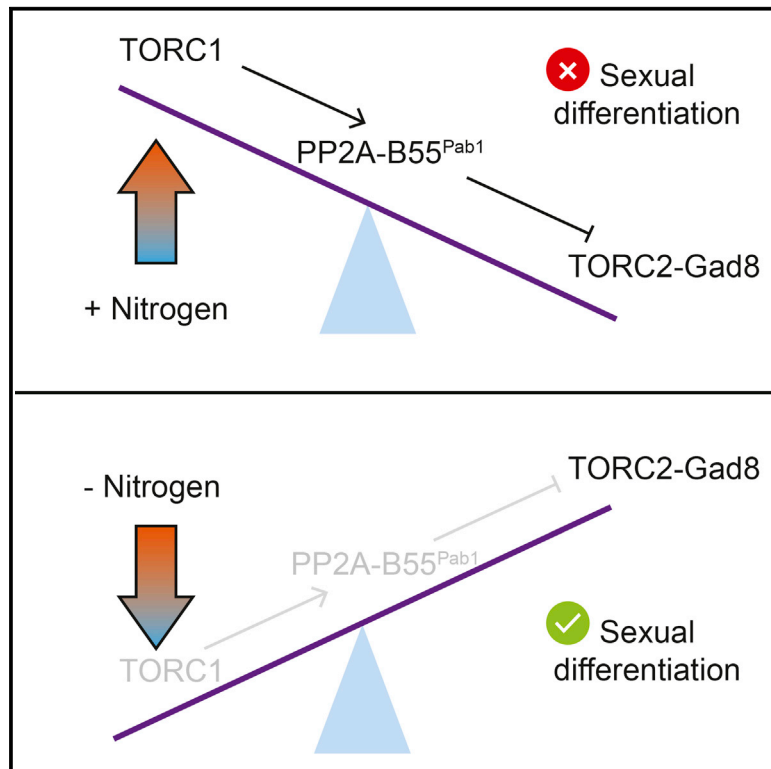


Current Biology

A PP2A-B55-Mediated Crosstalk between TORC1 and TORC2 Regulates the Differentiation Response in Fission Yeast

Graphical Abstract



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In Brief

TORC1 and TORC2 play opposite roles in the differentiation response of fission yeast. Here, Martín et al. show that PP2A-B55^{Pab1} coordinates the activities of these two modules in response to nitrogen availability, thus connecting nutritional status to cell-fate decisions.

Highlights

- PP2A-B55^{Pab1} regulates the differentiation response of fission yeast cells
- PP2A-B55^{Pab1} enables a crosstalk between TORC1 and TORC2
- TORC1 favors PP2A-B55^{Pab1} activity to prevent the hyperphosphorylation of Gad8
- TORC1 inactivation leads to PP2A-B55^{Pab1} inhibition, activation of Gad8, and differentiation

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A PP2A-B55-Mediated Crosstalk between TORC1 and TORC2 Regulates the Differentiation Response in Fission Yeast

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SUMMARY

Extracellular cues regulate cell fate, and this is mainly achieved through the engagement of specific transcriptional programs. The TORC1 and TORC2 complexes mediate the integration of nutritional cues to cellular behavior, but their interplay is poorly understood. Here, we use fission yeast to investigate how phosphatase activity participates in this interplay during the switch from proliferation to sexual differentiation. We find that loss of PP2A-B55^{Pab1} enhances the expression of differentiation-specific genes and leads to premature conjugation. *pab1* deletion brings about a transcriptional profile similar to TORC1 inactivation, and deletion of *pab1* overcomes the repression of differentiation genes in cells overexpressing TORC1. Importantly, we show that this effect is mediated by an increased TORC2-AKT (Gad8) signaling. Under nutrient-rich conditions, PP2A-B55^{Pab1} dephosphorylates Gad8 Ser546, repressing its activity. Conversely, TORC1 inactivation upon starvation leads to the inactivation of PP2A-B55^{Pab1} through the Greatwall-Endosulfon pathway. This results in the activation of Gad8 and the commitment to differentiation. Thus, PP2A-B55^{Pab1} enables a crosstalk between the two TOR complexes that controls cell-fate decisions in response to nutrient availability.

INTRODUCTION

Growing cells integrate a variety of cues in order to decide whether conditions are favorable for cell division or whether they have to halt their cell cycle and differentiate. Failure to do so has negative implications in the fitness of the organism. Notably, cancer pathogenesis is often associated with the poor capacity of cancer cells to differentiate.

The fission yeast *Schizosaccharomyces pombe* provides a good model to study how the nutritional status impinges on the differentiation response. *S. pombe* cells differentiate into conjugation-proficient forms if nutrients are scarce and a mating part-

ner is available. The process of conjugation and meiosis culminates in the formation of spores, which remain dormant until the nutritional conditions improve, when they germinate and resume their mitotic cycle (reviewed in [1]).

Central to these events lies the HMG-box transcription factor Ste11 [2]. Ste11 is essential for the expression of genes implicated in every step of the differentiation pathway [3]. Not surprisingly, Ste11 is subject to a very tight transcriptional regulation. In addition, posttranslational modifications prevent its untimely activation, only allowing it in response to starvation and during G1 phase of the cell cycle (reviewed in [4]).

Sensing whether the environment can provide the elements and the energy required for cell division is an essential aspect of the life cycle of any cell, and, consequently, the signaling pathways conveying this information are highly conserved through evolution. Particularly, target of rapamycin (TOR) signaling plays key roles connecting the environment with the molecular machinery that determines the behavior of the cell (reviewed in [5–7]).

Fission yeast contains two distinct TOR complexes, TORC1 and TORC2, each one with a different catalytic subunit (Tor2 for TORC1 and Tor1 for TORC2) (reviewed in [8]). The best characterized of the two is TORC1, which regulates ribosome biogenesis, protein translation, transcription, and autophagy, as well as cell-cycle progression [9–11]. The second TOR complex is less well understood. However, it is clear that the two TOR complexes have opposite effects in the differentiation response of fission yeast [11, 12], and a crosstalk between the two has been suggested [13].

Compared to protein kinases, the role of protein phosphatases in this context has been poorly explored, although they are excellent candidates to fine-tune cell-fate responses, and they are key players in the making of irreversible decisions during cell-cycle progression [14–16]. Here, we demonstrate that PP2A-B55 acting downstream of TORC1 modulates the activity of the TORC2-Gad8 module in order to prevent its differentiation-promoting functions under nitrogen-rich conditions.

RESULTS

Deletion of *pab1* Results in an Exacerbated Mating Response

PP2A and PP1 are the major phosphatases in the cell, and thus we focused on their potential role in sexual differentiation. To



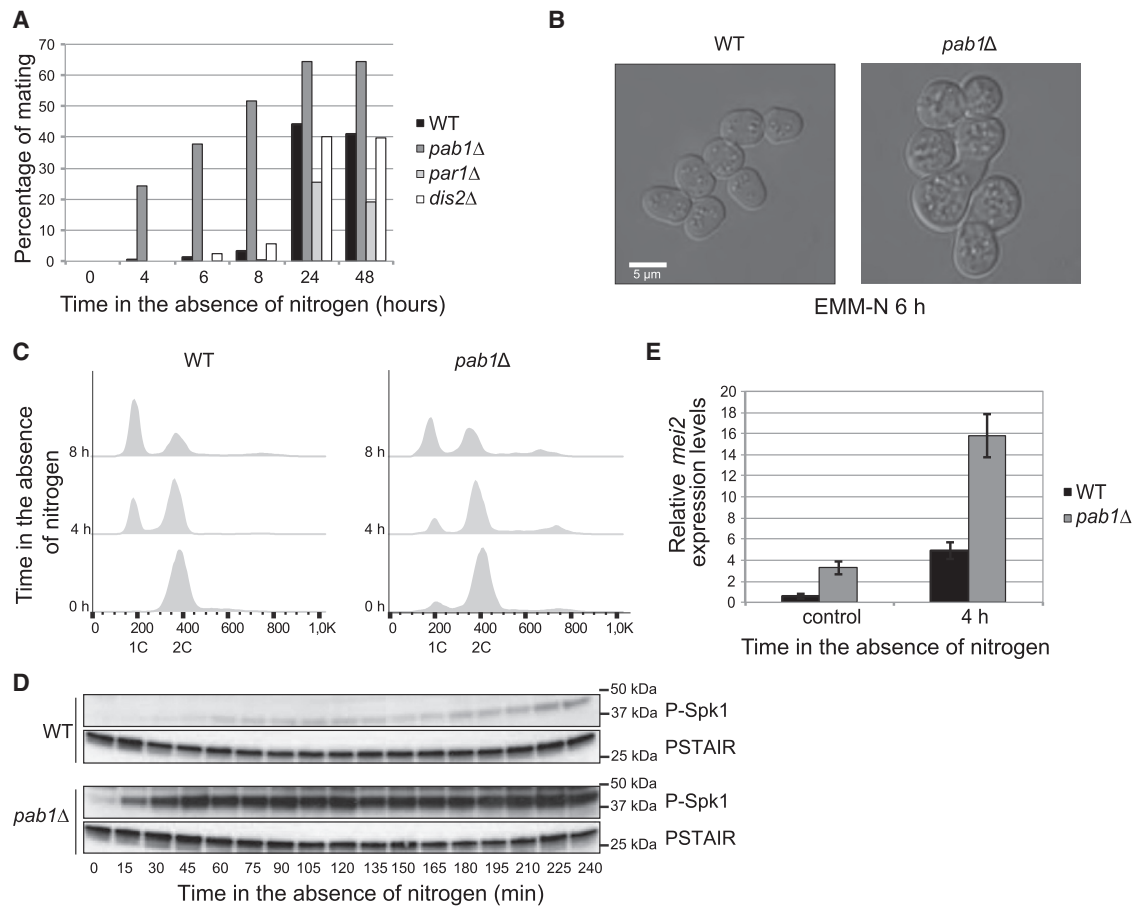


Figure 1. Deletion of *pab1* Results in an Exacerbated Mating Response

(A) Homothallic WT, *pab1Δ*, *par1Δ*, and *dis2Δ* cells were incubated at 25°C in the absence of nitrogen, and their mating ability was determined at 0, 4, 6, 8, 24, and 48 hr.

(B) DIC pictures of homothallic WT and *pab1Δ* cells in the absence of nitrogen for 6 hr.

(C) FACS analysis of the DNA content of homothallic WT and *pab1Δ* cells treated as in (A).

(D) Phosphorylation of the mating pheromone responsive MAPK Spk1 was used as a measure of the pheromone signaling in homothallic WT and *pab1Δ* cells starved for nitrogen. Cdc2 (PSTAIR) served as a loading control.

(E) mRNA expression of *mei2* in homothallic WT and *pab1Δ* cells that had been incubated for 4 hr in EMM (control) or EMM-N. Expression is relative to actin and was determined by qPCR. Mean and SEM of three biological replicates are shown.

See also Figure S1.

investigate the role of PP2A, we used deletion mutants in the genes encoding the two main regulatory subunits, B55 (also known as Pab1) and B56 (also known as Par1), which provide substrate specificity to the complex. For the analysis of PP1, we deleted the gene encoding the main catalytic subunit, Dis2.

Upon nitrogen depletion, homothallic wild-type (WT) cells rapidly initiated the mating response, and the first zygotes could be observed after 8 hr of starvation, with a peak of mating at 24 hr (Figure 1A). *dis2Δ* cells behaved almost indistinguishably to WT, whereas *par1Δ* cells showed a reduced and slower response. Strikingly, in the *pab1Δ* culture, mating products could be observed earlier and to a higher extent than in the WT strain, indicating an exacerbated response in this mutant (Figures 1A and B). This effect was only manifest in this condition (nitrogen starvation) as *pab1Δ* cells mated poorly upon glucose limitation.

Such phenotype could be due to an early G1 arrest upon nitrogen starvation. Cells with a short G2 phase require a longer resi-

dence in G1 in order to avoid excessive cell shortening, and therefore such cells are more prone to G1 arrest when nitrogen becomes limiting. PP2A-B55^{Pab1} inhibits entry into mitosis through the dephosphorylation of Wee1 and Cdc25 [17], [14], the two main regulators of Cdk during G2. As a result of a shorter G2 phase, the flow cytometry profile of exponentially proliferating *pab1Δ* cells showed a discrete G1 peak, indicating an expansion of this phase. Despite this, after nitrogen starvation the kinetics of G1 arrest was even slower than in a WT strain (Figure 1C), which might be due to a longer generation time.

In order to evaluate whether the premature mitotic entry could account for the increased conjugation efficiency of the *pab1Δ* strain, we compared it to that of a *wee1Δ* mutant. Similar to *pab1Δ* cells, the flow cytometry profile of *wee1Δ* cells showed a small G1 peak in nutrient-replete conditions. Upon nitrogen depletion, this strain arrested quickly in G1, but this did not correlate with an increased percentage of mating. Actually, its

conjugation efficiency was lower than that of a WT strain (Figure S1), an observation that had been documented before [18]. Hence, we conclude that although a premature mitotic entry prolongs G1 and can accelerate the G1 arrest, this cannot be the only reason for the mating phenotype observed in a *pab1Δ* mutant, especially if this cell-cycle effect occurs via Wee1 inhibition.

Further supporting our hypothesis of an additional, cell-cycle-independent, role of PP2A-B55^{Pab1} during differentiation, the activation of the mating pheromone cascade (as measured by phosphorylation of the MAPK Spk1) in the *pab1Δ* mutant was maximal just after 45 min of nitrogen starvation (Figure 1D). In the case of the WT strain, a faint signal became apparent only after 3 hr of treatment, a time when a large proportion of cells were already arrested in G1. In good agreement with these results, expression of the Ste11 target *mei2* under nitrogen-rich conditions was higher in the *pab1Δ* strain compared to the WT, and it was induced more rapidly and to a higher level upon shift to medium without nitrogen (Figure 1E).

Deletion of *pab1* Leads to Major Transcriptional Changes

Transcriptional adaptation is critical for an adequate mating response. For this reason and given the increased expression of *mei2* observed in *pab1Δ* cells, we decided to explore the transcriptional profile of this strain. Exponentially growing cells lacking *pab1* displayed striking changes in the set of genes that were basally expressed when compared to a WT strain. 240 genes were significantly upregulated at least 3-fold in the mutant, whereas 88 genes were more expressed in the WT strain (Figure 2A). Gene set enrichment analysis of the induced genes revealed over-representation of the GO biological processes “reproduction,” “meiotic cell cycle,” and “conjugation” among others (Figure 2A; Table S1) and for the gene expression terms “meiosis” and “reproduction modules,” “nitrogen depletion genes,” and “caffeine and rapamycin induced” (Table S1). Such genome-wide changes in the transcriptional profile could reflect a role of PP2A-B55^{Pab1} modulating the activity of the signaling pathways implicated in the mating response. In particular, given the enrichment in caffeine and rapamycin-induced genes, we paid special attention to genes affected by the two TOR signaling pathways (TORC1 and TORC2). Of the 240 genes upregulated in the *pab1Δ* mutant 63 were also found in the list of genes induced upon TORC1 inactivation (*tor2-ts6*) [10] (Figure 2B). As expected, these common genes showed further enrichment of the terms “Caffeine and rapamycin response,” “Conjugation,” “Meiosis reproduction module,” and “Ste11 targets.” Consistently, we could find significant overlap when comparing our list to the set of genes upregulated in a mutant of the SAGA complex component Gcn5, which regulates Ste11 [19] (Figure S2A). Additional comparisons to cell-cycle-regulated genes [20] were also done to rule out an effect of the cell-cycle distribution of our mutant in the expression profile (Figure S2B).

Finally, we compared the list of genes overexpressed in the *pab1Δ* mutant to the list of nitrogen-starvation-induced genes in a *tor1Δ* strain (TORC2 inactive) (Figure 2C). Consistent with previous findings that the two TOR complexes have opposite effects, many of the genes upregulated in *pab1Δ* and that are expressed in the WT strain upon nitrogen depletion depend on Tor1

for their induction, suggesting that the functions of both complexes converge in this response.

PP2A-B55^{Pab1} Functions Downstream of the TORC1 Preventing Sexual Differentiation

Having observed a considerable overlap between genes upregulated upon inactivation of Tor2 and genes upregulated in a *pab1Δ* strain, we decided to analyze in more detail the relationship between these proteins. Overexpression of *tor2* results in a profound mating defect, with no obvious effect on the ability to arrest the cell cycle in G1 upon nitrogen deprivation [9]. Deletion of *pab1* suppressed this defect to a great extent, allowing conjugation of the *tor2* overexpressing cells to the same level as for the WT strain (Figure 3A). Moreover, this correlated with a complete rescue of the expression of *mei2* and with an increased pheromone signaling in this strain (Figures 3B and 3C). These results place PP2A-B55^{Pab1} downstream of TORC1 as an important mediator of its functions. Yet, the fact that conjugation of *nmt1-tor2 pab1Δ* cells was not as high as in the *pab1Δ* mutant suggests that additional effectors of Tor2 also contribute to the regulation of the mating response.

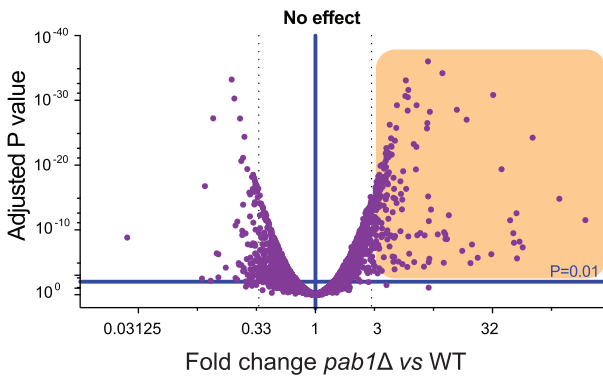
TORC1 is activated by the small GTPase Rhb1, which is, in turn, regulated by the Tsc1-Tsc2 GAP complex [21]. Deletion of *tsc2* impairs the inactivation of TORC1 after nitrogen starvation [22], and it was previously shown that *tsc2* mutants display a mating defect that depends on the cellular concentration at which cells were inoculated on a mating plate [23]. Under our experimental setup, *tsc2Δ* cells completely failed to mate (Figure 3D), and *mei2* expression was utterly abrogated (Figure 3E). Interestingly, we could observe this defect only in the nitrogen starvation response, as this mutant was proficient at mating under low glucose conditions (Figure S3A). Deletion of *pab1* could partially rescue this defect. The mating efficiency of the double mutant *tsc2Δ pab1Δ* was still lower compared to the WT strain, but, similar to the single *pab1Δ* mutant, these cells started to conjugate as early as after 4 hr of nitrogen starvation. Basal expression of *mei2* in the *tsc2Δ pab1Δ* homothallic strain was as high as in the *pab1Δ* mutant, and it increased upon nitrogen starvation, although not to the same level (Figure 3E). Similarly, we could observe early activation of the pheromone cascade, but it did not increase to the same extent as in the *pab1Δ* mutant (Figure 3F).

The fission yeast Tsc1-Tsc2 complex has been shown to have functions that are independent of TORC1 [24], and early studies suggested that the mating defect of the *tsc2Δ* mutant was the consequence of a deficient pheromone communication [23]. In agreement with a TORC1-independent conjugation defect in *tsc2Δ* cells, we could only marginally rescue the mating defect of this mutant by exogenously inactivating TORC1 with the temperature-sensitive allele *tor2-51* (data not shown).

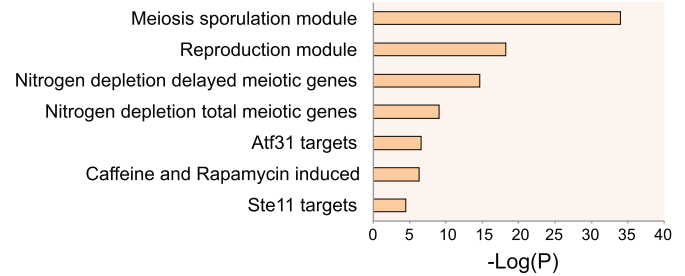
In order to evaluate whether a faulty pheromone signal transduction could be influencing the mating phenotype of the *tsc2Δ pab1Δ* mutant, we analyzed the effect of increasing the concentration of cells in the culture. While high cellular concentration did not have an impact in the mating efficiency of *tsc2Δ* cells, it improved the behavior of the *tsc2Δ pab1Δ* mutant, even at concentrations that were deleterious for the WT strain (Figure S3B).

Moreover, we analyzed *mei2* expression in heterothallic WT, *pab1Δ*, *tsc2Δ*, and *tsc2Δ pab1Δ* cells, in order to override the

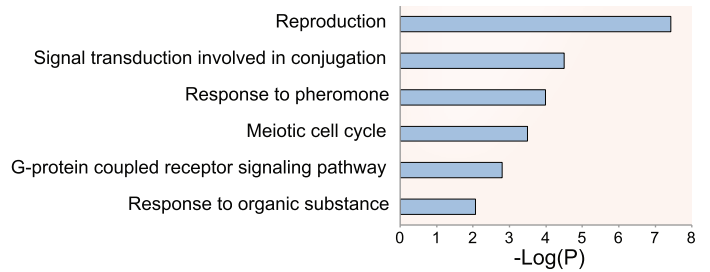
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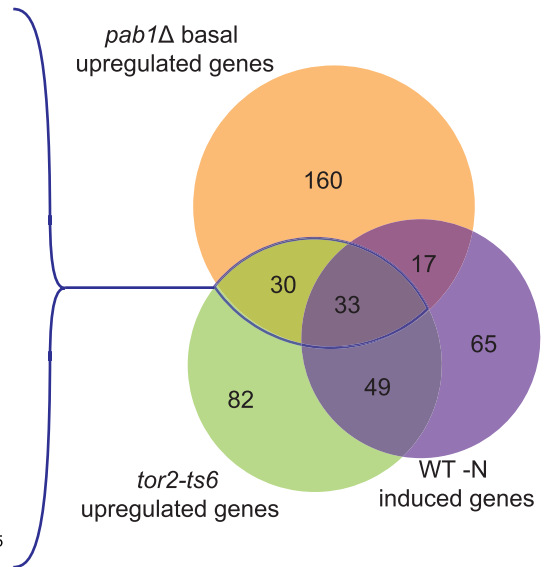
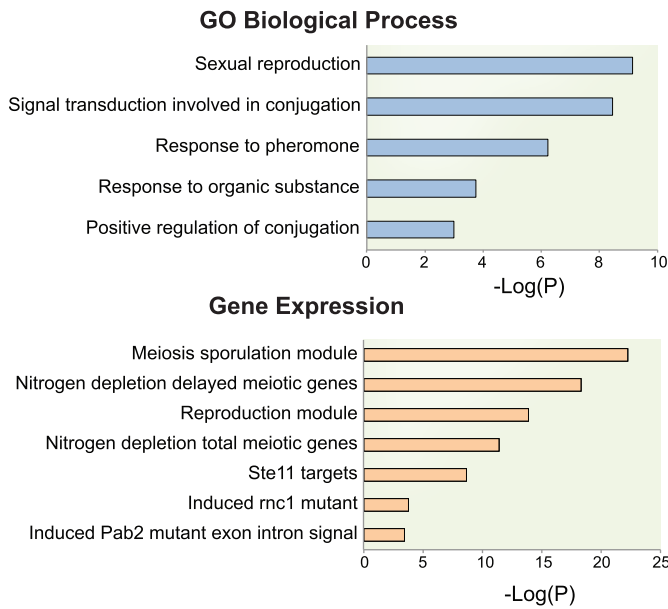
Gene Expression



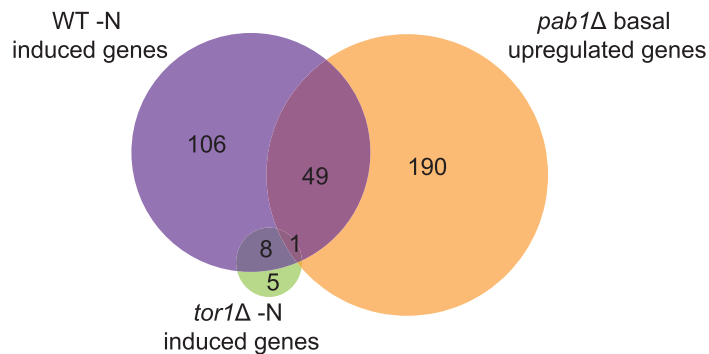
GO Biological Process



B



C



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induction due to activation of the pheromone pathway. In this background, *mei2* expression was generally lower, but now the double mutant *tsc2Δ pab1Δ* and the single *pab1Δ* mutant exhibited the same level of induction (Figure 3G). These results were also confirmed by the observation that heterothallic *tsc2Δ pab1Δ* and *pab1Δ* cells accumulated Ste11-GFP in the nucleus to the same extent, both in basal conditions and upon nitrogen depletion (Figure S3C). In WT and *pab1Δ* homothallic strains, Ste11-GFP nuclear accumulation was further enhanced and the cytoplasmic signal was reduced after nitrogen starvation. Nonetheless, the same did not hold true for the homothallic *tsc2Δ pab1Δ* mutant that behaved like its heterothallic counterpart (Figure S3C). Therefore, deletion of *pab1* only leads to a full rescue of the defects in *mei2* expression and Ste11 nuclear accumulation of *tsc2Δ* cells if pheromone signaling is prevented. These observations together with the partial rescue of the conjugation defect of the *tsc2Δ* mutant by either *pab1* deletion or TORC1 inactivation are indicative of additional targets of Tsc1/Tsc2 in the regulation of the pheromone signaling.

All in all, we conclude that PP2A-B55^{Pab1} is an instrumental element downstream of the signaling module composed of Tsc1/Tsc2-Rhb1-TORC1 regulating mating.

Nitrogen Starvation Leads to an Increased TORC2-Gad8 Signaling that Depends on the Inactivation of TORC1 and PP2A-B55^{Pab1}

After addressing the link between TORC1 and PP2A-B55^{Pab1}, we next explored the possibility of a crosstalk with the second TOR complex, TORC2, which is essential for the sexual differentiation pathway. While its role in promoting differentiation is central during nutritional stress, it should not take place under optimal growth conditions. Therefore, we hypothesized that nitrogen starvation would stimulate TORC2 signaling in order to favor its mating promoting activities.

To test this idea, we analyzed the level of phosphorylation of the AKT-like kinase Gad8 at Ser546 during nitrogen starvation. Gad8 is the only known effector of TORC2 and its phosphorylation is used as a readout of TORC2 signaling [25]. In a WT strain, the basal phosphorylation was low, and upon medium shift there was an initial drop before it started to increase, becoming maximal after 2 hr (Figures 4A and S4D). At later time points, the phosphorylation diminished, pointing at a transient nature of this signal (likely due to a negative feedback loop [26]). In a *pab1Δ* mutant, however, phosphorylation was already high in nitrogen-rich conditions, suggesting a negative role for PP2A-B55^{Pab1} on the TORC2-Gad8 pathway. Further reinforcing this idea, overexpression of *pab1* completely abrogated the

phosphorylation of Gad8 in response to nitrogen starvation (Figure 4B).

To confirm that this increased TORC2-Gad8 signaling in the *pab1Δ* mutant was a direct effect triggered by the loss of PP2A-B55^{Pab1} and not an adaptive mechanism of the cells bearing the *pab1* deletion, we constructed a conditional mutant of *pab1* (*nmt41-miniAID-pab1* [27, 28]). In this strain, addition of thiamine and auxin rapidly led to the almost complete disappearance of B55^{Pab1}, which was accompanied by an increase in the phosphorylation of Gad8 at Ser546, cell shortening, and the induction of *mei2* expression (Figures 4C–4E). Importantly, cell-cycle distribution was not affected in this mutant. If a G1 arrest was induced alongside B55^{Pab1} depletion, expression of *mei2* could be further potentiated (Figures 4F and 4G), indicating that the expansion of this cell-cycle phase in the *pab1Δ* mutant contributes to the transcription of mating genes.

Induction of Gad8 Ser546 phosphorylation prompted by the lack of nitrogen was a direct consequence of the inhibition of TORC1, as inactivation of TORC1 by means of the temperature-sensitive allele *tor2-51* [9] also produced the same effect (Figure 4H). Recently, Sergio Moreno's group has shown that in fission yeast TORC1 promotes the activity of PP2A-B55^{Pab1} through the repression of the PP2A-B55^{Pab1} inhibitory module constituted by the kinase Greatwall and its substrate Endosulfon (Igo1 in fission yeast) [15, 29]. Conversely, inactivation of TORC1 results in the repression of PP2A-B55^{Pab1} (Figure S4A) and accelerated mitotic entry [30]. In budding yeast, the homolog pathway (Rim15-Igo1/2) also controls PP2A-B55 in response to nutritional cues, affecting the expression of quiescence genes [31] and regulating cell-cycle progression through the stabilization of the CDK inhibitor Sic1 [32]. These observations led us to explore whether the Greatwall-Igo1 pathway is also required for the regulation of TORC2-Gad8. In order to address this possibility, we analyzed Gad8 phosphorylation upon inactivation of TORC1 (*tor2-51*) in *igo1Δ* cells. In this case, phosphorylation of Gad8 only marginally increased upon the temperature shift, but it was far from the level achieved in the single *tor2-51* strain. This also correlated with the inability of the double mutant to induce the expression of *mei2* (Figures 4H and 4I). Gad8 phosphorylation was not observed when mitotic entry was induced through Wee1 inactivation or in a WT strain under the same conditions (Figures 4H, 4I, and S4C), ruling out the possibility of its being a consequence of the cell-cycle effect elicited by the inactivation of TORC1 or by the temperature shift.

We then decided to analyze in more detail the kinetics of Gad8 phosphorylation upon TORC1 inhibition. In a WT strain, nitrogen starvation led to a fast drop in TORC1 activity (as judged by

Figure 2. Deletion of *pab1* Leads to Major Transcriptional Changes

(A) Volcano plot graph showing *pab1Δ* transcriptional profile in comparison to WT in nitrogen-rich conditions, the genes upregulated with an adjusted p value <0.01 and fold change >3 are highlighted. In the right panel, enrichment analysis of genes upregulated in *pab1Δ* by GO Biological Process and Gene expression is shown, (p = adjusted p value).

(B) Venn diagram illustrating the overlaps between *pab1Δ*-upregulated genes in nitrogen-rich conditions, WT-induced genes upon 4 hr of nitrogen starvation, and genes upregulated in a *tor2-ts6* strain at restrictive temperature. In both cases, there is significant overlap with a p value = 3.83e⁻²⁵ when comparing to the gene set induced in the WT strain in response to nitrogen starvation, and a p value = 1.75e⁻³⁷ when comparing to the gene set induced in the *tor2-ts6* strain. In the right panel, enrichment analysis of the genes shared in *pab1Δ* and *tor2-ts6* by GO Biological Process and Gene expression (p = adjusted p value).

(C) Venn diagram showing the overlaps between *pab1Δ*-upregulated genes in nitrogen-rich conditions, WT-induced genes, and *tor1Δ*-induced genes upon 4 hr of nitrogen starvation.

See also Figure S2.

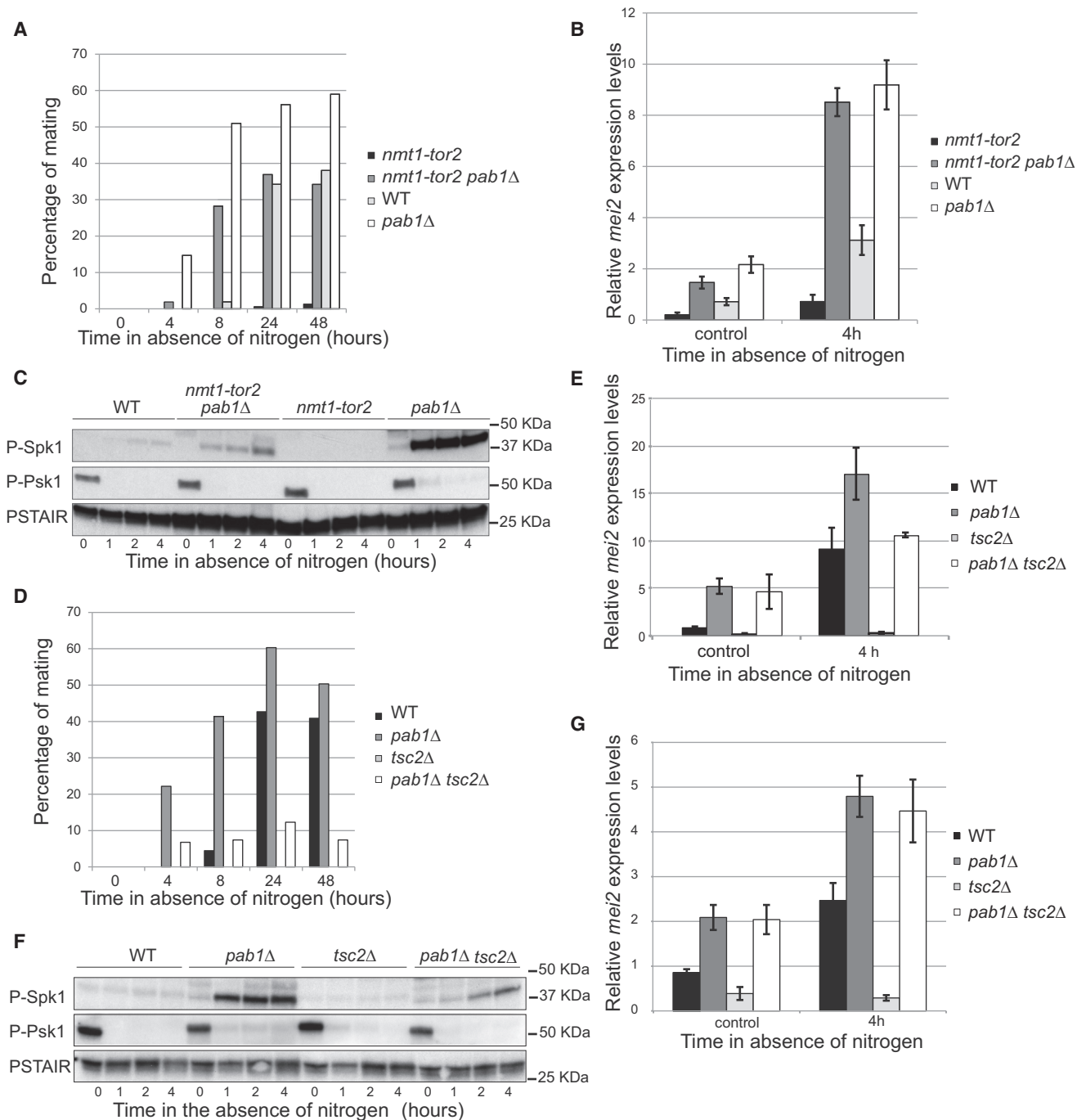


Figure 3. PP2A-B55^{Pab1} Functions Downstream of TORC1 Preventing Sexual Differentiation

(A) Homothallic WT, *pab1Δ*, *nmt1-tor2*, and *nmt1-tor2 pab1Δ* cells that had been grown in the absence of thiamine for 18 hr (to induce the overexpression of *tor2* in the *nmt1-tor2* strains) were incubated at 25°C in the absence of nitrogen, and their mating ability was determined at 0, 4, 8, 24, and 48 hr.

(B) mRNA expression of *mei2* in cells from (A) after incubation for 4 hr in EMM (control) or EMM-N. Expression is relative to actin and was determined by qPCR. Mean and SEM of three biological replicates are shown.

(C) Phosphorylation of the mating pheromone responsive MAPK Spk1 was used as a measure of the pheromone signaling in homothallic WT, *pab1Δ*, *nmt1-tor2*, and *nmt1-tor2 pab1Δ* cells (grown as in A) starved for nitrogen. Phosphorylated Psk1 was used as a measure of TORC1 activity. Cdc2 (PSTAIR) served as a loading control.

(D) Homothallic WT, *pab1Δ*, *tsc2Δ*, and *tsc2Δ pab1Δ* cells were incubated at 25°C in the absence of nitrogen and their mating ability was determined at 0, 4, 8, 24, and 48 hr.

(E) mRNA expression of *mei2* in cells from (D) after incubation for 4 hr in EMM (control) or EMM-N. Expression is relative to actin and was determined by qPCR. Mean and SEM of three biological replicates are shown.

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phosphorylation of the TORC1 substrate Psk1) and to the phosphorylation of Igo1 (Figure S4D). This did not lead to the immediate hyperphosphorylation of Gad8. Actually, Gad8 phosphorylation showed an initial drop before it started to increase and reached a maximal level after 105–120 min of treatment (Figure S4D). TORC2 activity has been shown to decrease in the early time points following nitrogen starvation [26], and this drop in Gad8 phosphorylation might be a reflection of this. In contrast, if the *tor2-51* allele was used to bring about TORC1 inactivation, Gad8 phosphorylation steadily increased as Igo1 phosphorylation became apparent (Figure S4E). Therefore, these experiments indicate that, while PP2A-B55^{Pab1} inhibition following TORC1 inhibition results in the hyperphosphorylation of Gad8, this event is delayed due to the inactivation of TORC2 in the early time points following nitrogen depletion.

All in all, we conclude that TORC1 and PP2A-B55^{Pab1} form a signaling module that prevents premature activation of the TORC2-Gad8 pathway during vegetative growth and that this regulation might explain the exacerbated mating phenotype in a *pab1Δ* strain.

Enhanced TORC2-Gad8 Signaling Contributes to the Premature Differentiation Response in *pab1Δ* Cells

To assess the above-mentioned hypothesis, we took two different approaches. First, we tested whether the constitutively active allele *ryh1QL* (an activator of TORC2 [25]) could rescue the *mei2* expression defect of the *tor2-51 igo1Δ* strain. Indeed, a triple mutant *tor2-51 igo1Δ ryh1QL* expressed *mei2* to the same level than the *tor2-51* mutant upon incubation at the restrictive temperature (Figures 5A and 5B).

Next, we investigated the effect of disrupting the TORC2-Gad8 pathway. We did this by treating the cells with the mTOR inhibitor Torin1, which targets both TOR complexes but that, in the context of nitrogen starvation, allowed us to observe the effect of inactivating TORC2, as TORC1 was already inhibited by the lack of nitrogen. Torin1 treatment quickly reduced Gad8 phosphorylation in both the WT and *pab1Δ* strains and completely abrogated the mating ability of both strains (Figures 5C and 5D). This was not a general effect of the drug, as it did not affect the mating ability of a *gcn5Δ* mutant or of a strain overexpressing *ste11* (Figures S5A and S5B). *Gcn5* and *Ste11* control sexual differentiation by directly modulating the expression of mating genes at the promoter level, and the fact that Torin1 did not inhibit conjugation in these strains indicates that they regulate events downstream of TORC2, as opposed to PP2A-B55^{Pab1}.

In line with this result, mutation of Gad8 Ser546 (*gad8Ser546Ala*) resulted in a decrease in the expression of *mei2* in the *pab1Δ* mutant, and in a major drop in its conjugation efficiency (to the same level as the single *gad8Ser546Ala* mutant) (Figures 5E and 5F). Similarly, a phospho-null mutant of the Gad8 substrate Fkh2 [33] (*fkh2Ser321Ala*) could also reduce *pab1Δ* mating ability to WT levels (Figure 5G). Mutation of Gad8 Ser546 had a stronger ef-

fect than mutation of Fkh2 Ser321 in the differentiation response of *pab1Δ* cells. In both cases, however, the effect of the mutation did not completely abrogate the phenotypes associated to *pab1* deletion (either *mei2* induction or mating), suggesting that PP2A-Pab1 and Gad8 regulate other targets involved in the mating response. Yet, the substantial reduction of the conjugation efficiency in the *pab1Δ gad8Ser546Ala* argues in favor of the idea that enhanced signaling of the TORC2-Gad8 module is a major contributor to the exacerbated mating phenotype of the *pab1Δ* mutant.

PP2A-B55^{Pab1} Negatively Regulates the Activity of the TORC2-Gad8 Pathway through the Direct Dephosphorylation of Gad8

Having characterized the events involving TORC1, PP2A-B55^{Pab1}, and TORC2-Gad8 from a genetic point of view, we wanted to analyze their interaction using a biochemical approach. Co-precipitation assays showed that PP2A-B55^{Pab1}, Tor1, and Gad8 could be found together in the cell (Figure 6A). PP2A-B55^{Pab1}-GFP localizes throughout the cell and is particularly enriched in the nucleus (Figure S6A), whereas Gad8 has been shown to localize mainly in the cytoplasm [25]. We also observed cytoplasmic localization of Gad8-mCherry, but inhibition of nuclear export with Leptomycin B led to an increased nuclear signal, indicating that a fraction of Gad8 shuttles between the nucleus and the cytoplasm (Figure S6B). While these results still leave open the question of where the interaction between these proteins occurs, they show that Gad8 localization in the cell is dynamic and consistent with its known nuclear functions.

We next addressed the possible mechanisms by which PP2A-B55^{Pab1} could affect the signaling of the TORC2-Gad8 module. We found that PP2A-B55^{Pab1} did not affect the interaction between Gad8 and TORC2, as neither the overexpression nor the depletion of B55^{Pab1} affected the interaction between the TORC2 component Sin1 and Gad8 (Figures 6B and 6C).

However, PP2A-B55^{Pab1} could proficiently reduce the phosphorylation of Gad8 at Ser546 in an in vitro assay, an effect that was reverted by the addition of okadaic acid (Figure 6D). Consistently, deletion of *pab1* also resulted in an enhanced activity of Gad8 toward a recombinant fragment of Fkh2 (Figure 6E). In this assay, Gad8S546A only phosphorylated the substrate feebly, and deletion of *pab1* did not have any significant effect on its activity. Therefore, these experiments confirm our genetic data and strongly suggest that PP2A-B55^{Pab1} can directly repress the activity of the TORC2-Gad8 module through the direct dephosphorylation of Gad8 at Ser546.

DISCUSSION

In fission yeast, nutritional sensing is intimately linked to sexual differentiation. Early studies of the fission yeast counterparts of mTOR, Tor2 as part of TORC1 and Tor1 as part of TORC2, revealed that the two play opposite roles in the mating response

(F) Phosphorylation of the mating pheromone responsive MAPK Spk1 was used as a measure of the pheromone signaling in cells in (D). Phosphorylation of Psk1 served as a readout for TORC1 activity. Cdc2 (PSTAIR) served as a loading control.

(G) mRNA expression of *mei2* in heterothallic WT, *pab1Δ*, *tsc2Δ*, and *tsc2Δ pab1Δ* cells treated as in (D) after incubation for 4 hr in EMM (control) or EMM-N. Expression is relative to actin and was determined by qPCR. Mean and SEM of three biological replicates are shown. See also Figure S3.

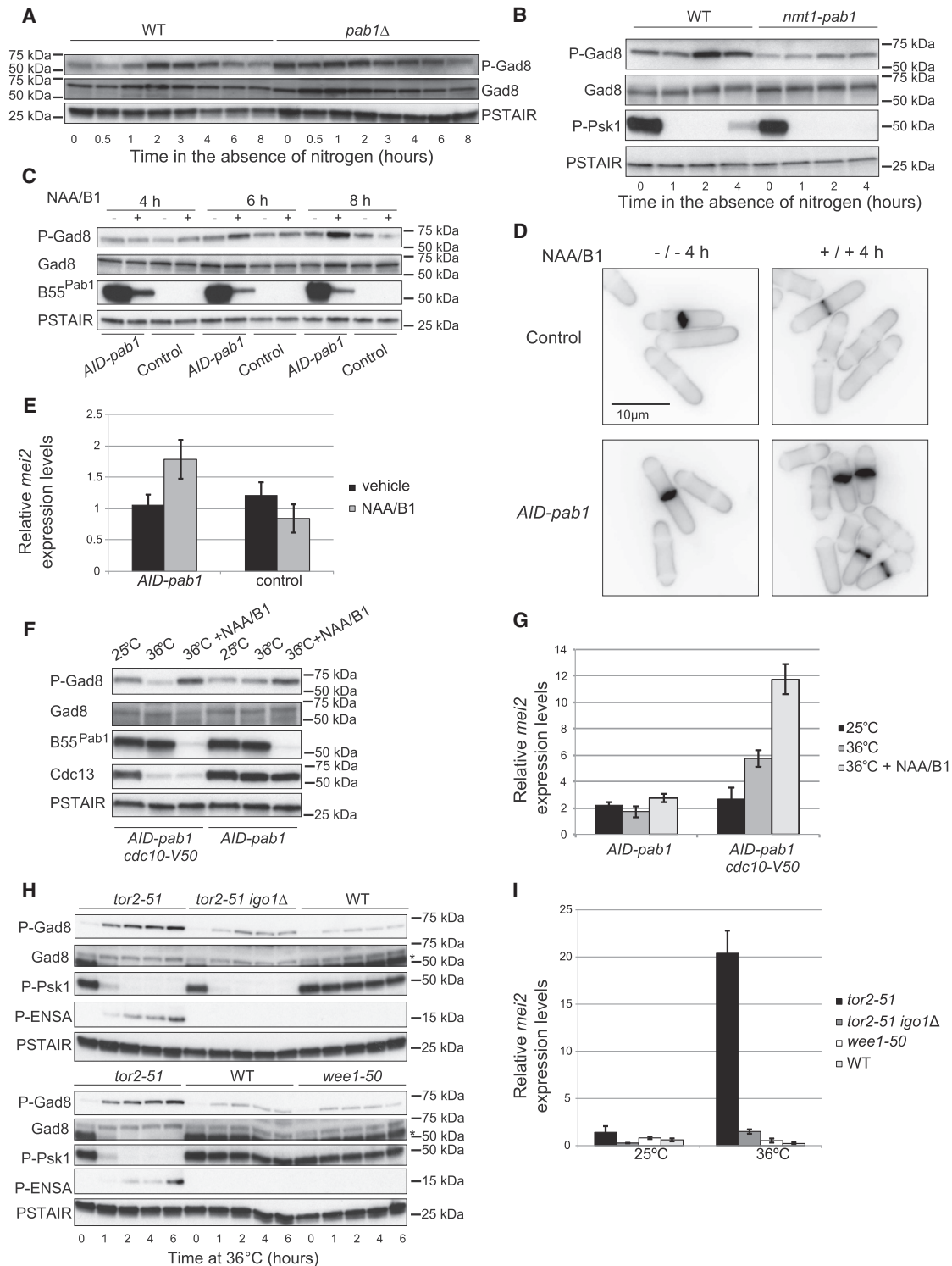


Figure 4. Nitrogen Starvation Leads to an Increased TORC2-Gad8 Signaling that Depends on the Inactivation of TORC1 and PP2A-B55^{Pab1}

(A) Homothallic WT and *pab1* Δ cells were incubated at 25°C in the absence of nitrogen, and samples were collected at the indicated time points. Phosphorylation of Gad8 at Ser546 was followed over the time course by western blot. Total Gad8 and Cdc2 (PSTAIR) served as loading controls.

(B) Homothallic WT and *nmt1-pab1* cells were grown in the absence of thiamine for 18 hr prior to shifting them to minimal medium without nitrogen (EMM-N). Samples were collected at the indicated time points and phosphorylation of Gad8 at Ser546 was monitored by western blot. Phosphorylation of Psk1 served as readout of TORC1 activity and total Gad8 and Cdc2 (PSTAIR) served as loading controls.

(C–E) Control cells (containing the auxin inducible degron background, *Padh15-skp1-At-Tir1-2NLS-Padh15-sk1-Os-Tir1*) and *nmt1-3PK-miniAID-pab1* cells in the same genetic background (referred to as *AID-pab1*) were treated with thiamine (0.5 μ M) and NAA (0.5 mM), and samples were collected at the indicated time

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[12]. TORC1 is active when in the presence of a rich nitrogen source, and its activity impedes the expression of genes required for differentiation [9, 10]. On the contrary, TORC2 activity is required for mating and cells deleted for *tor1* or its effector *gad8* are largely sterile [34]. Here, we show that the functions of the two TOR complexes are linked through the protein phosphatase PP2A-B55^{Pab1}.

Genetic studies show that PP2A-B55^{Pab1} lies downstream of TORC1, becoming downregulated as TORC1 activity drops (this work and [30]). Sergio Moreno's group showed that this leads to early mitotic entry due the premature CDK activation and to cell shortening, thus facilitating the extension of G1 [30]. TORC1 inhibition also results in major transcriptional changes and, expectedly, we observed significant overlap between the transcriptional profile of a *pab1Δ* and a *tor2-ts6* allele [10]. Still there was also a considerable amount of non-overlapping expression between our list of genes and the genes induced by TORC1 inactivation in the study carried by Matsuo and colleagues. This discrepancy can be explained from a technical point of view, as the latter study was done using microarrays while we used RNA sequencing, and this is a far more sensitive technology. Moreover, other differences in the experimental design could also have influenced the results. In our case, we analyzed cells permanently deleted for *pab1* grown at 25°C in Edinburgh minimal medium (EMM), while Matsuo et al. grew their cells in YES and transiently inactivated the *tor2-ts6* allele at 34°C.

Importantly, we observed that, as TORC1 becomes inactivated, the activity of the TORC2-Gad8 branch is enhanced through a mechanism that depends on PP2A-B55^{Pab1} inactivation (Figure 7). Nonetheless, during growth on nitrogen-rich medium (when TORC1 and PP2A-B55^{Pab1} are active) not all TORC2-Gad8 activity is affected by PP2A-B55^{Pab1}, as some Gad8-Ser546 phosphorylation can be detected. It was recently shown that glucose activates the TORC2-Gad8 module [35, 36]. As our experiments were done in the presence of glucose, the basal activity that we observe might be a reflection of this regulation. In addition to this, it is possible that only a certain pool of TORC2-Gad8 is subject to PP2A-B55^{Pab1} regulation. TORC2 and Gad8 are required for the cellular response to stress and DNA damage, gene silencing, and telomere maintenance [33, 37–39], but also for the expression of mating genes. This latter activity only has a purpose during nitrogen starvation, when the differentiation program is engaged. Coincidentally,

our RNA sequencing (RNA-seq) data revealed that this is the function of Tor1 that becomes enhanced upon loss of PP2A-B55^{Pab1}. Why and how PP2A-B55^{Pab1} regulates only specific functions of the TORC2-Gad8 pathway are outstanding questions, and in the future a thorough characterization of common interactors should help address them.

PP2A-B55^{Pab1} has been mainly studied in the context of cell-cycle regulation, and indeed we observed that loss of *pab1* has a strong effect on cell size and cell-cycle distribution. This on its own does not explain the exacerbated mating phenotype of *pab1Δ* cells, because depletion of B55^{Pab1} could lead to the induction of mating genes even in cells already arrested in G1. Interestingly, we found that a longer residence in G1 was instrumental for the expression of differentiation genes. Hence, downregulation of PP2A-B55^{Pab1} results in a double effect: on the one hand, it favors the G1 arrest; on the other hand, it enhances the activity of TORC2-Gad8 resulting in the expression of mating genes.

In budding yeast, downregulation of PP2A-B55^{Pab1} by the Greatwall-Endosulfon pathway promotes transcription of genes involved in quiescence upon nutritional stress [31], as well as gametogenesis [40]. While the function of budding yeast TORC2 has not been linked to sexual differentiation, overexpression of its substrate YPK2 (homolog to Gad8) induces filamentous growth, which constitutes another form of differentiation that is initiated upon nutritional stress [41].

YPK2 and Gad8 belong to the family of AGC kinases. TORC2 signaling through these kinases is widely conserved across species, with mTORC2 regulating AKT, SGK1, and PKC α . Of the three, Gad8 resembles SGK1 the most. mTORC2 and SGK1 have been involved in the regulation of T cell differentiation [42–44], as well as in adipocyte differentiation [45]. However, SGK1 functions differ depending on the cell type and its overexpression has also been shown to promote cell proliferation and to dictate resistance to anti-AKT therapies in breast cancer cell lines [46]. Importantly, a large cohort study revealed that deletion of *PPP2R2A* (the gene encoding the human B55 alpha subunit of PP2A) was often associated to breast cancer occurrence [47]. Although the mechanisms of action in mammalian cells are undoubtedly more complex, it is most likely that the crosstalk revealed in this work is conserved through evolution and could help explaining the role of PP2A-B55 and SGK1 in these different cellular contexts.

points. (C) Gad8 phosphorylation at Ser546 was monitored by western blot. Depletion of B55^{Pab1} was detected by western blot against its N-terminal 3PK tag. Total Gad8 and Cdc2 (PSTAIR) served as loading controls. (D) Mock-treated and thiamine and NAA-treated cells were stained with Calcofluor to visualize the septa. (E) mRNA expression of *mei2* after 6 hr in the presence of thiamine and NAA. Expression is relative to actin and was determined by qPCR. Mean and SEM of three biological replicates are shown.

(F and G) *nmt41-3PK-miniAID-pab1* and *nmt41-3PK-miniAID-pab1 cdc10-V50* cells were incubated at 36°C and treated with thiamine (0.5 μ M) and NAA (0.5 mM) for 6 hr or mock treated. Control cells were kept at 25°C for the same time. (F) Gad8 phosphorylation at Ser546 was monitored by western blot. Depletion of B55^{Pab1} was detected by western blot against its N-terminal 3PK tag. Cdc13 was used to monitor the cell-cycle arrest. Total Gad8 and Cdc2 (PSTAIR) served as loading controls. (G) mRNA expression of *mei2* in cells from (F). Expression is relative to actin and was determined by qPCR. Mean and SEM of three biological replicates are shown.

(H and I) *tor2-51*, *tor2-51 igo1Δ*, WT, and *wee1-50* cells were grown at 25°C (permissive temperature) and then shifted to 36°C in order to inactivate TORC1 and Wee1. Samples were collected at the indicated time points. For comparability between strains, the samples corresponding to the *tor2-51* mutant and the WT strain were run in the two gels. (H) Gad8 phosphorylation at Ser546 and Igo1 phosphorylation at Ser64 (P-ENSA) were detected by western blot. Psk1 phosphorylation was used as readout of TORC1 activity. Total Gad8 and Cdc2 (PSTAIR) served as loading controls. The asterisk in the Gad8 panels indicates remaining signal from the phosphorylated Psk1 western blot. (I) mRNA expression of *mei2* at permissive temperature and after incubation for 4 hr at 36°C. Expression is relative to actin and was determined by qPCR. Mean and SEM of three biological replicates are shown.

See also Figure S4.

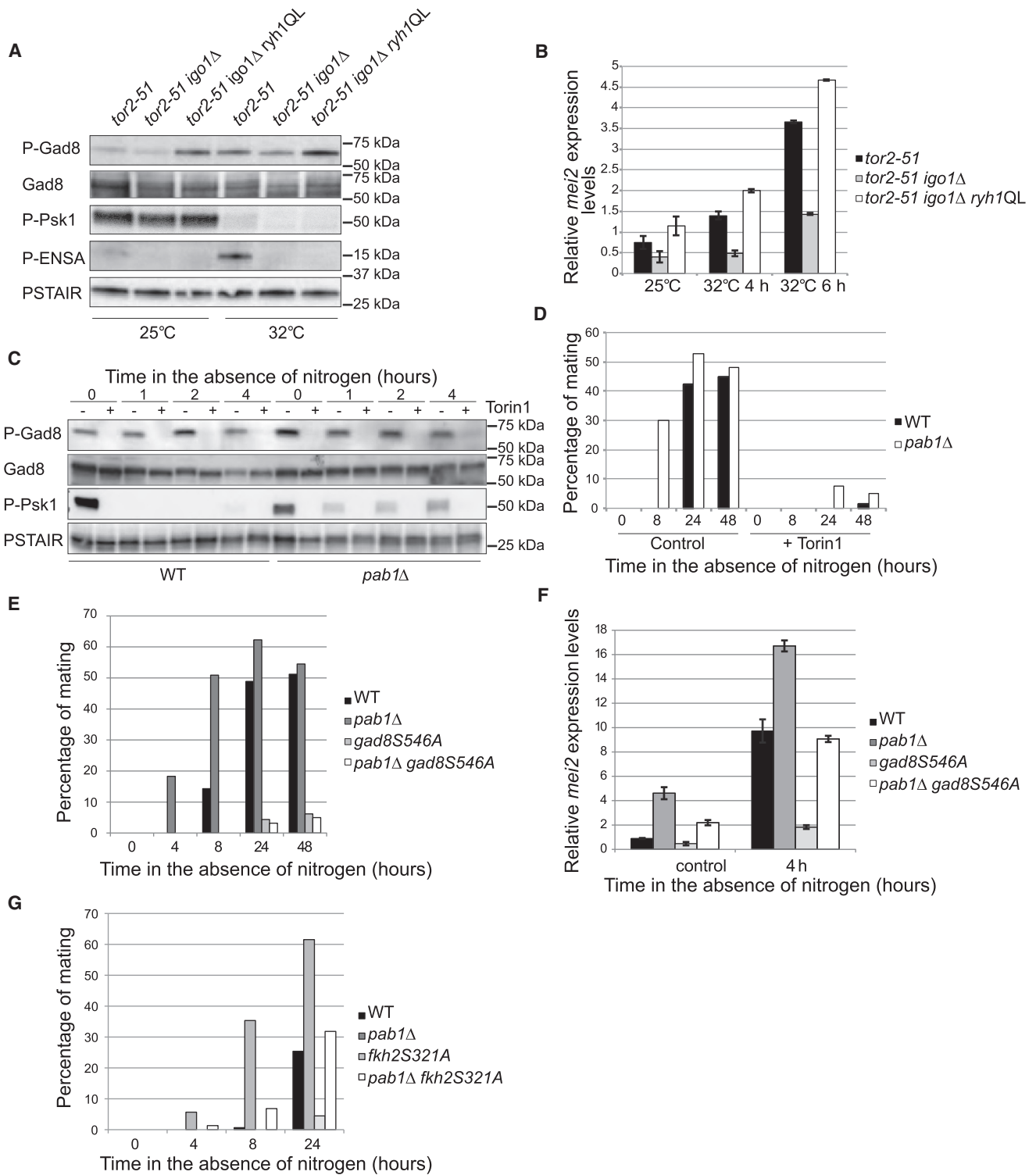


Figure 5. Enhanced TORC2-Gad8 Signaling Contributes to the Premature Differentiation Response in *pab1Δ* Cells
 (A and B) *tor2-51*, *tor2-51 igo1Δ*, and *tor2-51 igo1Δ ryh1QL* were incubated at 25°C (permissive temperature) or 32°C for 4 hr in order to inactivate TORC1. (A) Gad8 phosphorylation at Ser546 and Igo1 phosphorylation at Ser64 (P-ENSA) were detected by western blot. Psk1 phosphorylation was used as readout of TORC1 activity. Total Gad8 and Cdc2 (PSTAIR) served as loading controls. (B) mRNA expression of *mei2* at permissive temperature and after incubation for 4 and 6 hr at 32°C. Expression is relative to actin and was determined by qPCR. Mean and SEM of three biological replicates are shown. (C and D) Homothallic WT and *pab1Δ* cells were incubated at 25°C in the absence of nitrogen and in the presence of Torin1 (25 μM) or in its absence (treated with DMSO instead), and samples were collected at the indicated time points. (C) phosphorylation of Gad8 at Ser546 was monitored by western blot. Phosphorylation

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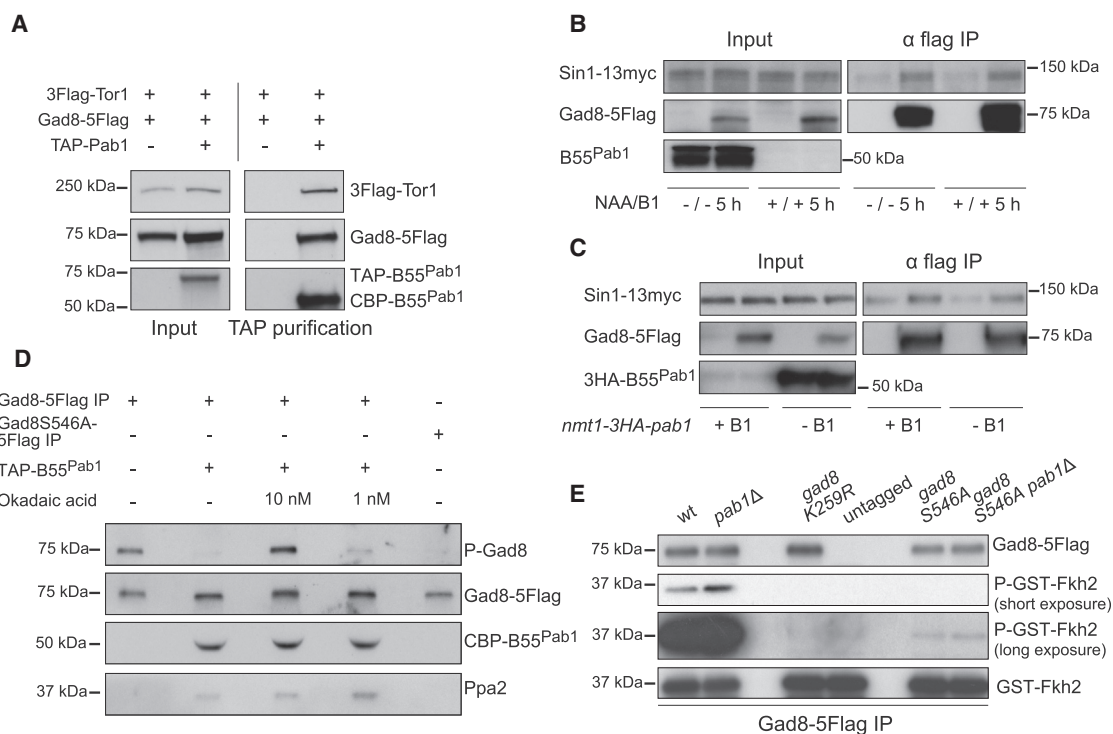


Figure 6. PP2A-B55^{Pab1} Interacts with Tor1 and Gad8 and Directly Dephosphorylates Gad8 at Ser546

(A) N-terminally TAP-tagged B55^{Pab1} was purified from cells expressing flag-tagged versions of Tor1 (3flag-Tor1) and Gad8 (Gad8-5flag). Western blot against the flag tag was used to detect Gad8 and Tor1 that co-purify with Pab1. A strain expressing 3flag-Tor1 and Gad8-5flag was used as a negative control in the TAP-purification. B55^{Pab1} was monitored by western blot against calmodulin-binding peptide (CBP).

(B) *nmt1-3PK-miniAID-pab1 sin1-13myc gad8-5flag* cells and *nmt1-3PK-miniAID-pab1 sin1-13myc* cells (all containing the auxin inducible degron background) were mock treated or treated with thiamine (15 μM) and NAA (0.5 mM) for 5 hr in order to deplete B55^{Pab1}. Gad8 was immunoprecipitated via its C-terminal 5flag tag, and the interaction between Sin1 and Gad8 was subsequently assessed by western blot against the flag tag and the myc tag.

(C) *nmt1-3HA-pab1 sin1-13myc gad8-5flag* cells and *nmt1-3HA-pab1 sin1-13myc* cells were grown in the absence of thiamine for 18 hr in order to induce the overexpression of *pab1*. Gad8 was immunoprecipitated via its C-terminal 5flag tag, and the interaction between Sin1 and Gad8 was subsequently assessed by western blot against the flag tag and the myc tag. Pab1 overexpression was monitored by western blot against the 3HA tag.

(D) PP2A- B55^{Pab1} purified from *par1Δ* cells via an N-terminal TAP tag in B55^{Pab1} was used in a phosphatase assay using as substrate immunopurified Gad8-5flag. Phosphorylation of Gad8 at Ser546 in the reactions was monitored by western blot. Okadaic acid (1 and 10 nM) was added in two of the reactions in order to show that the dephosphorylation observed was PP2A dependent. Immunoprecipitated Gad8 Ser546Ala was also used as control for the non-phosphorylated form of Gad8. Western blots against the flag tag (Gad8), the CBP tag (B55^{Pab1}), and against Ppa2 (the major catalytic subunit of the complex) served as controls for the purifications and showed the even presence of each component in the reactions.

(E) Gad8-5flag or Gad8S546A-5flag was immunopurified from WT and *pab1Δ* cells and used in a kinase assay against a recombinant fragment of Fkh2 (Gln291-Pro370) fused to GST. Gad8K259R-5flag (kinase dead) was used as negative control. Phosphorylation of this fragment was determined by western blot using an antibody that recognizes the phosphorylated AKT consensus sequence (anti-PAS) and was used as readout of Gad8 activity. Western blots against the flag tag (Gad8) and the GST tag (recombinant Fkh2) served as controls for the purifications.

See also Figure S6.

Here, we show that activation of Gad8 results from the repression of PP2A-B55^{Pab1} activity by the Greatwall-Endosulfon pathway. Interestingly, a recent study showed that overexpression of Greatwall brings about the phosphorylation of

AKT Ser473 during tumor formation by enhancing the degradation of the phosphatase PHLPP [48]. While the mechanism of action described in the current work is different from this, both works underscore the importance of Greatwall in

of Psk1 served as readout of TORC1 activity. Total Gad8 and Cdc2 (PSTAIR) served as loading controls. (D) The mating counting indicates that Torin1 completely abrogates the mating ability of both WT and *pab1Δ* cells.

(E and F) Homothallic WT, *pab1Δ*, and *pab1Δ gad8Ser546Ala* cells grown in EMM were shifted to medium devoid of nitrogen (EMM-N) at 25°C and samples were collected at the indicated time points. (E) mating counting indicates that mutation of Gad8 Ser546Ala completely abrogates the exacerbated conjugation of *pab1Δ* cells. (F) mRNA expression of *mei2* before and upon shift to EMM-N. Expression is relative to actin and was determined by qPCR. Mean and SEM of three biological replicates are shown.

(G) Homothallic WT, *pab1Δ*, *fkh2Ser321Ala*, and *pab1Δ fkh2Ser321Ala* cells were incubated at 25°C in the absence of nitrogen, and their mating ability was determined at the indicated time points. The mating counting indicates that mutation of Fkh2 Ser321 to Ala reduces the enhanced mating phenotype of *pab1Δ* cells.

See also Figure S5.

Working model

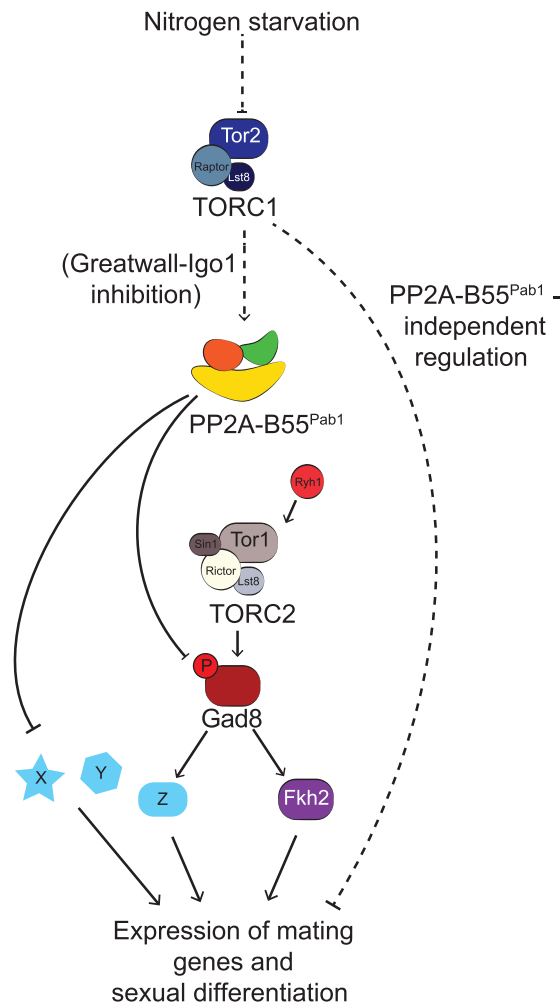


Figure 7. Working Model

PP2A-B55^{Pab1} represses the activity of the module TORC2-Gad8 by directly reverting the phosphorylation of Gad8 at Ser546. In nitrogen-rich conditions, this mechanism prevents untimely expression of mating genes. Upon nitrogen starvation, TORC1 inactivation leads to the inhibition of PP2A-B55^{Pab1}, through a mechanism that depends on the Greatwall-Endosulfen pathway (Ppk18-Igo1). Consequently, PP2A-B55^{Pab1} can no longer exert its negative effect on the TORC2-Gad8 module, which results in the increased phosphorylation of Gad8 at Ser546 and its enhanced activity toward Fkh2Ser321. Phosphorylation of Fkh2 (and probably other unknown targets of Gad8) leads to the induction of the expression of mating genes. Other unknown targets of PP2A-B55^{Pab1} might also participate in the transcriptional regulation of mating genes and sexual differentiation. The relative importance of Gad8 compared to other targets cannot be evaluated with current data. This effect is aided by other events also regulated by TORC1, e.g., cell shortening and G1 arrest (see main text for details).

regulating AKT signaling through the repression of protein phosphatases.

Finally, is TORC2-Gad8 the only target of PP2A-B55^{Pab1} in the mating response? Most likely other targets of PP2A-B55^{Pab1} also influence it. Clearly, the cell-cycle role of PP2A-B55^{Pab1} contributes to it. Moreover, the fact that a *pab1Δ gad8 Ser546Ala*

mutant still showed some induction of *mei2* (although its conjugation ability was dramatically reduced) would also indicate it, at least for some aspects of this response. Actually, for a robust effect, one would anticipate that Gad8 and PP2A-B55^{Pab1} share substrates, forming a coherent feedforward loop. In the future, more exhaustive studies of PP2A-B55^{Pab1} and Gad8 targets will be fundamental to understand how these signaling nodes modulate the differentiation process.

EXPERIMENTAL PROCEDURES

Cell Culture and Growth

The strains used in this study are listed in [Supplemental Experimental Procedures](#). All strains were prototroph, and all experiments were performed using early exponential cells grown in EMM containing NH₄Cl 93.5 mM as the source of nitrogen without supplemented amino acids [49]. For nitrogen starvation experiments, cells were collected by filtration and washed with three volumes of EMM without NH₄Cl (EMM-N) before resuspending them in EMM-N. Control cells were washed with EMM and resuspended in fresh EMM.

When using temperature-sensitive alleles, cells were grown at 25°C before shifting them to the restrictive temperature of 36°C in all cases except for the triple mutant *tor2-51 igo1Δ ryh1QL*, in which Tor2 was inactivated at 32°C, as the *ryh1QL* allele does not tolerate the temperature shift to 36°C.

Mating Assays

Mating assays were performed in liquid culture. Briefly, early exponential cells grown at 25°C in EMM were collected by centrifugation and washed with three volumes of EMM-N before resuspending them in EMM-N to a final concentration of 5 × 10⁶ cells/mL. These cultures were subsequently incubated at 25°C, and samples were collected at the indicated time points. After gentle sonication the number of zygotes (Z) and tetrads (T) per 300 non-mating cells (C) were counted and mating efficiency was calculated using the following formula:

$$\frac{(Z+T) \times 2}{(Z+T) \times 2 + 300} \times 100.$$

Flow Cytometry Analysis

DNA content was determined through fluorescence-activated cell sorting (FACS) analysis of propidium-iodide-stained cells according to published protocols [50].

Cell Imaging

For blankophor staining, 3 mL of cells in exponential phase were washed and resuspended in 3 μL of 50 μg/mL blankophor (Bayer) and 2 μL of PBS. Cell imaging was carried out using a Cell Observer High Speed fluorescence microscope (Zeiss) equipped with a Hamamatsu ORCA-Flash 4.0 camera and a Plan/Apo 100× (numerical aperture [NA] 1.46) oil objective. For fluorescence imaging GFP-specific and m-Cherry specific filters were used. All images were acquired with Zen software and treated using Fiji software (<http://fiji.sc/>) and Adobe Photoshop CS6 (Adobe Systems).

Genetic Manipulation, Strain Construction, and Plasmids

Gene deletion, promoter exchange, and gene tagging were carried out using PCR cassettes amplified from pFA6a derivative plasmids. More information about plasmid construction, specific phosphomutants, and the NTAP-tagged *pab1* strain can be found in the [Supplemental Experimental Procedures](#). Double mutants were constructed by genetic cross and tetrad dissection.

Additional information on protein and RNA methods can be found under [Supplemental Experimental Procedures](#).

ACCESSION NUMBERS

The accession number for the raw data files reported in this paper is ArrayExpress: E-MTAB-4106.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, Supplemental Experimental Procedures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2016.11.037>.

AUTHOR CONTRIBUTIONS

R.M., M.P., and N.C. performed experiments. R.M., M.P., M.N.-A., and S.L.-A. generated reagents. S.L.-A., R.M., and M.P. designed experiments. J.M. analyzed the RNA-seq data. All authors contributed to the interpretation of the results. S.L.-A. wrote the manuscript.

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