presence or absence of 10 mM 3-amino-triazole (3AT). After 5 days incubation, cells on the surface were washed off as described (Roberts and Fink, 1994; Guldal and Broach, 2006) for invasive growth and for

adhesive growth assays, surface cells were washed off with a gentle stream of H_2O as described (Braus et al., 2003; Guldal and Broach, 2006). Growth in the presence of Calcofluor white and Congo red was carried out as described (Ram and Klis, 2006). Induction of a Fus1LacZ reporter was assayed as described (Nern and Arkowitz, 1998; Nern and Arkowitz, 1999). Quantitative matings were carried out as described (Nern and Arkowitz, 1998; Nern and Arkowitz, 1999) with the wild-type (RAY876) and enfeebled (RAY1563) tester strains.

FLO11–LacZ assays

Strains carrying the YEp355 FLO11-LacZ plasmid (B3782; Rupp et al., 1999) were grown overnight in SC-His-Trp-Ura medium containing glucose. Exponentially growing cells (10 OD_{600}) were transferred to medium (20 ml) with or without glucose. After 7 hr incubation, NaF and NaN_3 (10 mM final concentration each) were added to cultures. LacZ activity determinations were carried out with culture extracts as described (Rose and Botstein, 1983) and activity was normalized to the total protein (Bradford, 1976).

Immunoprecipitation and kinase assays

*Mss4*Δ strains (RAY1940 and RAY1942) carrying p414MSS4pMSS4GFP and p414MSS4pmss4f12GFP were grown exponentially in SC-Trp medium and cells (50 OD_{600}) were harvested by centrifugation. All subsequent steps were carried out at 4°C. Cells were lysed by agitation with glass beads in buffer A (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 40 µg/ml each of leupeptin, chymostatin, pepstatin A, aprotinin, and antipain) containing 0.1% Triton X-100. Immunoprecipitation was carried out as described (Nern and Arkowitz, 1999) using 10,000 ***g*** clarified cell extracts and anti-GFP IgG-Sepharose (from protein A Sepharose [Pharmacia] incubated with anti-GFP sera and then cross-linked with dimethylpimelimidate). Anti-GFP IgG-Sepharose (20 µl) was incubated for 1 hr with clarified cell extracts (1 mg protein) followed by four washes with buffer A containing 0.1% Triton X-100. The amount of immunoprecipitated Mss4p was quantified by SDS-PAGE followed by immunoblot and detection with anti-Mss4p sera. The remaining resin was used for kinase assays as described (Desrivières et al., 1998; Homma et al., 1998). Kinase buffer (40 µl of 50 mM Tris-HCl pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 50 µM ATP) was added to resins and assays were initiated by addition of 5 µCi [γ-^{32}P]ATP and 80 µM of PtdIns(4)*P*. After 1 hr at 30°C reactions were stopped by addition of $CHCl_3$/MeOH (187 µl 1:2 v/v). The lipids were extracted by addition of 2.4 M HCl (45 µl) and $CHCl_3$ (190 µl). The lower phase was removed and washed with 184 µl of 1 M HCl/MeOH/$CHCl_3$ (47:48:3 v/v/v) and then dried. Lipids were analyzed by TLC (Reggiori et al., 1997) on silica gel 60 plates (Merck) impregnated with 1.2% potassium oxalate and dried at 100°C for 1 hr. Samples were separated with a $CHCl_3$/MeOH/$(CH_3)_2CO$/CH_3COOH/H_2O (42:30:12:12:12 v/v/v/v/v) solvent system. Autoradiography was carried out and radioactivity was quantified using a phosphoimager (FujiBAS 1000; Fujifilm).

Phosphatidylinositol labeling

In vivo labeling was performed essentially as described (Desrivières et al., 1998). Exponentially growing cultures (20 OD_{600}) (RAY1986 or RAY1991) grown in SC-Trp-His were resuspended in fresh medium (10 OD_{600}/ml) and incubated with shaking for 30 min. [^{3}H]Inositol (20 µCi) was added to each culture prior to incubation for an additional 40 min, followed by addition of 6 ml of pre-warmed medium and then another 80 min incubation. NaF and NaN_3 (10 mM final concentration each) were then added to each culture. Lipid extraction and analysis was carried out as described (Guillas et al., 2001). Immediately after NaF/NaN_3 addition, cells were harvested by centrifugation for 5 min at 3000 rpm, resuspended in ice cold water, and centrifuged for 5 min at 10,000 rpm. Cells were then suspended in 400 µl of ice cold $CHCl_3$/MeOH (1:1 v/v) and disrupted by vortexing with glass beads for 5×1 min. Following centrifugation for 5 min at 10,000 rpm, the supernatant was recovered. Pellets extracted twice more with 400 µl $CHCl_3$/MeOH/H_2O (10:10:3 v/v/v). The organic phases were pooled and dried using a speed vac. Lipids were subsequently desalted by partitioning between *n*-butanol and water followed by a back extraction of the butanol phase with water (Krakow et al., 1986). The desalted lipids were then analyzed using 0.2 mm silica gel TLC plates ($CHCl_3$/MeOH/H_2O; 55:45:10 v/v/v solvent system). Radioactivity was detected and quantitated by fluorography (Benghezal et al., 1995). For phosphate labeling, [^{33}P]$H_2PO_4^-$ was used at 1 µCi/OD_{600} cells and lipid extraction carried out similarly. Lipids were analyzed by TLC ($CHCl_3$/MeOH/25% NH_4OH/H_2O; 90:70:4:16 v/v/v/v) (Munnik et al., 1996).

Microscopy and image analyses

Differential interference contrast images were acquired with a Leica DMR wide-field microscope (NA 1.32×63 Plan-Apo objective) and colony morphology images were acquired with a Leica MZ6 dissection scope (×10 magnification) (Bassilana et al., 2005). Images were captured with a Princeton Instruments Micromax charge coupled device camera or an Andor Technology Neo sCMOS camera using IPLab (Scanalytics Inc.) v3.5 software or Solis (Andor Technology) v4 software, respectively. The length of the major cell axis was determined by fitting an ellipsoid to the mother cell using ImageJ. Actin was visualized using Alexa-Fluor-488–phalloidin (Pringle et al., 1989) on a DeltaVision deconvolution microscopy (Olympus IX-70) system (Applied Precision) with a NA 1.4 ×60 objective. Images (30×0.15 µm *z*-sections) were deconvolved using softWoRx software and maximum projections generated. All GFP and RFP images (except PtdIns(4,5)P_2 analyses) were acquired on an Andor Technology Revolution-XD spinning-disk laser confocal microscope (UplanSApo 1.4 NA 100× objective) controlled by v2 iQ2 software with 488-nm or 561-nm laser excitation and 140×140×400 nm voxel size (Vernay et al., 2012). Sum projections were generated by ImageJ. Bars are 5 µm.

For PtdIns(4,5)P_2 distribution, wild-type and *mss4-f12* cells were grown overnight at in SC-ura medium containing Fructose (4%) and Galactose (4%), back-diluted the following day into same medium containing Galactose and grown for an additional 6 hr. Cells were washed with PBS prior to analyses on a Zeiss LSM 510 META confocal (Axiovert 200M microscope, Plan-Apo 1.4 NA 63× objective and 488-nm laser excitation) (Bassilana et al., 2005). Voxel size was 140×140×700 nm and the pinhole was set to 1 airy unit.

The semi-automatic MATLAB program, BudPolarity (BP) was used as described (Vernay et al., 2012). Three-dimensional images were converted into 2D images by sum projection. Cell morphology was defined by an entered intensity threshold and the major axis of each cell was determined by an ellipsoid fit. Fluorescence intensity is integrated along the major axis (normalized) to follow concentration. Another Matlab program (HyphalPolarity) was used to determine average intensities of the plasma membrane and cytoplasm (all signal interior to the plasma membrane excluding the vacuole) from the central *z*-section for each cell (Vernay et al., 2012).

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Author contributions

I.G. and R.A.A. conceived and designed the experiments. I.G., A.V., J.-J.V. and R.A.A. performed the experiments. I.G., A.V. and R.A.A. analyzed the data. I.G. and R.A.A. wrote the manuscript.

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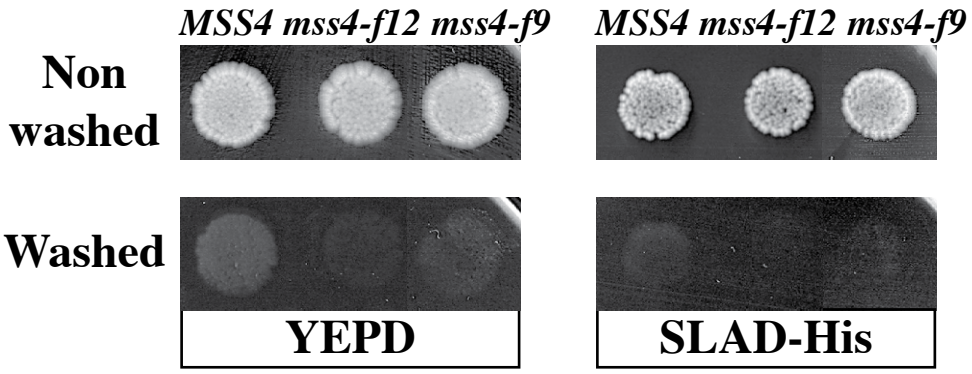

Fig. S1. The *mss4-f9* and *mss4-f12* mutants are defective for haploid invasive growth in glucose and low ammonia media. Indicated strains were spotted on YEPD and SLAD-His media and grown for 5 days. Cells on the surface of the agar were washed off revealing cells that had invaded the agar surface. Spots are from same agar plate. Similar results were observed in 2 independent experiments and representative images are shown.

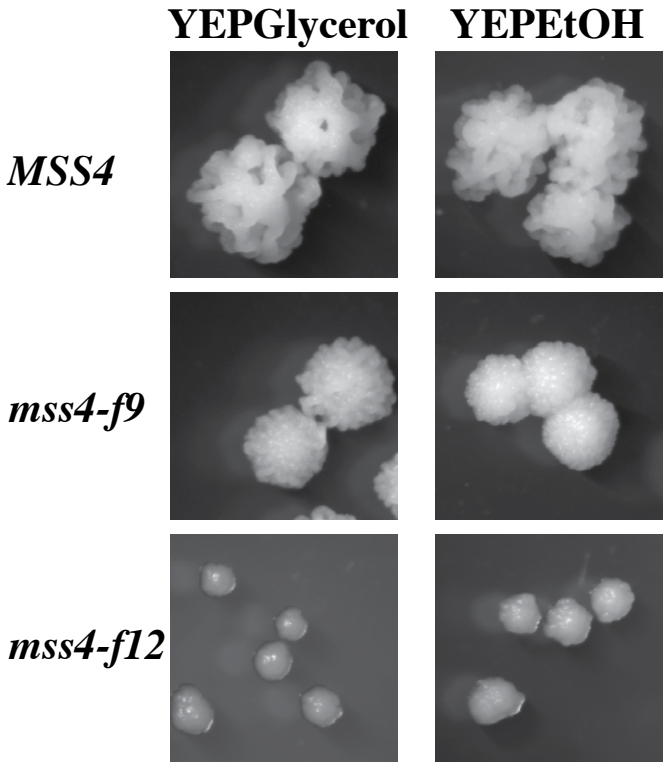

Fig. S2. *mss4* mutants are defective in colony crenelation on glycerol and EtOH containing media. Enlarged images of colonies of indicated strains grown on YEPGlycerol and YEPEtOH media for 5 days are shown. Similar results were observed in 3 independent experiments and representative images are shown.

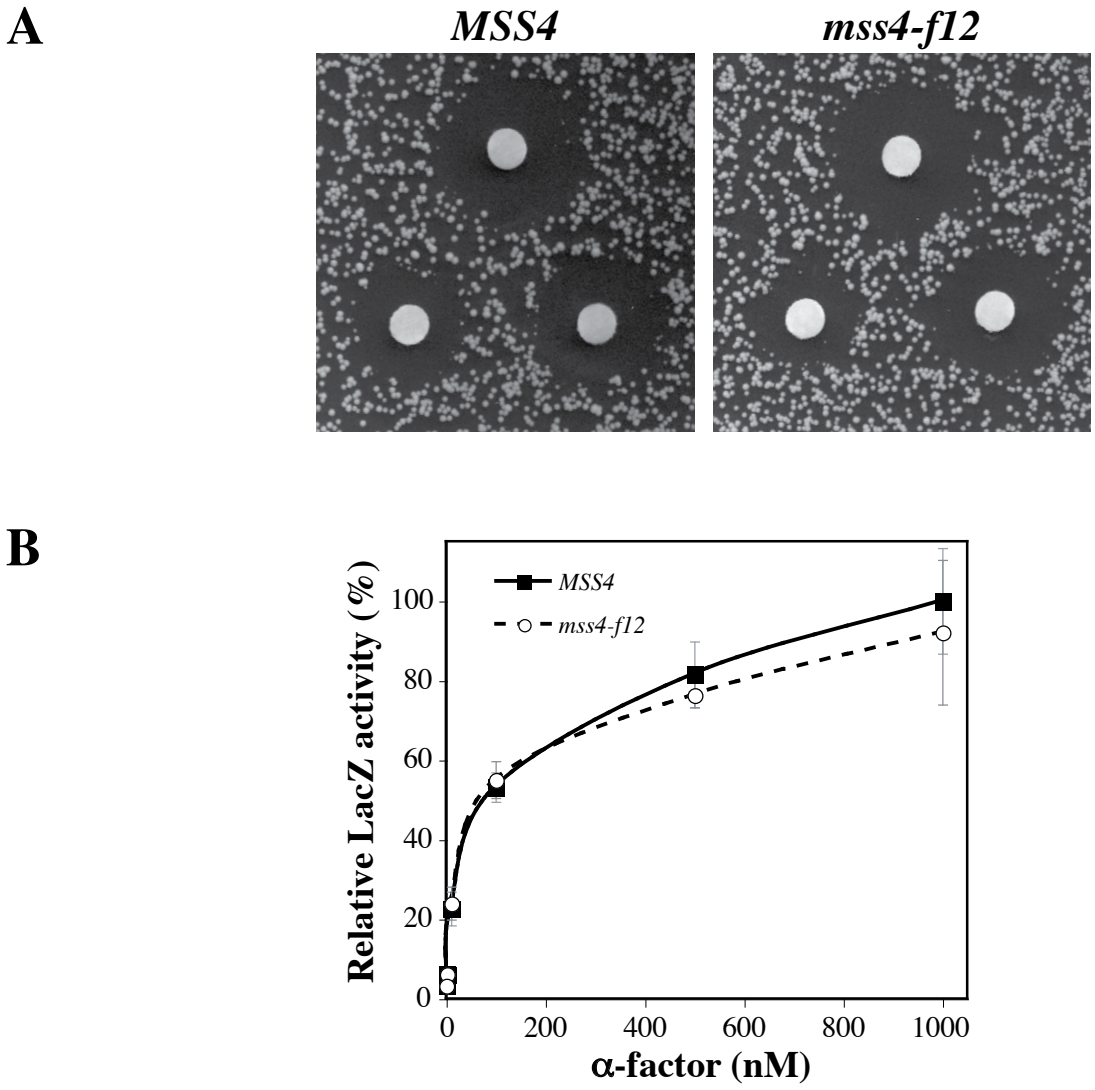

Fig. S3. *mss4-f12* cells respond to mating pheromone. *mss4-f12* cells arrest growth in the presence of mating pheromone similar to wild-type cells. α-factor (1, 0.5, and 0.2 μg) was spotted on filters placed on a lawn of the indicated *MAT**a*** strain. Plates were incubated for 2 days. Measurements of the halo diameter indicated <5% difference between *mss4-f12* and *MSS4* halos. Similar results were observed in 2 independent experiments with 2 independent *mss4-f12* and *MSS4* strains. B) *mss4-f12* cells induce the mating-specific *FUS1* gene similar to wild-type cells. Cells containing a *FUS1-lacZ* plasmid (pSG231) were incubated with the indicated α-factor concentration for 1 h and LacZ activity was determined. Values were normalized to the wild-type treated with 1000 nM α-factor (0.48 β-galactosidase units [μg protein]$^{-1}$ min^{-1}) and are the averages of two independent experiments each with two clones, each in duplicate and triplicate determinations, s.d. indicated.

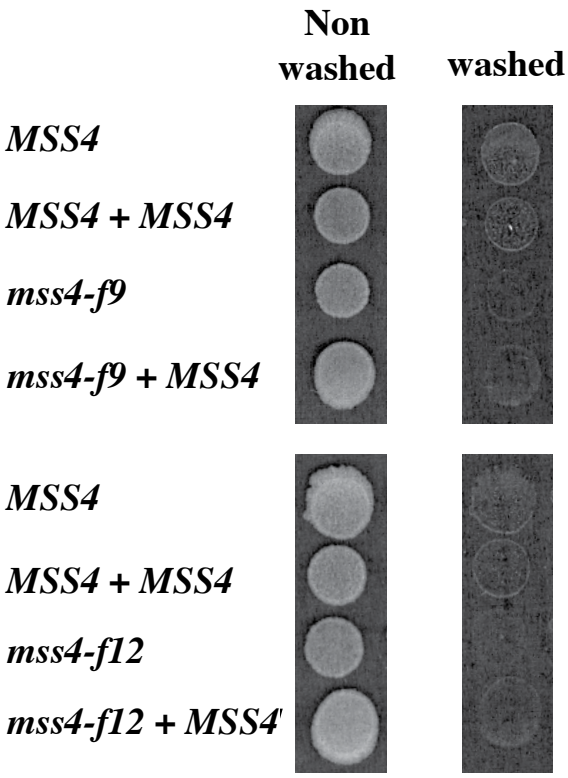

Fig. S4. *Mss4* mutant invasive growth defect is recessive. Complementation of the invasive growth defect was examined using an *mss4*Δ strain carrying either *MSS4*, *mss4-f9* or *mss4-f12* (RAY1986, RAY1991 and RAY2005, respectively) with or without the p415MSS4pMSS4. Indicated strains were grown on YEPD media for 5 days and invasive growth was assessed as in Fig. S1. Similar results were observed in 2 independent experiments and representative images are shown.

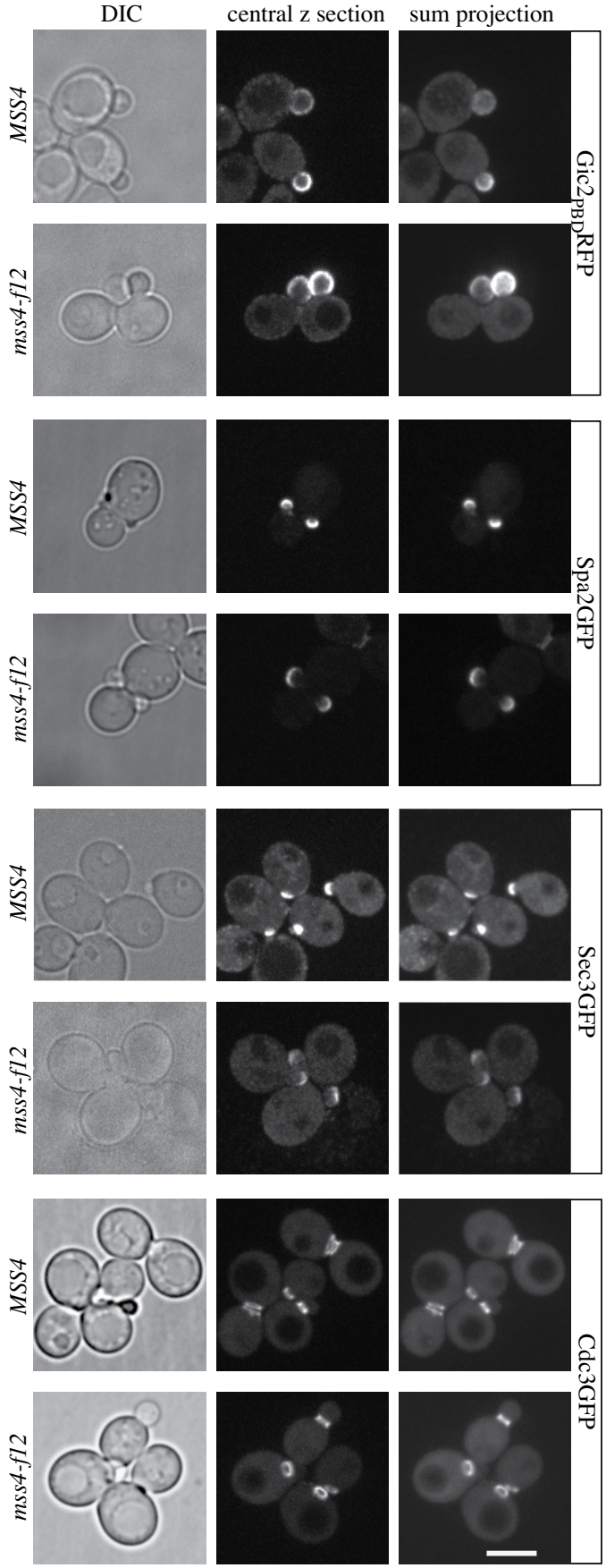

Fig. S5. Active Cdc42p, polarisome, exocyst and septin subunits localization is unaffected in *mss4-f12* cells. Indicated strains expressing the indicated RFP/GFP fusion protein were grown in selective media with 2% glucose. Spinning disk confocal fluorescence and DIC images were taken and central z-sections and sum projections (8-12 z-sections) of representative cells are shown. Similar results were observed in 3 independent experiments.

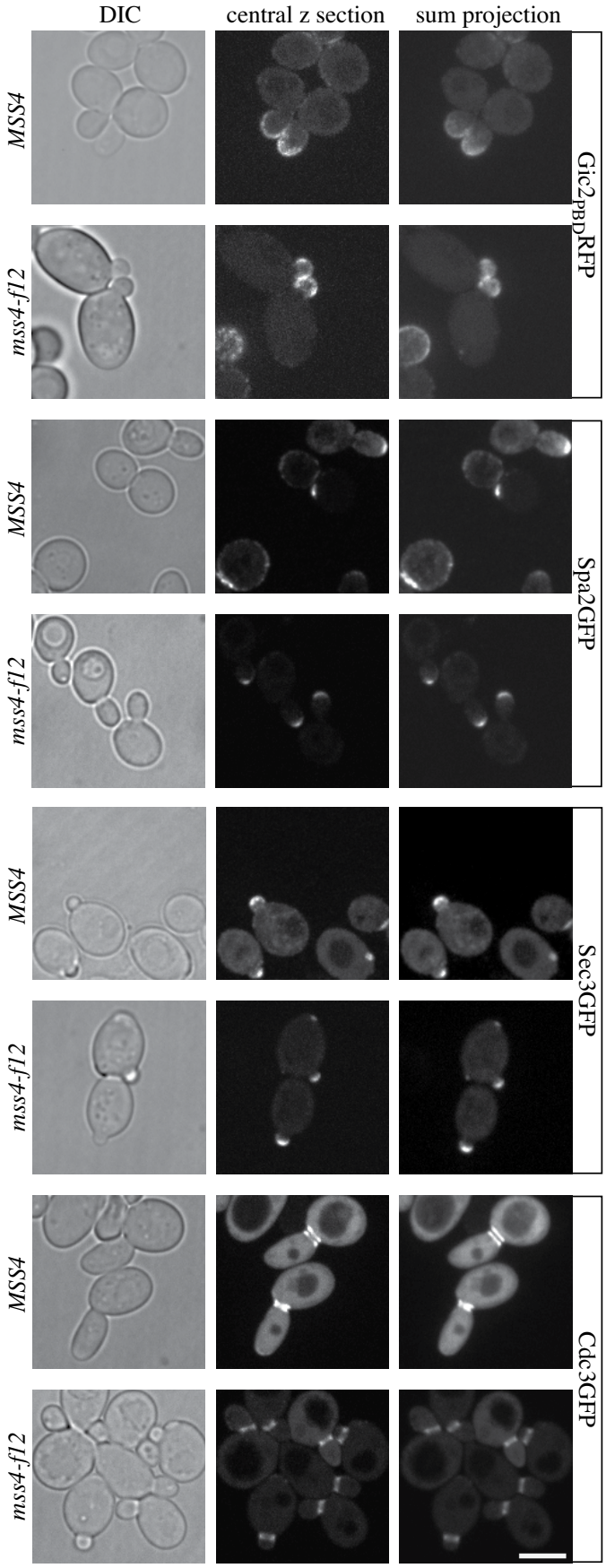

Fig. S6. Active Cdc42p, polarisome, exocyst and septin subunits localization is unaffected in *mss4-f12* cells in limiting glucose. Indicated strains expressing the indicated RFP/GFP fusion protein grown in selective media were back diluted into media containing 0.2% glucose and grown for an additional 6-7 hr. Spinning disk confocal fluorescence and DIC images were taken and central z-sections and sum projections (8-12 z-sections) of representative cells are shown. Similar results were observed in 2 independent experiments.

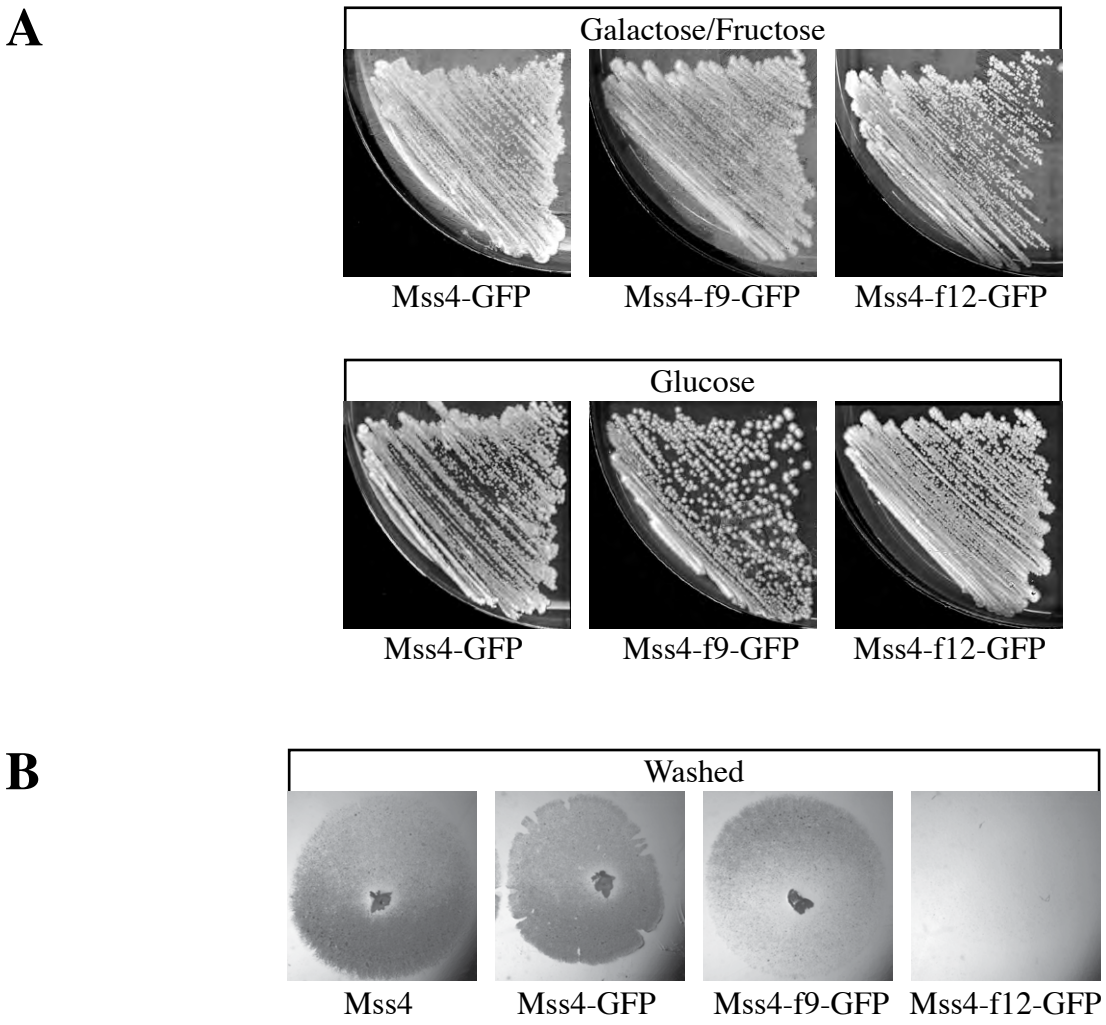

Fig. S7. Mss4, Mss4-f9 and Mss4-f12 GFP fusions are functional. A) RAY1885 carrying Mss4-GFP, Mss4-f9-GFP or Mss4-f12-GFP were grown on galactose or glucose containing media for 2 days. B) An *mss4*Δ strain carrying Mss4, Mss4-GFP, Mss4-f9-GFP or Mss4-f12-GFP was grown on YEP0.2%D media for 5 days and invasive growth was assessed as in Fig. S1.

Table S1. Yeast strains used in this study

Strain	Genotype	
10560-6B	*MAT ura3-52 trp1::hisG leu2::hisG his3::hisG*	(Roberts et al., 1997)
JY426	*MAT**a**, leu2-3,-112 ura3-52 his4-34 fus1- 1 fus2- 3*	Cold Spring Harbor
SEY6211	*MAT**a**, leu2-3,-112 ura3-52 his3- 200 trp1- 901 ade2 suc2- 9*	S. Emr
RAY876	SEY6211 *URA3*	This study
RAY1563	JY426 with pRS406GFPBud1	This study
RAY1885	10560-6B *mss4- 1::HIS5Sp* with p416GALpMSS4	This study
RAY1940	10560-6B *mss4- 1::HIS5Sp* with p414MSS4pMSS4GFP	This study
RAY1941	10560-6B *mss4- 1::HIS5Sp* with p414MSS4pmss4f9GFP	This study
RAY1942	10560-6B *mss4- 1::HIS5Sp* with p414MSS4pmss4f12GFP	This study
RAY1945	*MAT /**a** ura3-52/ura3-52 trp1::hisG/trp1::hisG leu2::hisG/leu2::hisG his3::hisG/his3::hisG mss4- 1::HIS5Sp/mss4- 1::HIS5Sp* p414MSS4pMSS4	This study
RAY1949	*MAT /**a** ura3-52/ura3-52 trp1::hisG/trp1::hisG leu2::hisG/leu2::hisG his3::hisG/his3::hisG mss4- 1::HIS5Sp/mss4- 1::HIS5Sp* p414MSS4pmss4f12	This study
RAY1986	10560-6B *mss4- 1::HIS5Sp* with p414MSS4pMSS4	This study
RAY1990	10560-6B *mss4- 1::HIS5Sp* with p414MSS4pMSS4	This study
RAY1991	10560-6B *mss4- 1::HIS5Sp* with p414MSS4pmss4f12	This study
RAY1992	10560-6B *mss4- 1::HIS5Sp* with p414MSS4pmss4f12	This study
RAY1993	10560-6B *mss4- 1::HIS5Sp URA3::GALpGFP-PH^{Plc}-PH^{Plc}-GFP* with p414MSS4pmss4f12	This study
RAY1999	10560-6B *mss4- 1::HIS5Sp URA3::GALpGFP-PH^{Plc}-PH^{Plc}-GFP* with p414MSS4pmss4f12	This study
RAY2001	10560-6B *mss4- 1::HIS5Sp URA3::GALpGFP-PH^{Plc}-PH^{Plc}-GFP* with p414MSS4pMSS4	This study
RAY2003	10560-6B *mss4- 1::HIS5Sp URA3::GALpGFP-PH^{Plc}-PH^{Plc}-GFP* with p414MSS4pMSS4	This study
RAY2005	10560-6B *mss4- 1::HIS5Sp* with p414MSS4pmss4f9	This study
RAY2006	10560-6B *mss4- 1::HIS5Sp* with p414MSS4pmss4f9	This study
RAY2012	*MAT**a** ura3-52 trp1::hisG leu2::hisG his3::hisG mss4- 1::HIS5Sp*	This study

	p414MSS4pMSS4	
RAY2013	*MAT**a** ura3-52 trp1::hisG leu2::hisG his3::hisG mss4- 1::HIS5Sp* p414MSS4pMSS4	This study
RAY2014	*MAT**a** ura3-52 trp1::hisG leu2::hisG his3::hisG mss4- 1::HIS5Sp* p414MSS4pmss4f12	This study
RAY2015	*MAT**a** ura3-52 trp1::hisG leu2::hisG his3::hisG mss4- 1::HIS5Sp* p414MSS4pmss4f12	This study

Table S2. Primers used in this study

#	Primer name	Primer sequence	Mutation	Restriction site
1	Mss4pBamHI	CGCGGATCCATGTCAGTCTTGCGATCAC		
2	Mss4mNotI	ATAGTTAGCGGCCGCACTCAGTCTTTATAATTTTTC		
3	Mss4pSalI	CGCCTTGTCGACCATCGTGAGTTAAGG		
4	Mss4pAatIInostop	CCTAACCAGAAAAATTATAAAGACGTCGTGCGGCCGCCACCGCGGTGG		
5	Mss4mAatIInostop	CCACCGCGGTGGCGGCCGCACGACGTCTTTATAATTTTTCTGGTTAGG		
6	AatIIpGAyeGFP	AAAGACGTCGGAGCAGGTGCTGGTGCTGG		
7	ADHtNotI	TCCCCGCGGTGGCGGCCGCGTTATCCCTAGCGGATCTG		
8	Mss4C12pT1717C	GATTTAAAAGGTCCCACATGGGGCCGTTTTACCAATCTAGATAAAG	S573P	XbaI
9	Mss4C12mT1717C	CTTTATCTAGATTGGTAAAACGGCCCCATGTGGGACCTTTTAAATC	S573P	XbaI
10	Mss4KOp	GTTTACACCCCCCGAGACAGTTGCCCTATATCGCTTTTCCCTATCAATAGTTTCTAACTCCGGTGGCGGCCGCTCTAG		
11	Mss4KOm	ATTAAATCAAAGTAGATTAGACTGAGTACATAGACGATAGGTTATTTACCTGTGCCCTACCCTCGAGGTCGACGGTATC		
12	Mss4pNheI-3	TCCCTATCAATAGTTTCGCTAGCATGTCAGTCTTGCGATCACAAC		NheI
13	Mss4mNheI-3	GTTGTGATCGCAAGACTGACATGCTAGCGAAACTATTGATAGGGA		NheI
14	Mss4pBglII607	TTTACTAAACAAGCGCGTTTCGAGGAGATCTTCCAGAATATCGGC		BglII
15	Mss4mBglII607	GCCGATATTCTGGAAGATCTCCTCGAAACGCGCTTGTTTAGTAAA		BglII
16	Mss4pAatII1401	GATTATTTGGTTTCGTTGACGTCCAAATACATTTTGAGTGAGTTGA		AatII
17	Mss4mAatII1401	TCAACTCACTCAAAATGTATTTGGACGTCAACGAAACCAAATAATC		AatII

18	Mss4pXhoI1804	GAAAGATTTAAATTGGCTCGAGGAAGGTCAGAAAATTAAATTCGG		XhoI
19	Mss4mXhoI1804	CCGAATTTAATTTTCTGACCTTCCTCGAGCCAATTTAAATCTTTC		XhoI
20	Mss4C9pA1768T	GGCGAAAGATTGATCATATAGGCCTGTGATGAGAGATCTAAATTGGC	R590*	BglII
21	Mss4C9mA1768T	GCCAATTTAGATCTCTCATCACAGGCCTATATGATCAATCTTTCGCC	R590*	BglII
22	Mss4pAatII1767	GGATAAAGAAAGGTTGGCGAAAGACGTCTCATATAGGCCTGTGATGAAAGATTTAAATTGGC	R590D S591V	AatII
23	Mss4mAatII1767	GCCAATTTAAATCTTTCATCACAGGCCTATATGAGACGTCTTTCGCCAACCTTTCTTTATCC	R590D S591V	AatII
24	Mss4pAatIIstop	CCTAACCAGAAAAATTATAAAGACGTCTGAGTGCGGCCGCCACCGCGGTGG	*780V 781*	AatII
25	Mss4mAatIIstop	CCACCGCGGTGGCGGCCGCACTCAGACGTCTTTATAATTTTTCTGGTTAGG	*780V 781*	AatII
26	Mss4C22pT1697A	CCACCACACTTAGACATTCACAATACTTATGATTTAAAAGGATCCATATGGGG	I566N	BamHI
27	Mss4C22mT1697A	CCCCATATGGATCCTTTTAAATCATAAGTATTGTGAATGTCTAAGTGTGGTGG	I566N	BamHI
28	Mss4C22pC1721T	CTTATTTCCACCACATCTAGACATTCACAATACTTATGATTTAAAAGGTTCC	T574I	XbaI
29	Mss4C22mC1721T	GGAACCTTTTAAATCATAAGTATTGTGAATGTCTAGATGTGGTGGAAATAAG	T574I	XbaI
30	MSS4clone9p	GCGAAAGATAGATCATATAGGCCTGTGATGAGAGATTTAAATTGGCTTGAAGAAGGTCAG	K597R	
31	MSS4clone9m	CTGACCTTCTTCAAGCCAATTTAAATCTCTCATCACAGGCCTATATGATCTATCTTTCGC	K597R	

Table S3. Plasmids used in this study

Plasmid	Vector	Insert	Source
pGEX-6P	pGEX-6P		Amersham
p406	pRS406		(Sikorski and Hieter, 1989)
p415	pRS415		(Sikorski and Hieter, 1989)
p416	pRS416		(Sikorski and Hieter, 1989)
pCM188	pCM188		(Gari et al., 1997)
pBSLoxPHisSpLoxp	pBS	LoxPHis5SpLoxP	(Nern and Arkowitz, 1998)
p406GALp	pRS406	GAL1-10 promoter	This study
p416GALp	pRS416	GAL1-10 promoter	This study
p416GalCdc24HAGFP	pRS416Gal	Cdc24HAGFP	(Nern and Arkowitz, 2000a)
p416GALpMSS4	pRS416GALp	MSS4	This study
pCM188TetpMSS4	pCM188	Tetp-MSS4	This study
p414MSS4pMSS4	pRS414	MSS4pMSS4	This study
p415MSS4pMSS4	pRS415	MSS4pMSS4	This study
p414MSS4pMSS4-AII	pRS414	MSS4pMSS4-AII	This study
p414MSS4pMSS4GFP	pRS414	MSS4pMSS4GFP	This study
p414MSS4pmss4f9GFP	pRS414	MSS4pmss4f9 GFP	This study
p414MSS4pmss4f12GFP	pRS414	MSS4pmss4f12GFP	This study
pExpARG-pADH1GFP-PH^{Plc}-PH^{Plc}-GFP	pExpARG-pADH1	GFP-PH^{Plc}-PH^{Plc}-GFP	(Vernay et al., 2012)
p406GALpGFP-PH^{Plc}-PH^{Plc}-GFP	pRS406	GFP-PH^{Plc}-PH^{Plc}-GFP	This study
p414MSS4pMSS4*	pRS414	MSS4pMSS4*	This study
p414MSS4pmss4f9	pRS414	MSS4p-mss4-f9	This study
p414MSS4pmss4f12	pRS414	MSS4pmss4-f12	This study

p414MSS4pMSS4-590AII	pRS414	MSS4pMSS4-590AII	This study
p414MSS4pMSS4-590AII-AII	pRS414	MSS4pMSS4-590AII-AII	This study
p414MSS4pmss4f9	pRS414	MSS4pmss4f9	This study
pGEX-6PMSS4	pGEX-6P	MSS4	This study
Flo11 lacZ (B3782)	YEp355	3 kbp-*FLO11::LacZ* in YEp355	(Rupp et al., 1999)
pSG231	*URA3* CEN	FUS1-LacZ	(Trueheart et al., 1987)
YIp211-GIC2-PBD-RFP	YIp211	GIC2-PBD-1.5tdTomato	(Tong et al., 2007)
p406GFPBud1	pRS406	GFPBUD1	(Nern and Arkowitz, 2000b)
p406Spa2GFP	pRS406	Spa2	(Arkowitz and Lowe, 1997)
p316Sec3GFP	pRS316	Sec3GFP	(Finger et al., 1998)
p316Cdc3GFP	pRS316	Cdc3GFP	(Caviston et al., 2003)
pSL1509	*URA3* CEN	*ste11-4*	(Stevenson et al., 1992)