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Amino Acid Transporter Genes Are Essential for FLO11-Dependent and FLO11-Independent Biofilm Formation and Invasive Growth in Saccharomyces cerevisiae

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Abstract

Amino acids can induce yeast cell adhesion but how amino acids are sensed and signal the modulation of the FLO adhesion genes is not clear. We discovered that the budding yeast Saccharomyces cerevisiae CEN.PK evolved invasive growth ability under prolonged nitrogen limitation. Such invasive mutants were used to identify amino acid transporters as regulators of FLO11 and invasive growth. One mutant invasive had elevated levels of FLO11 mRNA and a Q32STOP mutation in the SFL1 gene that encodes a protein kinase A pathway regulated repressor of FLO11. Glutamine-transporter genes DIPS and GNP1 were essential for FLO11 expression, invasive growth and biofilm formation in this mutant. Invasive growth relied on known regulators of FLO11 and the Ssy1-Ptr3-Ssy5 complex that controls DIPS and GNP1, suggesting that DIPS and GNP1 operate downstream of the Ssy1-Ptr3-Ssy5 complex for regulation of FLO11 expression in a protein kinase A dependent manner. The role of DIPS and GNP1 appears to be conserved in the S. cerevisiae strain 1278b since the dipS gnp11/1278b mutant showed no invasive phenotype. Secondly, the amino acid transporter gene GAP1 was found to influence invasive growth through FLO11 as well as other FLO genes. Cells carrying a dominant loss-of-function PTR3<sup>GAP1</sup> allele had increased transcription of the adhesion genes FLO1, 5, 9, 10, 11 and the amino acid transporter gene GAP1. Deletion of GAP1 caused loss of FLO11 expression and invasive growth. However, deletions of FLO11 and genes encoding components of the mitogen-activated protein kinase pathway or the protein kinase A pathway were not sufficient to abolish invasive growth, suggesting involvement of other FLO genes and alternative pathways. Increased intracellular amino acid pools in the PTR3<sup>GAP1</sup>-containing strain opens the possibility that Gap1 regulates the FLO genes through alteration of the amino acid pool sizes.


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Introduction

Nitrogen is a vital metabolite in living organisms and nitrogen metabolism governs major developmental decisions in Saccharomyces cerevisiae. For example, nitrogen starvation triggers diploid cells to undergo meiosis [1] and can lead to quiescence of both haploid and diploid cells [2]. Low concentrations of nitrogen induce pseudohyphal growth through elongated growth and polar budding [3] while propagation of haploid cells on rich complex medium leads to biofilm formation and invasive growth [4,5].

Biofilm, pseudohyphal growth and invasive growth are dependent on the cell wall adhesin gene FLO11 (MUC1), which confers cell-surface adhesion [6,7]. Cellular access to nitrogen is linked to FLO11 expression through the cyclic AMP-protein kinase A (cAMP-PKA) pathway and the general transcription factor Gen1 [8,9]. However, a central question is how the yeast cell senses extracellular nitrogen in the form of amino acids and the signal is transmitted to activation of FLO11.

Nitrogen in the form of ammonium induces transcription of FLO11 via the high-affinity ammonium transporter Mep2 and several observations support the hypothesis that Mep2 is an ammonium receptor [8,10]. However, the ability to transport ammonium appears to be essential for the signaling function of Mep2 [11] suggesting that Mep2 is a transceptor or transports ammonium to an intracellular receptor. Mep2 is proposed to induce expression of FLO11 via the cAMP-PKA pathway, based on the findings that exogenous cAMP, dominant active RAS2 or GPA1 alleles are able to restore pseudohyphal differentiation in a Δmep2/Δmep2 strain [8].

Lorenz and Heitman observed that pseudohyphal growth on proline and glutamine-based medium was not dependent on MEP2 [8] and hypothesized that amino acid transporters could have a function similar to Mep2. In the current work we have
explicitly tested the role of amino acid transporters in the regulation of FLO11, since these transporters have not yet been shown to play a role in biofilm formation, pseudohyphal or invasive growth.

Glutamine transport is mediated by Gnp1, Agp1, Gap1 and Dip5 [12–15] while proline is transported by Put4 and the general amino acid transporter Gap1 [16]. Transcription of Gnp1, AGP1 and DIP5 is induced by extracellular amino acids through the Ssy1-Ptr3-Ssy3 (SPS) complex and deletion of Ssy1 leads to a 5–15 fold reduction in DIP5 and GAP1 expression [13,17,18]. Counter intuitively, deletion of Ssy1 or PTR3 lead to increased invasive growth in the Σ1278b strain background [17] while the opposite would be expected if invasive growth only depended on GAP1, AGP1 or DIP5. A cause of the invasive growth in the ssy1 and ptr3 mutants may be increased expression of GAPI and higher levels of intracellular amino acids.

Here we investigated the importance of the amino acid transporters Gap1, Dip5 and Gap1 in regulation of FLO11-dependent invasive growth. We hypothesize that (i) the general amino acid permease Gap1 is essential for invasive growth and FLO11 expression under conditions where GAP1 is highly expressed and (ii) that Gap1 and Dip5 are essential for FLO11 dependent invasive growth conditions where GAP1 is not expressed.

Results

Nitrogen-limited S. cerevisiae Populations Evolve Invasive Growth

To investigate the role of amino acids transporters for invasive growth and FLO11 regulation, we initially tested the phenotype of gap1, dip5 and gap1 mutants in the Σ1278b strain background but neither of these single gene deletions led to the loss of the invasive growth phenotype (Fig. S1). In an alternative approach we selected invasive mutants and used these as background for further analysis of amino acid regulation of FLO11. Invasive mutants appeared in populations of the normally noninvasive S. cerevisiae (CEN.PK113-7D) when propagated under nitrogen limitation (Fig. 1A–D). Clones were isolated from two haploid CEN.PK113-7D populations of 10¹⁰ cells after 250 generations in continuous bioreactors, limited for ammonium or glutamine respectively.

Clones Sfl1C320STOP and Ptr3647:C...N Induce Invasive Growth

To characterize clones from nitrogen-limited populations, we compared the genomes of six clones to the wildtype using DNA tiling arrays, revealing 12 to 88 single nucleotide polymorphisms (SNPs) per genome. We characterized the SNPs that affected ORFs by PCR and Sanger sequencing and verified up to two mutations in ORFs per clone (Table 1). Mutant alleles identified in the descendants were subsequently introduced into the wildtype strain to test if these caused the invasive growth phenotype. All gave rise to invasive phenotypes, except for a deletion of the general amino acid permease gene GAPI (Fig. 2A–C).

We focused our work on two of the invasive clones from the glutamine-limited population. The clone Sfl1C320STOP (Table 1) carries two mutant alleles: the nonsense mutation gfl1C320STOP that leads to a truncation of Sfl1 at residue 320 and the pho90ΔD14176 mutation that substitutes threonine with alanine at residue 672 of Pho90. Clone Ptr3647:C...N (Table 1) carries a partial substitution of the PTR3 ORF with the long terminal repeat (LTR) of a Ty1 element from residue 1941 of PTR3. This leads to substitution of the 31 C-terminal amino acids of Ptr3, starting from residue 647, with 11 novel residues CWNKNPLSSIN encoded by the Ty1 element.

Fig. 1. Invasive growth of haploid descendants after prolonged nitrogen limitation. Descendants of CEN.PK113-7D in A, glutamine-limited culture, and C, ammonium-limited culture, grown on rich complex medium (YPD) for 3 days at 30°C and flushed with water to determine the proportion of adhering clones (B and D). Of descendants from the glutamine-limited population, 80% were invasive (n>200). doi:10.1371/journal.pone.0041272.g001

GAP1 Transcription is Induced in Ptr3647:C...N

Ptr3 is part of the SPS complex that responds to the presence of amino acids in the environment and induces transcription of amino acid transporter genes including AGP1, GNP1 and DIP5 [13,17,18], which encode glutamine transporters. Transcription profiling of the Ptr3647:C...N clone revealed that DIP5 and other Ptr3-regulated transporter genes (GNP1, PTR2, BAP2, BAP3, TAT1) had strongly reduced mRNA levels relative to the wildtype (Fig. 2D). The general amino acid permease gene GAPI on the other hand, had a 10-fold increase in transcription level. The Ptr3647:C...N clone also had increased transcript levels of FLO genes, which are involved in cell-surface and cell-cell adhesion and could potentially explain the adhesive phenotype. FLO3 and FLO9 were mildly induced while FLO1 was upregulated 10-fold (Fig. 2D). Northern blot analysis showed that FLO11 had increased mRNA levels in the Ptr3647:C...N mutant showing that also FLO11 had been induced (Fig. 3B). We found a clear discrepancy between FLO11 mRNA levels found in the Northern blot and the array analysis (Fig. 2D). This difference could be caused by binding of unspecific mRNA species to the 60-mer probe used in the array analysis. The 621 bp long probe used for Northern blot analysis was specific for FLO11, and hence, we made the assumption that Northern blot analysis was more representative for the FLO11 mRNA levels.
GAP1 is Essential for FLO11 Expression and Invasive Growth in Ptr3647::C\ldots N

To test if increased GAP1 expression was causative for FLO expression and invasive growth we deleted GAP1. The gap1Ptr3647::C\ldots N mutant lost the ability to grow invasively while deletion of the glutamine transporter genes DIP5 or GNP1 in Ptr3647::C\ldots N did not affect invasiveness (Fig. 3A). Northern blot analysis showed that FLO11 mRNA could not be detected in the gap1Ptr3647::C\ldots N mutant (Fig. 3B), suggesting that GAP1 was essential for FLO11 expression and invasive growth in this mutant.

GAP1 also Induce Invasive Growth Independent of FLO11

We next tested if the regulation of invasive growth in the Ptr3647::C\ldots N clone was dependent on FLO11 and known
regulators of FLO11 [19]. Invasive growth was still maintained after gene-deletion of either FLO11, the mitogen-activated protein kinase (MAPK) transcription factor gene TEC1, or the PKA-pathway genes TPK2 and CYR1. The same result was obtained when we deleted the transcription factor genes GCN4 and MSS11 as well as the SPS-component gene SSY1 (Fig. 3C–E). Only deletion of the PTR3647::C…N allele with a ptr3::KanMX cassette led to loss of invasive growth. Hence, besides FLO11, a second response was responsible for invasive growth in Ptr3647::C…N. The response was dependent on high GAP1 expression and not dependent on the MAPK-, PKA- and SPS-pathways.

Intracellular Amino Acid Pools are Increased in PTR3647::C…N

To explain the role of Gap1 for invasive growth, we hypothesized that Gap1 could either i) change the balance in the intracellular pools of certain amino acids, ii) affect the activity of invasive growth regulators, and/or iii) act as receptors, eliciting a signal transduction pathway causing invasiveness. To test the first hypothesis, amino acid pool sizes were determined for clones Ptr3647::C…N and Sfl1 Q320STOP (Fig. 4A). Compared to the wildtype strain, glutamine pools were almost two-fold higher in the Ptr3647::C…N clone while the pool of the nitrogen-rich vacuolar amino acids lysine, histidine and arginine was as high as 2.5-fold higher than in the wildtype. We hypothesized that high levels of glutamine induced invasive growth. Both Ptr3647::C…N and Sfl1 Q320STOP were invasive when grown on medium with 100 mM glutamine as the only nitrogen source, while the wildtype strain remained noninvasive. The strains were not invasive on ammonium (Fig. 4B) suggesting that the effect was glutamine specific.

PTR3647::C…N is a Dominant Loss-of-function Allele

The transcript profile of Ptr3647::C…N was similar to transcript profiles of ptr3 deletion mutants, with severe reductions in transcript levels of SPS-regulated amino acid and peptide transporters GNP1, PTR2 and DIP5 (Fig. 2D; [17]). Hence, PTR3647::C…N behaves as a loss-of-function allele when expressed on YPD. Reconstitution of the PTR3647::C…N allele in the wildtype strain (Fig. 2A–C) and mating of the resultant invasive clone with a wildtype PTR3 strain, CEN.PK110-16D, revealed that the PTR3647::C…N allele was dominant for invasive growth (data not shown). Moreover, the finding that SSY1 deletion did not suppress the invasive phenotype of Ptr3647::C…N (Fig. 3C–E) suggested that the PTR3647::C…N allele is epistatic to ssy1 and supported that it is dominant. Taken together, these data suggest that the PTR3647::C…N allele must be a dominant loss-of-function allele.

GNP1 and DIP5 are Essential for FLO11 Expression and Invasive Growth in SFL1 Q320STOP

The transcript profile of the invasive mutant Sfl1 Q320STOP revealed increased transcript levels of the adhesion genes FLO5, FLO9, FLO10 (Fig. 2D) and FLO11 (Fig. 5B). SFL1 encodes a

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Figure 3. Gap1-dependent FLO11 expression and invasive growth in Ptr3647::C…N. Amino acid permease gene product in invasive growth ability in Ptr3647::C…N was tested in a ura3 PTR3647::C…N background (RB428) by introducing gene deletions dip5 (RB468), gnp1 (RB469) or gap1 (RB547). Cells were grown on rich complex medium (YPD) for 2 day at 30°C (A, upper panel) and flushed with water to expose invasive growth (A, lower panel). Northern blot of FLO11 mRNA for strains in A are shown in B (upper panel). RNA for ribosomal subunits 18S and 28S served as loading controls (B, lower panel). Genes of modulating proteins in invasive growth were deleted in the RB428 mutant: flo11 (RB457), tec1 (RB460), tpk2 (RB461), cyr1 (RB465), gcn4 (RB472), mss11 (RB474), ssy1 (RB477) and ptr3 (RB492). Cells were grown on YPD for 3 days at 30°C (C). The plate was flushed with water to test for invasive growth (D). Distribution of deletion strains in C and D are in E.
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PKA-regulated suppressor of FLO11 [20–22] and the sfl1Q320STOP mutation causes a truncation of the C-terminal part of Sfl1 that normally interacts with the co-suppressor Snf6 [23]. Because the Snf6-Sfl1 complex is essential for Sfl1-mediated FLO11 repression, the sfl1Q320STOP allele could be responsible for derepression of FLO11. The Sfl1Q320STOP clone also carried a SNP in the phosphate transporter gene PHO90 (Table 1), however the role of the pho90T672A allele in invasive growth regulation was unclear.

We next tested the role of glutamine transporter genes for invasive growth of Sfl1Q320STOP. Both a gnp1Δ Sfl1Q320STOP and a dip5Δ Sfl1Q320STOP mutant lost their adhesion properties (Fig. 5A) and Northern blot analysis supported these data, showing that FLO11 mRNA levels were reduced in these strains (Fig. 5B). Thus, GNP1 and DIP5 were essential for expression of FLO11 and invasive growth in a PKA pathway dependent manner. Deletion of GAP1 in the Sfl1Q320STOP strain had no effect on invasive growth and only led to a partial reduction of FLO11 expression (Fig. 5B), presumably because (i) GAP1 was not highly expressed as in Ptr3647C::N and (ii) GNP1 and DIP5 were expressed and responsible for invasive growth.

We also found that GNP1 and DIP5 were essential for invasive growth in the S. cerevisiae strain Σ1278b. In Σ1278b, deletion of both GNP1 and DIP5 was required for loss of invasive growth (Fig. S1). Based on these results, the glutamine transporter genes appear to be a general requirement for invasive growth in the S. cerevisiae strains Σ1278b and CEN.PK.

Mutations in FLO11, MAPK, SPS and PKA Pathway Genes Suppress Sfl1Q320STOP Invasiveness

Expression of FLO11 in the Sfl1Q320STOP clone indicated that the FLO11 promoter was in a PKA pathway regulated derepressed state. Northern blot analysis revealed that FLO11 was in fact transcribed in the Sfl1Q320STOP clone and deletion of FLO11 showed that it was responsible for invasive growth (Fig. 5). The absence of Sfl1 repression is known to allow access of transcriptional activators to the FLO11 promoter [20–22]. Individual deletions of the genes TEC1, TPK2, CYR1, GCN4 and MSS11, which encode known regulators of FLO11 expression [17,19], led to the loss of invasive growth in all cases, except for the deletion of MSS11 (Fig. 5C–E). Hence, regulation of invasive growth in the Sfl1Q320STOP clone, CEN.PK background, was similar to regulation in Σ1278b. This result suggests that GNP1 and DIP5 induce FLO11 and invasive growth through identical pathways in the two strain backgrounds involving Gcn4, the MAPK- and PKA-pathways. We tested if regulators of GNP1 and DIP5 were also essential for invasive growth. Ssy1-Ptr3-Ssy5 induces the expression of GNP1 and DIP5 in response to amino acids [13,17]. We found that both PTR3 and SSY1 were essential for invasive growth in the Sfl1Q320STOP clone (Fig. 5), suggesting that the SPS complex regulates invasive growth through GNP1 and DIP5.

Amino acid pools in the Sfl1Q320STOP mutant and in the Sfl1Q320STOP dip5 mutant were almost identical to the wildtype strain with a slight increase in the glutamine pool. Thus, Sfl1 and Dip5 did not appear to influence invasive growth through pool sizes (Fig. 4A).

Dip5 Interacts with Gnp1 and Mep2

Interestingly, individual deletions of DIP5 and GNP1 resulted in complete loss of FLO11 expression and invasive growth in Sfl1Q320STOP (Fig. 5A). The finding that two amino acid transporter genes are essential for invasive growth under the same condition indicated corporative behavior between the transporters in the CEN.PK background. We tested the physical interaction between Dip5 and Gnp1, using the split-ubiquitin assay. In accordance with previous findings [24], we found that Dip5 interacts with itself (Fig. 6). Dip5 also interacted with the glutamine transporter Gnp1 and the ammonium permeases Mep1 and Mep2 but did not interact with the glucose transporter Hxt1 (Fig. 6). Physical interaction between Dip5 and Gnp1 might cause a functional dependence for activity of both transporters and explain why deletion of either DIP5 or GNP1 led to complete loss of FLO11 expression in the CEN.PK background.
DIP5 is Essential for Biofilm Formation and Pseudohyphal Growth

Invasive growth shares many regulative constituents with biofilm and pseudohyphal growth [19]. We found that the Sfl1Q320STOP mutant formed biofilm on a polystyrene surface and that this was dependent on DIP5 and GNP1 (Fig. 7A). A diploid form of Sfl1Q320STOP, homozygous for the sfl1 mutation, formed pseudohyphal growth on synthetic low ammonium medium (SLAD); however, a sfl1Q320STOP/sfl1Q320STOP dip5/dip5 mutant lost the ability to form pseudohyphae (Fig. 7B). This showed that DIP5 was essential for the pseudohyphal phenotypes.

We attempted to construct sfl1Q320STOP/gnp1 haploids to test if GNP1 was required for pseudohyphal growth but were unsuccessful in mating sfl1Q320STOP gnp1 haploids.

Discussion

The ammonium transporter Mep2 has been shown to be essential for expression of FLO11 and filamentous growth of S. cerevisiae under nitrogen limitation [8]. Our results suggest that amino acid transporters can have a similar function as Mep2, since they are essential for transcription of the cell surface glycoprotein gene FLO11, for biofilm as well as invasive growth in S. cerevisiae. We show that GAP1 is essential for invasive growth and that invasiveness can be maintained even after deletion of FLO11. The highly expressed GAP1 phenotype correlates with an increased amino acid pool and an increased expression of FLO11 and other FLO genes. In cases where GAP1 is not expressed, we find that...
GNP1 and DIP5 are essential for invasive growth and that they probably exhibit this role through PKA-pathway components and FLO11 expression.

GNP1 and DIP5 induction of FLO11 is likely to be dependent on an active PKA-pathway, since this regulation is observed in a Sfl1Q320STOP mutant. The transcription factor Sfl1 represses transcription of FLO11 when activity of the PKA pathway is low [20,22]. In a ∑1278b background, Sfl1 is removed from the promoter when phosphorylated by an active PKA subunit, Tpk2, leading to FLO11 transcription [20–22]. The PKA pathway is partially inactive in the CEN.PK strain background due to a point mutation in the adenylate cyclase gene, cyr1K1876M [25]. Therefore, the wildtype CEN.PK strain is likely to have permanent low PKA activity that keeps the FLO11 promoter in the repressed state.

Mutant Sfl1Q320STOP lost the Sfl1 domain that interacts with the co-repressor Ssn6 [23] and the FLO11 promoter was likely converted to a state amenable to transcription similar to the state found when the PKA pathway is active.

Invasive growth of the Sfl1Q320STOP mutant appeared to be regulated by the same pathways described for the ∑1278b background, including the essential constituents of the PKA-, MAPK- and general amino acid control (GCN) pathways (Fig. 5).

The role of the SPS complex (Ptr3, Ssy1 and Ssy5) in regulating invasive growth is so far unclear [17]. Our results suggest that the SPS complex acts on FLO11 expression by inducing GNP1, DIP5 and potentially other amino acid transporter genes (Fig. 8A model). The model is supported by the fact that the function of GNP1 and DIP5 in FLO11 regulation appears to be conserved between CEN.PK and ∑1278b (Fig. S1).

In the Ptr5Δ46-4C.ΔN strain, GAP1 activity was essential for FLO11 expression (Fig. 5).
The phenotype of this strain appeared to be similar to previously reported recessive ptr3 and ssy1 mutants [17]. Ljungdahl and coworkers found that loss of Ptr3 activity leads to increased GAP1 transcript levels, reduced levels of GNP1 and PTR2 mRNA, invasive growth and increased pools of glutamate, glutamine, histidine, arginine and several other amino acids when grown on YPD [17]. However, in contrast to the recessive ptr3 alleles, the dominant loss-of-function PTR3647::C…N allele complemented an ssy1 deletion, revealing that it was epistatic to SSY1 (Fig. 3C–E). We propose that Ptr3647::C…N leads to an inactive SPS complex, under the tested conditions, causing reduced transcription of GNP1 and DIP5 (Fig. 8B model). GAP1 transcription is not under direct control of the SPS complex but might be induced in Ptr3647::C…N as a result of altered activity of the TOR-regulated nitrogen catabolite repression pathway [27], giving rise to a higher intracellular amino acid pool. The glutamine transporter genes GNP1 and DIP5 are not essential for invasive growth in Ptr3647::C…N probably because these genes have very low expression levels (Fig. 2D).

Figure 8. Models of FLO gene regulation by amino-acid transporters. Extracellular amino acids induce the SPS complex at the plasma membrane (PM) and the activated complex elicits gene expression of amino acid transporters DIP5 and GNP1. Dip5 and Gnp1 activate FLO11 transcription in a manner dependent on activity of the PKA-pathway (A). Inactive SPS signaling indirectly leads to increased transcript levels of GAP1. Gap1 increases the amino acid pool concentration, which in turn triggers a PKA independent signal for induction of FLO genes (B). PM, plasma membrane. NM, nuclear membrane.

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In addition to Gap1’s role as an amino acid transporter, Gap1 has been reported to induce the PKA pathway, leading to rapid degradation of trehalose and glycogen in nitrogen-starved cells [20]. Hence, Gap1 could be acting on the FLO11 promoter via the PKA pathway. However, deletion of the PKA pathway genes CR1 and TPK2 in PrsΔ^{647/-} were not sufficient for loss of invasive growth (Fig. 3C-E). Deletion of other regulatory factors essential for invasive growth in Δ^{1276b} was also not sufficient to eliminate invasive growth by PrsΔ^{647/-}, suggesting a novel GAP1-dependent regulatory mechanism. This mechanism acts independent of the PKA-pathway and could regulate transcription of FLO genes other than FLO11 (Fig. 3B model). We found that deletion of FLO11 was not sufficient to abolish invasive growth, so alternative adhesion proteins must be involved. S. cerevisiae contains a number of FLO genes that confer cell-cell and cell-surface adhesion [29,30]. The FLO1, 5, 9 and 10 genes are normally inactive in laboratory strains though FLO1, FLO5 and FLO10 are known to have an impact on S. cerevisiae adhesion [29,31–33]. A likely candidate is FLO11, which was 10-fold induced in the PrsΔ^{647/-} mutant relative to the wildtype and correlated with a high GAP1 and FLO11 expression (Fig. 2D).

In summary, our data demonstrate that amino acid transporters are involved in biofilm development and invasive growth. We propose that at least two modes of action exist on invasive growth regulation; a FLO11-dependent modulated by Dip5, Gnp1 and Gap1 and a FLO11-independent, regulated by Gap1. The former, Dip5 and Gnp1, are likely amino acid transporters or transceptors that directly activate a cytosolic signaling pathway linked to PKA-pathway components, which in turn induce FLO11. Our observations support that both permeases operate downstream of amino acid sensing complex, SPS, for regulation of FLO11. The latter, Gap1, may potentially induce invasive growth through increased amino acid pool levels that elicit a novel signal-cascade for gene expression of other FLO genes.

Materials and Methods

Strains

Strains used are listed in Table 2. The wildtype strain CEN.PK113-7D and the isogenic strains CEN.PK113-5D (ura3) and CEN.PK110-16D (tpi1) were kind gifts from Peter Kotter, Frankfurt am Main, Germany. The two populations of CEN.PK113-7D and two populations of S288c evolved in glucose limitation were kind gifts from Maitreyi Dunham, Seattle, USA. The 10506-2B parental strain (Δ^{1276b}) and dip5 (RB598), gap1 (RB399) and gap1 (RB600) mutants in the Δ^{1276b} background were kind gifts from Owen Ryan and Charles Boone, Toronto, Canada [34]. Descendants of CEN.PK113-7D exposed to glutamine limitation for 250 generations were isolated and three clones were chosen for analysis (Table 1). Descendants of CEN.PK113-7D exposed to ammonium limitation for 400 generations were isolated and three clones were chosen for analysis (Table 1).

Tiling array analysis of descendants revealed potential SNPs and deletions. SNPs in ORFs were identified by PCR amplification of 500 bp around the potential SNP and Sanger sequencing of the product, while deletions were identified as described previously [35]. Mutations in the three clones from the glutamine limited culture were i) sf11^{C358G} and pho90^{A2014G} (clone Sfl1^{C358G}); ii) and PrsΔ^{647/-} (clone Sfl1^{C358G}), allele PrsΔ^{647/-} and iii) YKRC811-Agap1-YKRC812. The three clones from ammonium limitation carried the mutations: i) gap1^{C358G} and mep2^{A2014G}; ii) YKRC811-Agap1-YKRC812 and gdh1^{G163R} and iii) no SNPs were detected in the last clone. The resulting protein modifications are described in Table 1. The PrsΔ^{647/-} insertion initially appeared as a SNP in PTR3 in the tiling array analysis but several primer combinations around residue 1941 of PTR3 failed to give a PCR product. The mutation was identified by digestion of genomic DNA from the PrsΔ^{647/-} clone with XhoI followed by ligation with T4 ligase and inverted primers identical to regions just upstream of residue 1941 for inverse PCR. Sanger sequencing of the PCR product revealed insertion of a long terminal repeat (LTR) identical to YMBCTy1-1, YJRTy1-2, YGRWTy1-1 and YOLWTy1-1 (http://www.yeastgenome.org/), given that PrsΔ^{647/-} had an insertion through transposition of one of these Ty1s.

The PrsΔ^{647/-} allele was reconstituted into the wildtype strain background (CEN.PK113-5D isogenic to CEN.PK113-7D except for URA3) by transformation with a chimeric PrsΔ^{647/-} PCR product, using the primers 5’-CATGATACATTGAGGAG-3’ (identical to nucleotides 983–1001 of the PrsΔ ORF) and 5’-tcgaatgactatggttcgcagtcagattgctgaGTATTCCATT- GAGATTCC-3’ (identical to nucleotides 215–234 of the Ty1 LTR and 213143–213173 of chromosome XI downstream of the stop codon of wild type Prs). The PCR product was co-transformed into CEN.PK113-5D with a URA3 plasmid. A prototrophic transformant able to adhere and invade agar had the correct insertion of the PrsΔ^{647/-} mutation, identified by PCR and Sanger sequencing. The resultant strain was named RB567, gdh1^{C358G} and pho90^{A2014G} were reconstituted into CEN.PK113-5D by co-transformation of a URA3 plasmid with PCR products carrying gdh1^{C358G} or pho90^{A2014G}. Correct clones were identified through prototrophic, invasive growth and Sanger sequencing and the resultant mutants denoted RB484 and RB389. sf11^{C358G} was not successfully introduced into CEN.PK113-5D; therefore a full sf1 deletion was used as substitute (RB595). YKRC811-Agap1-YKRC812 mutants (RB318, RB351, RB347) were selected on D-histidine [36] and YKRC811-Agap1-YKRC812 deletions were confirmed by PCR as described [35].

Auxotrophic ura3 Sfl1^{Q320STOP} and PrsΔ^{647/-} strains were selected on medium with 5-FOA [37] and the genotype confirmed by complementation with URA3-based plasmids to generate strains RB20 and RB428. PCR-based gene deletions were made as described [38], using primers to theloxP::KanMX::loxp cassette in pUG6 with 5’-flanking sequences of the 40 bp immediately upstream and downstream of the targeted ORF. Deletion mutants were selected onYPD with 200 mg L⁻¹ geneticin. Correct insertion of the pUG6-based PCR fragment was confirmed by diagnostic PCR on genomic DNA from geneticin-resistant clones using primers for the KanMX cassette and the promoter sequence of the targeted gene (see Table 2 for deletion strains).

A MATa Sfl1^{Q320STOP} ura3 strain (RB52) was obtained by mating a MATa sf11^{Q320STOP} pho90^{G725A} ura3 strain, RB20, and CEN.PK110-16D and sporulating the resultant diploid. Diploid homozygous mutants were obtained by crossing the haploid mutants RB20 (MATa sf11^{Q320STOP} pho90^{G725A} ura3) and RB52 (MATa Sfl1^{Q320STOP} ura3). Standard yeast genetics and molecular biology methods were used [37].

Chemostat Cultivation

CEN.PK113-7D MATa was grown in modified minimal medium at dilution rates of 0.20 h⁻¹ as described [39]. Cultures were fed with modified minimal medium containing ammonium (in duplicated culture experiments) or glutamine at a concentration corresponding to 6 mM nitrogen and 230 mM glucose (calculated on nitrogen and carbon atom bases, respectively), which ensured that growth was limited by nitrogen source with all other nutrients.
Table 2. *S. cerevisiae* strains and populations used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>CEN.PK113-7D [MATα (generated from progenitor CEN.PK113-7D)]</td>
<td>[47]</td>
</tr>
<tr>
<td>CEN.PK113-5D</td>
<td>CEN.PK113-5D [MATα ura3-52 (generated from progenitor CEN.PK113-7D)]</td>
<td>[47]</td>
</tr>
<tr>
<td>CEN.PK110-16D</td>
<td>CEN.PK110-16D [MATα trp1]</td>
<td>[47]</td>
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<td>10560-2B</td>
<td>CEN.PK113-5D [MATα ura3-52 leu2::hisG his3::hisG]</td>
<td>[48]</td>
</tr>
<tr>
<td>THY.AP4</td>
<td>CEN.PK113-5D [MATα leu2 lacZ::trp1 lexA::HIS3 lexA::ADE2]</td>
<td>[46]</td>
</tr>
<tr>
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<td>CEN.PK113-5D [MATα leu2 trp1 his3 loxP::ade2]</td>
<td>[46]</td>
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<td>this study</td>
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<tr>
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<td>this study</td>
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<td>this study</td>
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<td>this study</td>
</tr>
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<td>this study</td>
</tr>
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<td>this study</td>
</tr>
<tr>
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<td>this study</td>
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<td>this study</td>
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<td>this study</td>
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<td>this study</td>
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<td>this study</td>
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<td>CEN.PK113-5D [MATα sfl1A]</td>
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<td>CEN.PK113-5D [MATα dip5::KanMX can1::STE2p-SpHIS5 lyp1::STE3p-LEU2 his3::HisG leu2::hisG]</td>
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<tr>
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<td>[34]</td>
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in excess. After steady state was reached, cellular dry weight, metabolite concentrations and gas profiles were monitored as described [39] every thirteenth generation. All parameters were constant throughout cultivation including biomass yield, glutamine limitation: 0.22±0.01 g dry weight/g glucose consumed and ammonium limitation: 0.22±0.01 and 0.21±0.01 g dry weight/g glucose consumed. Dry weight was determined in the efflux medium to test if cells accumulated because of flocculation in the bioreactor. Residual concentrations of glutamine were below detection level (<30 μM). In the ammonium-limited chemostat, the residual ammonium concentration was less than 50 μM throughout fermentation. Glucose and nitrogen consumption and metabolites (biomass, ethanol and glycerol) during chemostat cultivation showed no changes and the C:N ratio was thus constant over time.

Culture Media

Media for the chemostat cultivation was as described [39]. Standard yeast media, YPD and SC were as described [37]. All standard media were variations of synthetic dextrose medium with 1.7 g L−1 yeast nitrogen base without amino acids and ammonium sulphate, 20 g L−1 glucose, and 18 g L−1 agar. Low ammonium SLAD contained 0.05 mM ammonium sulphate as sole nitrogen source, while synthetic glutamine dextrose medium was supplemented with 100 mM glutamine as the sole nitrogen source.

Gene Expression Analysis

Cells grown in liquid YPD were harvested in mid-exponential phase by addition of an equal volume crushed ice and centrifugation. RNA was prepared by acid phenol extraction and hybridized to Agilent 60-mer yeast ORF expression microarrays as described [40]. Data were acquired using an Agilent scanner and extracted with Agilent software using standard settings. Ratios of mRNA levels in the descendants to the progenitor were sorted using a 3-fold cutoff (Fig. S2). The 106 resultant Log2 transformed transcript ratios were clustered by centered Pearson correlation. All raw microarray data are available from the Princeton Microarray Database [http://puma.princeton.edu].

Tiling Arrays and SNP Identification

Affymetrix Yeast Tiling arrays 1.0 were used. Total genomic DNA was hybridized to probes spaced every 5 bases and the data were analyzed as described [41,49].

Northern Blots

Northern blot analysis was performed with RNA from cells in YPD in mid-exponential phase. Total RNA was isolated using an acid-phenol extraction protocol [42] and 10 μg separated by electrophoresis, blotted and hybridized as described [43] with the following modifications: a probe was prepared from a PCR-amplified 621 bp FLO11 DNA fragment from the 5′-end of FLO11 (≤60% homologous to other regions in the S. cerevisiae genome). The probe was radioactively labeled with [γ-32P]-CTP using a Prime-It II Random Primer Labeling Kit (Agilent Technologies, USA) and purified from unincorporated nucleotides using Illustra ProbeQuan G-50 Micro Columns (GE Healthcare, UK). The membrane was pre-hybridized with Ambion ULTRAhyb (Life Technologies Ltd., UK) for 2 hours at 42°C before addition of labeled probe.

Intracellular Amino Acid Pools

Intracellular amino acids were extracted with trichloroacetic acid as described [44] and quantified by reverse-phase HPLC using an LKB-Alpha Plus amino-acid analyser [45].

Split-ubiquitin Interaction Assay

The split-ubiquitin interaction assay was essentially as described [46]. S. cerevisiae genes were amplified from genomic DNA [S288c] using gene-specific primers acaagtttgtacaaaaaagcaggctctccaaccac-cATG\_19-25 ORF and tcgcagcaccaacaacctcagttacagagttggg-ta\_19-27 ORF without stop. The Dip5 PCR product was cloned in vivo by homologous recombination in the THY.AP4 strain, carrying the plasmid pMet-YCg_t, to form the plasmid pMet-Dip5-Cgate. The THY.AP5 strain, carrying the plasmid pNXgate33-3HA, was transformed with PCR products of either HXT1, MEP1, MEP2, Dip5 or GnP1 to form strains with the plasmids pNY-HXT1-gate33-3HA, pNY-MEP1-gate33-3HA, pNY-MEP2-gate33-3HA, pNY-Dip5-gate33-3HA and pNY-GnP1-gate33-3HA. THY.AP4 and THY.AP5 are CEN.PK derivatives and transformants of THY.AP4 were selected on SC-leucine and transformants of THY.AP5 were selected on SC-tryptophan. Plasmids were isolated and amplified in Escherichia coli by standard methods and DNA insertion confirmed by PCR. Protein-protein interactions between Dip5, fused to the N-terminal half of ubiquitin (Dip5-CubPLV), and Hxt1, Mep1, Mep2, Dip5, or Gnp1, fused to the N-terminal half of ubiquitin (NubG-X), were determined by mating strain THY.AP4 (Dip5-CubPLV) to strain THY.AP5 (NubG-X). Diploids were selected on SC-leucine and -tryptophan and interaction between Dip5-CubPLV and each strain NubG-fusion was determined by growth after 3 days on reporter plates (SC+500 μM methionine and without leucine, tryptophan, adenine and histidine).

Pseudohyphal Transition, Invasive Growth and Biofilm Formation

Pseudohyphal growth assays were as described [3] and performed using diploid mutants. Invasive and adhesive growth was tested by incubating haploid cells on solid medium at 30°C for 2 days. Plates were photographed and a gentle stream of water used to rinse non-invasive cells from the plate. Plates were dried for 30 minutes and photographed again. Biofilm formation in polystyrene 96-wells plates was tested as described [3]. Biofilm formation assay was conducted with tp1/ auxotrophs (CEN.PK110-16D, RB52, RB157, RB196, RB209, 10560-2B; Table 2).

Supporting Information

Figure S1 Amino acid permeases are essential for invasive growth in S\textit{f}1\textit{Q}^{228STOP}, P\textit{tr}r\textit{g}^{474C…N} and \textit{Ss}1276b. The role of amino acid permeases on invasive growth was tested in several strains: a ura3 auxotroph version of the CEN.PK wildtype (CEN.PK113-5D), the mutants S\textit{f}1\textit{Q}^{228STOP} ura3 (RB26) and P\textit{tr}r\textit{g}^{474C…N} ura3 (RB429) and the \textit{Ss}1276b strain (10560-2B). In the \textit{ura3} S\textit{f}1\textit{Q}^{228STOP} background we deleted dip5 (RB317), gnp1 (RB197) and gnp1 (RB531). In the \textit{ura3} P\textit{tr}r\textit{g}^{474C…N} background we deleted dip5 (RB468), gnp1 (RB469) and gnp1 (RB547). In the \textit{Ss}1276b background, we tested invasive growth ability of dip5 (RB598), gnp1 (RB599), gnp1 (RB600) and gnp1 (RB157). All strains were grown as dot spots on rich complex medium (YPD) for 1 day at 30°C (A) and flushed with water to expose invasive growth (B). (TIF)

Figure S2 Microarray analysis of descendants after prolonged nitrogen limitation. Six single clones were collected after chemostat cultivation with a single limited nitrogen source of either glutamine or ammonium. All other nutrients were in excess. After growth to mid-exponential phase in liquid YPD, microarray analysis was performed. Gene expression data are shown for the glutamine-limited descendants S\textit{f}1\textit{Q}^{228STOP},
and Agrp1 (RB16) and for the ammonium-limited gap1 gdh D206E (RB16), Agrp1 gdh D206E (RB17) and no confirmed SNPs for RB15 ( ). Asterisk denotes descendants that grow invasively. Transcripts are clustered using Pearson correlation and data are presented as log₂-transformed ratios. The color code bars illustrate the fold-change in gene expression in descendants relative to the progenitor strain CEN.PK113-7D. (THF)

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Author Contributions
Conceived and designed the experiments: BR. Performed the experiments: RT SA DG. Analyzed the data: BR HDMI EB DO. Contributed reagents/materials/analysis tools: BR. Wrote the paper: BR HDMI.

References
