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Crosstalk between cAMP-PKA and MAP kinase pathways is a key regulatory design necessary to regulate *FLO11* expression

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Abstract

Signal transduction pathways crosstalk with one another and play a central role in regulation of cellular events. Crosstalk brings complexity to the system, and hence, a systematic analysis of these crosstalks helps in relating the signaling network structure to its function. Here, we present a modular steady state approach to quantify the network comprising of cAMP-PKA and MAP kinase pathways involved in the regulation of *FLO11*, a gene which is required for pseudohyphae growth in *Saccharomyces cerevisiae* under nitrogen starvation. These two pathways crosstalk by converging on the same target, i.e., *FLO11* and through Ras2p, an upstream activator of both cAMP and MAPK pathway. Analysis of crosstalk at the gene level revealed that cAMP-PKA and MAPK pathways are indispensable to *FLO11* expression. The dose response was highly sensitive and primarily controlled by cAMP-PKA pathway. We demonstrate that the highly sensitive response in the cAMP-PKA pathway was due to crosstalk and inhibitor ultrsensitivity, key regulatory designs present at the downstream of cAMP-PKA pathway. The analysis of the role of Ras2p in the crosstalk between the cAMP-PKA and MAPK pathways indicated that crosstalk essentially helped in amplification of the Gpa2p signal, another upstream activator of the cAMP-PKA pathway. However, the effect of crosstalk due to Ras2p on *FLO11* expression was minimal under normal activation levels of Ras2p. Whereas, the crosstalk itself can bring about *FLO11* expression under the hyperactivated Ras2p conditions thereby eliminating the requirement for the other activator Gpa2p. We also observed the presence of system level properties such as amplification, inhibitor ultrasensitvity and bistability, which can be attributed to the regulatory design present in the *FLO11* expression system. These system level properties might help the organism to respond to varying nutritional status.

Keywords: Pseudohyphae growth; Crosstalk; Ultrasensitvity; Bistability; Signaling

1. Introduction

Sensing of the nutrients to respond rapidly to changes in the nutritional status is essential for the survival of micro-organisms. The signaling network utilized for this purpose senses the change, further transmitting the signal into the nucleus to regulate specific genes. The end result is a phenotypic response to the changing nutritional status [1]. The signaling network is typically complex comprising of different pathways and interactions. To obtain insight into the working of the intracellular networks, it is essential to analyze the roles and contributions of various interactions responsible in eliciting a specific phenotypic response. Here, we present a modular steady state approach to quantify the network comprising of cAMP-PKA and MAP kinase pathways involved in the regulation of *FLO11*, a gene which is required for pseudohyphae growth in *Saccharomyces cerevisiae* under nitrogen starvation.

S. cerevisiae can utilize a wide range of nitrogen sources, but not all of these are utilized with equal efficiency [2]. Nitrogen sources such as ammonia, glutamate and glutamine act as good nitrogen sources as they can support much higher growth rates than nitrogen sources considered to be poor, such as proline, arginine or urea. Under the limitation of nitrogen source, diploid yeast cells respond by switching to a filamentous growth called as pseudohyphae [3]. The switch from the budding yeast form to the pseudohyphal growth is accompanied by changes in distinct cellular processes in which the

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cells elongate, adopt a unipolar budding pattern, remain physically connected in chains and invade the agar [2]. In this phenotypic state, a cell wall protein encoded by *FLO11* is shown to be responsible for cell–cell adhesion, substrate adhesion and agar invasion [4].

FLO11 promoter is one of the largest and most complex in *S. cerevisiae* genome that integrates various signaling pathways [5]. The regulatory network that mediates *FLO11* gene expression involves two parallel pathways (Fig. 1). The first pathway includes a MAPK cascade which positively regulates the *FLO11* transcriptional activators Ste12p and Tec1p [6]. The second pathway implicated in *FLO11* expression is cAMP-PKA pathway, which positively regulates *FLO11* transcriptional activator Flo8p [1]. Both the transcriptional activators have distinct binding site on *FLO11* promoter [5]. Since the transcriptional activators of both the pathways are responsible for *FLO11* expression, the interactions from these transcriptional activators at gene level contribute crosstalk between the two pathways.

In MAPK pathway, the upstream signals consist of activation of Cdc42p by Ras2p (Fig. 2) [1]. The Cdc42p signal and Bmh1/2p then activate the core MAPK cascade [6,7]. The components of this cascade include the Ste20p, Ste11p, Ste7p, Kss1p kinases and the transcription factor Ste12p. In addition, another transcription factor, Tec1p, forms a heterodimer with Ste12p and regulates the expression of *FLO11* [1,4]. In cAMP-PKA pathway, the upstream signal consists of activation of adenylate cyclase by Gpa2p and Ras2p [1]. The synthesis of cAMP by adenylate cyclase positively modulates the PKA activity which in turn controls the activation of Flo8p, a transcriptional activator of *FLO11* [1]. It can be noted that Ras2p provides a positive feedback to the activation of MAPK pathway (Fig. 2). Ras2p also activates the cAMP-PKA pathway by adenylate cyclase activation thus constituting a second level of crosstalk at the upstream of the two pathways.

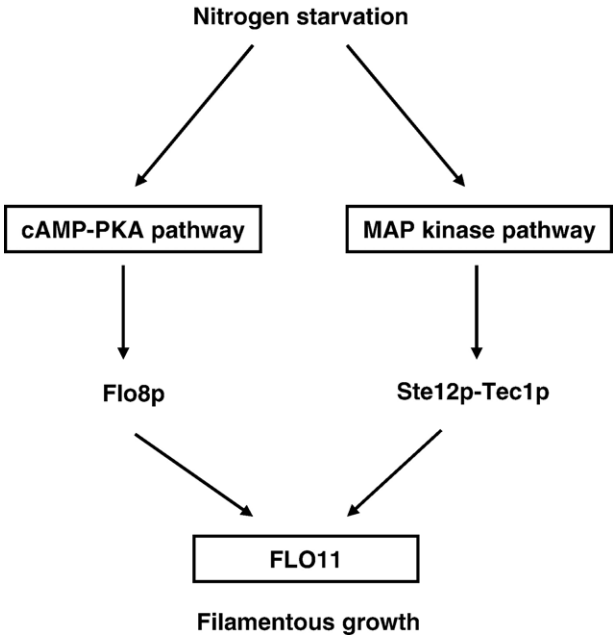

Fig. 1. The pathways involved in the regulation of *FLO11* in response to nitrogen starvation in yeast.

In the context of *S. cerevisiae* response to nitrogen starvation, there are some intriguing questions regarding the actual role played by these regulatory pathways and their components. What are the roles of the two crosstalks present to regulate on *FLO11* expression? For example, if both cAMP-PKA and MAPK pathway can activate *FLO11*, then which of these pathways exercise major control over *FLO11* expression? Furthermore, what is the contribution of Ras2p and Gpa2p in activating adenylate cyclase during nitrogen starvation leading to elevated intracellular cAMP levels?

The major objective of this work focuses on answering the above-mentioned intriguing questions with the help of steady state modeling of cAMP-PKA and MAPK pathways with respect to *FLO11* regulation. A steady state model was developed, to generate dose–response curves for fractional expression of *FLO11* to varying input protein stimuli. Also, we investigated the effect of inherent signaling structure made up of phosphorylation cycles, allosteric interactions and feedback loops on the system level response of upstream regulators on *FLO11* expression. The quantification of the crosstalk at the gene level revealed that *FLO11* expression was more sensitive with respect to the upstream regulator, adenylate cyclase of cAMP-PKA pathway, than the upstream regulator, Cdc42p of MAPK pathway. Further, Gpa2p was essential for activation of adenylate cyclase and the crosstalk by Ras2p contributed to the amplification of the signal from Gpa2p. The positive feedback loop on Ras2p in MAPK pathway exhibits a bistable response with respect to the protein Bmh1/2p. These input–output relationships were further tested at various concentration of signaling components and system parameters in order to monitor the range over which the feasible response persisted. System level properties such as bistability, robustness, signal amplification and ultrasensitivity were also observed in the *FLO11* signaling pathways.

2. Materials and methods

The regulatory network used for the steady state modeling is shown in Fig. 2. The schematic representation of various interactions in cAMP-PKA and MAPK pathway which has been very well reviewed [1], is provided in Supplementary Figs. S1 and S2. In cAMP-PKA pathway (Supplementary Fig. S1), adenylate cyclase acts as a receiver of the signal from the most upstream components and thereafter directing the physiological changes. Ras2p and Gpa2p are shown to be the upstream activator of adenylate cyclase. Mep2p, a high-affinity ammonium permease, serves as an ammonium sensor to regulate pseudohyphal growth and functions as an upstream regulator of Gpa2p [8–12]; however, the exact interaction mechanism remains unclear. The activated adenylate complexes produce cAMP (cyclic adenosine mono phosphate) from ATP which activates cAMP-dependent protein kinases PKA [1]. PKA complex consists of a tetramer of two regulatory units (Bcy1p) and two catalytic subunits in the inactivated state. The catalytic subunits are Tpk1p, Tpk2p and

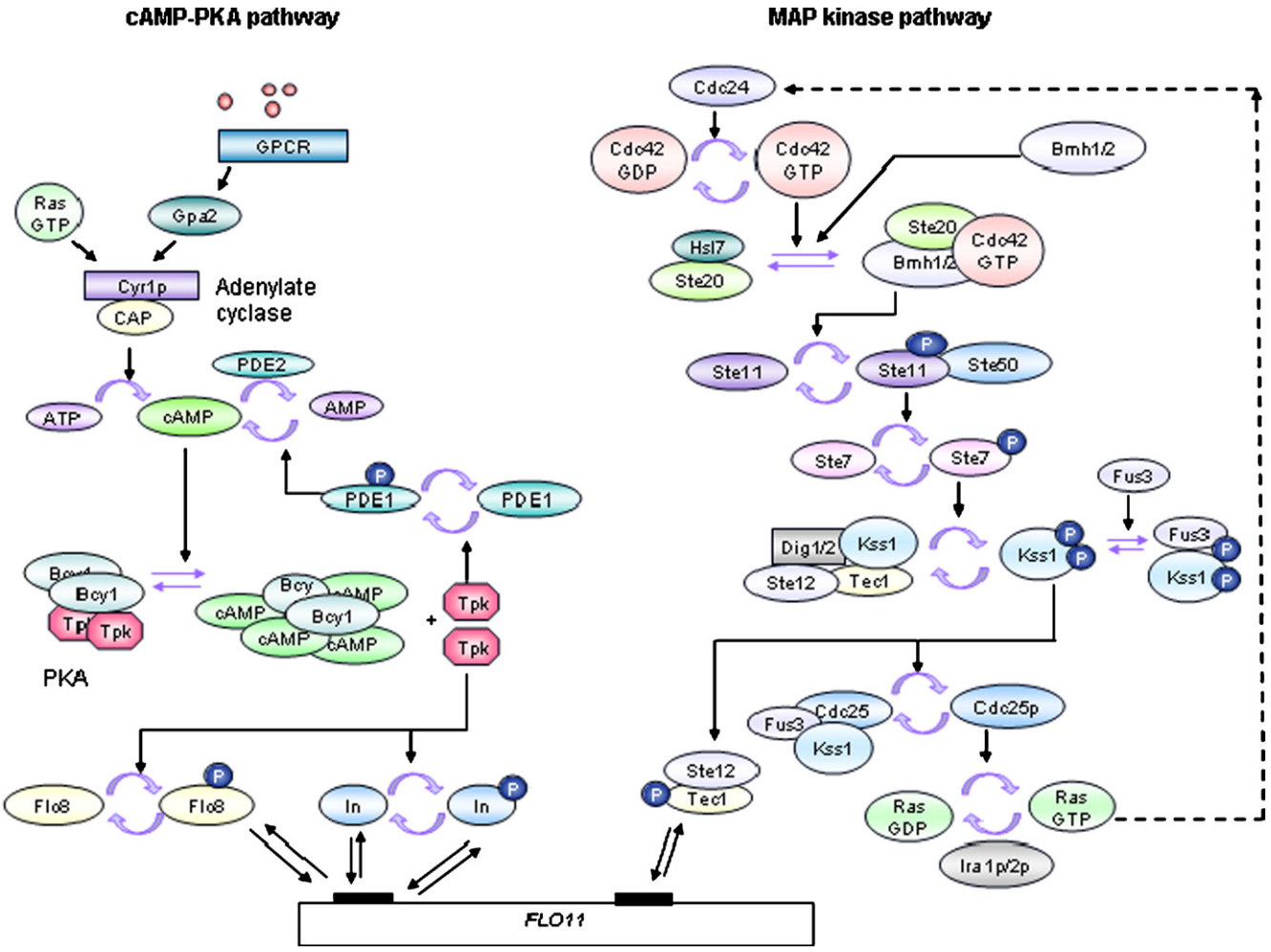

Fig. 2. A simplified molecular mechanism showing the signaling network involved in the filamentous growth in yeast. *cAMP-PKA signaling pathway*: The Cdc25 is activated by the Kss1 which in turn activates Ras2. Activated Ras2 and the Gpa2 combine with Adenylate cyclase to form the activated complex, which provides cAMP. cAMP combines with the PKA complex to release its catalytic subunits Tpk1/2/3. Tpk2 phosphorylates Flo8 and Tup1 inhibitor complex to activate the *FLO11* gene, which will cause filamentous growth. *MAP kinase signaling pathway*: The Cdc24 is activated by the Ras2, which in turn activates Cdc42 to its GTP bound form. Activated Cdc42 stabilized by the external signal from Msb2 relieves the Ste20 from the Hsl7 inhibition to form its activated form. Activated Ste20 activates MAPKKK Ste11 which in turn activates the MAPKK Ste7. Activated Ste7 phosphorylates MAPK Kss1p to relieve it from the Dig1/2p inhibition. Activated Kss1 then activates the *FLO11* activator Ste12-Tec1 which will cause filamentous growth. Further, there is a positive feedback on Ras2 via activation of Cdc25 by Kss1.

Tpk3p [1]. The cAMP binds to the regulatory units to release the catalytic subunits that act as a signal to the next module. Hydrolysis of cAMP by cAMP-phosphodiesterases Pde1p and Pde2p restores PKA to the inactive state. Pde1p is activated by the cAMP-PKA pathway, which hydrolyze cAMP to AMP; thus, there is a negative feedback on the levels of cAMP. The high-affinity Pde2p is only involved in the basal level control of the cAMP [13]. The activation of Pde1p has been suggested to be by direct phosphorylation or by the activation of some intermediate kinase [13]. In the current analysis, it was assumed that Pde1p is directly activated by Tpk1p and Tpk3p. Sfl1p-Tup1p-Ssn6p complex acts as a negative regulator of *FLO11* gene, which is phosphorylated by Tpk2p to inactivate its negative effect [14]. The analysis presented here assumes a mechanism in which the phosphorylation of the Sfl1p-Tup1p-Ssn6p by Tpk2p leads to decrease in the free Sfl1p-Tup1p-Ssn6p concentration resulting in dissociation of *FLO11*-Sfl1p-Tup1p-Ssn6p complex and rendering *FLO11* activation. Tpk2p also phosphorylates Flo8p, the transcriptional activator of *FLO11* [10].

The core MAPK module gets the signal from the Cdc42p which is activated by the upstream signal Cdc24p mediated by the Ras2p activation (supplementary Fig. S2) [1]. Cdc24p activates Cdc42p by facilitating the formation of its GTP bound form [1]. GAPs, Bem3p, Rga1p and Rga2p stimulate the hydrolysis of the Cdc42p-bound GTP to return it to an inactive GDP-bound state. Recently Paul et al. [15] identified a new component of the Cdc42p and MAPK-dependent signaling pathway that acts specifically in regulating filamentous growth in yeast termed as Msb2p. This interacts more strongly with the activated form of Cdc42p than with wild-type Cdc42p *in vivo*; thus, they suggested that Msb2p may stabilize Cdc42p in its activated form. The current analysis assumed that Cdc42p was already bound to Msb2 and hence exists in a stable form. The interaction of activated Cdc42p with Ste20p removes a negative regulator, Hsl7p to activate Ste20p [1]. Bmh1/2p is shown to

associate with Ste20p and is essential for the filamentous growth [1]. In this manner, Ste20p can activate the MAP kinase cascade formed by Ste11p, Ste7p and Kss1p. The Kss1p in its unphosphorylated form is able to interact with the transcription factor Ste12p [16] and with the negative regulators Dig1p (Rst1p) and Dig2p (Rst2p), thereby potentiating the Dig-mediated block of Ste12p activity. When Kss1p is phosphorylated by Ste7p, it is able to phosphorylate Ste12p and Dig1/2p, the Dig proteins dissociate and this allows derepression of the target genes to which Ste12p may bind. Ste12p activates the transcription of *TEC1* and also interact with the encoded protein [16]. Transcriptional activators Ste12p and Tec1p bind cooperatively to filamentous growth response elements (FREs), which have been identified in the *FLO11* promoter [5]. It is assumed that Ste12p and Tec1p are preassociated in a complex to activate the *FLO11*. Cherkasova et al. [17] have demonstrated that Kss1p and Fus3p act upstream of Ras2p to regulate its activation. The phosphorylated Kss1p activates Cdc25p which in turn activates Ras2p (Ras2p-GTP formation). The fractional transcriptional expression of *FLO11* was quantified by Eq. (1).

$$f_{\mathrm{FLO11}} = \frac{\mathrm{FLO11\_Flo8p\_Ste12p\_Tec1p}}{\mathrm{FLO11_t}} \tag{1}$$

FLO11_Flo8p_Ste12p_Tec1p represents *FLO11* with both Flo8p and Ste12p-Tec1p complex bound to it and $\mathrm{FLO11_t}$ is the total *FLO11* concentration. Binding of these activators to *FLO11* depends on activation of Flo8p and Ste12p-Tec1p complex, which are in turn under the control of cAMP-PKA and MAPK pathways, respectively. The fractional activation of Flo8p and Ste12p-Tec1p complex will give the measure of fractional activation of these pathways and could be varied to study the effect of these pathways on *FLO11* expression. The fractional activation of Flo8p is given by Eq. (2).

$$f_{\mathrm{Flo8p}} = \frac{[\mathrm{Flo8p}]_{\mathrm{p}}}{[\mathrm{Flo8p}]_{\mathrm{t}}} \tag{2}$$

where f_{Flo8p} is the fraction of the total Flo8p concentration in the phosphorylated state. Similarly, the fraction of the total Ste12p-Tec1p complex concentration in the activated (phosphorylated) state is given by Eq. (3).

$$f_{\mathrm{Ste12p\_Tec1p}} = \frac{[\mathrm{Ste12p\_Tec1p}]_{\mathrm{p}}}{[\mathrm{Ste12p\_Tec1p}]_{\mathrm{t}}} \tag{3}$$

The framework reported by Goldbeter and Koshland [18] was used to model the network at steady state and accordingly an equivalent rate constant and Michaelis–Menten constant nomenclature scheme was applied. The steady state equations for covalent modification cycles, equilibrium relationships for allosteric interactions, and mass balance equations for total species are listed in the supplementary material (Section A1). These equations were solved numerically using Fsolve program of MATLAB (The Math-Works Inc.).The simulations were carried out for estimating the fractional *FLO11* expression for particular Flo8p and Ste12p-Tec1p complex concentration. The accuracy of the simulation was verified by numerically checking the mass balance of all of the species. All component enzyme concentrations are represented with respect to whole cell volume. Most of the kinetic/equilibrium constants were taken from the literature. The reactants like ATP and PPi concentrations were assumed to be constant. The gene activator/inactivator dissociation constants were assumed to be in the range of values reported by Malkhey et al. [19]. The total component concentrations, rate constants, the Michaelis–Menton constant and dissociation constants are listed in the supplementary material (Section A2).

3. Results

3.1. Influence of cAMP-PKA pathway and MAP kinase pathway on FLO11 expression: crosstalk at gene level

The steady state model was used to quantify the *FLO11* expression with respect to transcriptional activators Flo8p and Ste12-Tec1p complex, which are the immediate upstream components from cAMP-PKA and MAPK pathways, respectively. Simulations were performed in which the concentrations of both the activators were varied individually while keeping the other fixed at a specific concentration to calculate the effect of the individual pathways on the fractional expression of *FLO11*. The *FLO11* expression increased with increase in activated Flo8p concentration, but the response was fairly invariant to different fixed concentrations of activated Ste12p-Tec1p complex (Fig. 3a). The response yielded a Hills coefficient of 4 with a half saturation constant ($k_{0.5}$) of 67 nM. On varying the activated Ste12p-Tec1p complex concentration at different fixed Flo8p concentration, the *FLO11* expression response was hyperbolic in nature with maximum expression of *FLO11* highly dependent on the amount of activated Flo8p concentration (Fig. 3b). The response curve saturated below 90% *FLO11* expression at lower activated Flo8p concentrations indicating that Flo8p concentration was limiting. Only beyond 135 nM of Flo8p concentration, 90% expression could be achieved, yielding a Hill coefficient of 0.8 and a half saturation constant ($k_{0.5}$) of 10 nM.

The network was also analyzed for *FLO11* expression with respect to the upstream activators, i.e., adenylate cyclase from the cAMP-PKA pathway and Cdc42p from the MAPK pathway. Fig. 4a shows the output response with respect to variation in adenylate cyclase concentration at different fixed concentration of Ste12p-Tec1p complex. It was clear from the figure that the overall response was not dependent on the activated Ste12p-Tec1p complex concentration. This behavior was similar to that observed with downstream element of the same pathway (Fig. 3a). The response with respect to adenylate cyclase concentration had a Hill coefficient of 4 with a half saturation constant ($k_{0.5}$) of 15 nM .This indicated that the response was amplified by 4-fold (since the $k_{0.5}$ for Flo8p was

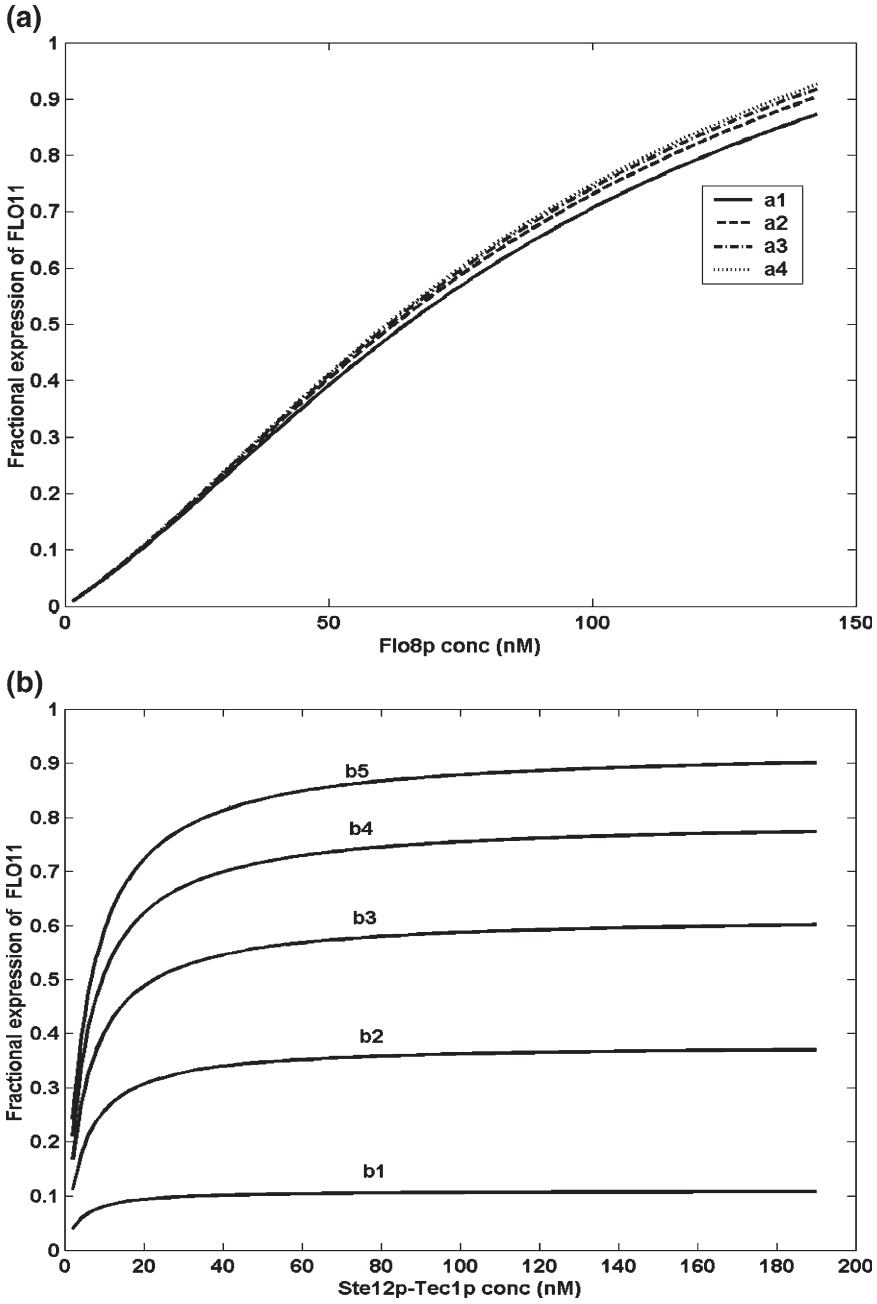

Fig. 3. Fractional response of *FLO11* with varying downstream regulators Flo8p (cAMP-PKA pathway) and Ste12p-Tec1p (MAPK pathway). (a) *FLO11* activation with changing Flo8p concentration at various fixed values of Ste12-Tec1p concentrations, a1 (60 nM), a2 (100 nM), a3 (140 nM) and a4 (180 nM). (b) *FLO11* activation with changing Ste12pTec1p concentration at various fixed values of Flo8p concentration, b1 (15 nM), b2 (45 nM), b3 (75 nM), b4 (105 nM) and b5 (135 nM).

67 nM), with respect to upstream element as compared to the downstream element. Furthermore, the dose–response curves for *FLO11* expression with respect to Cdc42p were also evaluated (Fig. 4b). The response was highly dependent on the activated Flo8p concentration as seen in the case of the downstream element of MAPK pathway (Fig. 3b). Even in this case, Flo8p concentration was limiting and 135 nM of Flo8p was essential for greater than 90% expression of *FLO11*. The concentration required for 90% expression of *FLO11* with respect to the downstream activator Ste12p-Tec1p complex was 10 nM, whereas for the upstream activator Cdc42p, it was only 5 nM. Hence, 2-fold amplification was observed with respect to the upstream activator.

Fig. 5 shows a dose–response surface of *FLO11* expression over the entire concentration range of the upstream components Cdc42p (MAPK pathway) and adenylate cyclase (cAMP-PKA pathway). It was clear from the surface plot that the *FLO11* expression primarily varies with the adenylate cyclase concentration over the entire range (0–30 nM) and was less dependent on Cdc42p. However, at lower values of Cdc42p (less than 10 nM), the expression gets restricted to 20–40% over the entire range of adenylate cyclase concentration. The

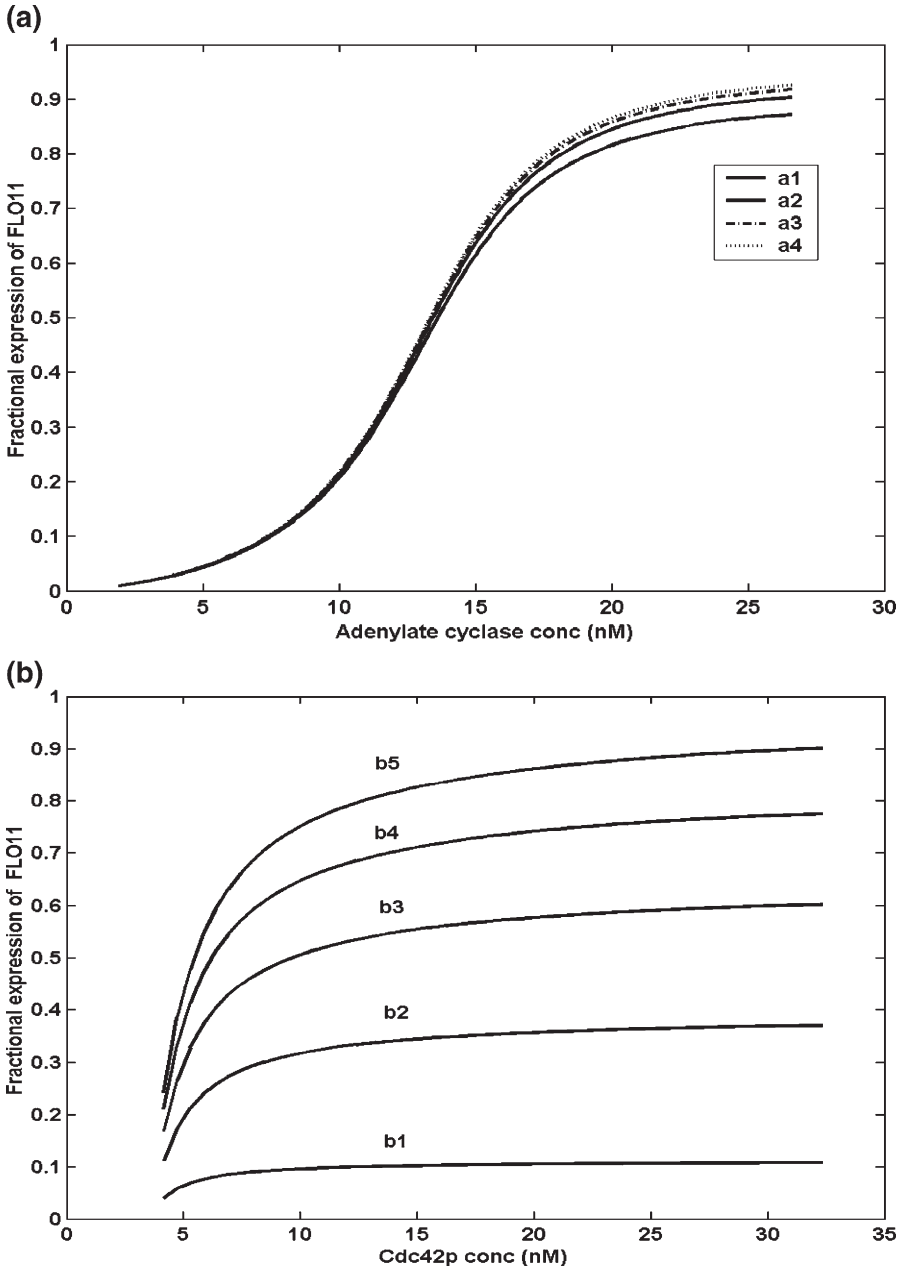

Fig. 4. Fractional response of *FLO11* with respect to upstream regulators Adenylate cyclase (cAMP-PKA pathway) and Cdc42p (MAPK pathway). (a) *FLO11* activation with changing upstream activator adenylate cyclase concentration at various fixed values of Ste12p-Tec1p concentrations, a1 (60 nM), a2 (100 nM), a3 (140 nM) and a4 (180 nM) (b) *FLO11* activation with changing upstream activator Cdc42p concentration at various fixed values of Flo8p concentration, b1 (15 nM), b2 (45 nM), b3 (75 nM), b4 (105 nM) and b5 (135 nM).

fractional expression of *FLO11* was represented by a multiplicative product of two separate functionalities involving adenylate cyclase (adc) and Cdc42p as described below:

$$f_{\mathrm{FLO11}} = \left[\frac{\mathrm{adc}^{\mathrm{nh1}}}{\mathrm{adc}^{\mathrm{nh1}} + k_{\mathrm{adc}}^{\mathrm{nh1}}}\right]\left[\frac{\mathrm{cdc42p}^{\mathrm{nh2}}}{\mathrm{cdc42p}^{\mathrm{nh2}} + k_{\mathrm{cdc42p}}^{\mathrm{nh2}}}\right] \quad (4)$$

where, k_{adc} (average value $\approx$ 14 nM) and k_{cdc42p} (average value $\approx$ 2 nM) are half saturation constants for the fractional activation of cAMP-PKA pathway with respect to adenylate cyclase and fractional activation of MAPK pathway with respect to Cdc42p, respectively. The exponents $\eta h_1 = 4$ and $\eta h2 = 0.8$ represent approximate Hill coefficients for the dose–response curves of fractional *FLO11* expression to adenylate cyclase and Cdc42p concentration, respectively.

3.2. Contribution of Ras2p and Gpa2p in activating adenylate cyclase during filamentous growth: crosstalk due to Ras2p

Previously, Ras1p and Ras2p were thought to be the main regulators of cAMP in *S. cerevisiae* [1]. During nitrogen starvation, the Ras2p, in addition to activating adenylate cyclase, induces filamentous growth by stimulating the

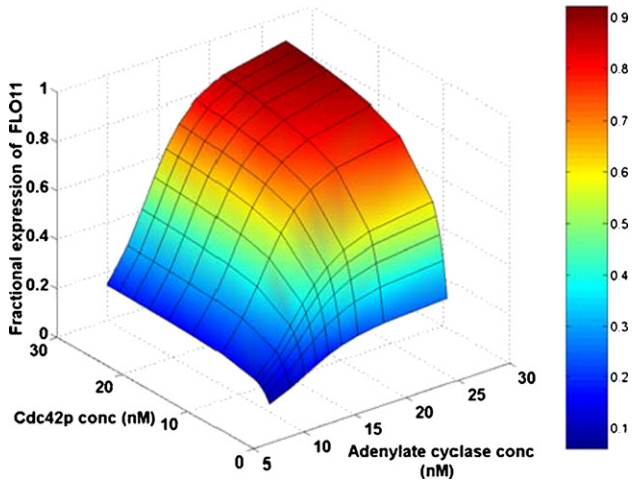

Fig. 5. The surface plot of *FLO11* expression over the entire concentration range of the upstream components Cdc42p (MAPK pathway) and Adenylate cyclase (cAMP-PKA pathway). The concentrations of *FLO11* activator Flo8p (10–150 nM) and Ste12p-Tec1p (1–200 nM) were independently varied to simulate *FLO11* activation. Simulated dose–response surface obtained was plotted with respect to the corresponding concentrations of the upstream regulators Cdc42p (MAP kinase pathway) and Adenylate cyclase (cAMP-PKA) pathway to get the global response. The color variation represents the fractional activation of *FLO11*; red represents activation levels greater than 90% whereas deep blue represents activation levels less than 10%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MAPK pathway [1]. Genetic studies have shown that Gpa2p also plays a role in the regulation of cAMP levels in yeast filamentous growth [20,21]. At this juncture, it is relevant to raise the question regarding the relative contributions of Ras2p and Gpa2p in the regulation of elevated cAMP levels observed during filamentous growth (Fig. 6a). Since Ras2p is involved in the activation of adenylate cyclase (cAMP-PKA pathway) and MAPK pathway, the effect of crosstalk between these pathways were also studied. Colombo et al. [22] have shown that a maximum of 3-fold change in Ras2p activation was observed on glucose addition to derepressed cells. This 3-fold change was assumed to be the maximum fold change possible in Ras2p activation in the current analysis. Also, Ras2p was assumed to be activated only by MAPK pathway through feedback mechanism [18,23].

Based on these assumptions, the simulations were carried out by varying the concentration of *FLO11* activators Flo8p (cAMP-PKA pathway) and Ste12p-Tec1p complex (MAPK pathway) independently to simulate adenylate cyclase distribution in Gpa2p and Ras2p bound forms (Figs. 6b and c). The different color zones shown in Figs. 6b and c represent variation in fractional *FLO11* expression. For *FLO11* expression greater than 90% (red Zone), the fraction of the adenylate cyclase in the Gpa2p bound form was about 40–50% of the total adenylate cyclase concentration (Fig. 6b). Whereas the fraction of adenylate cyclase in the Ras2p bound form was only 20–25% of the total adenylate cyclase concentration (Fig. 6c). This analysis indicated that both Ras2p and Gpa2p can activate adenylate cyclase but the major percentage of the activation was through Gpa2p binding. The preferential activation of adenylate cyclase by Gpa2p was due to five-fold higher binding constant of Gpa2p binding to adenylate cyclase as compared to Ras2p [24,26].

The model was also used to study an in-silico Δ*gpa2* mutant, by eliminating the activation of adenylate cyclase by Gpa2p. The response curves (Fig. 7a) indicated that there was a reduced *FLO11* expression in spite of the 3-fold increase in Ras2p activation. This fold change in the activation of Ras2p was not sufficient to express *FLO11*. However, a 6 fold change in the activation of Ras2p (hyper-activated) brought about by increasing the activated Ras2p concentration was able to overcome Δ*gpa2* mutation and express *FLO11* (Fig. 7b). A 6-fold change in the Ras2p is reported in the case of dominant $RAS2^{Val19}$ mutant strain [22]. However, by the introduction of Gpa2p in hyper-activated Ras2p condition in our simulation, it was observed that Ras2p competes with Gpa2p binding to adenylate cyclase. In such a case, both would equally contribute to the total activation of adenylate cyclase (data not shown).

To further investigate the role of Ras2p in the crosstalk between cAMP-PKA and MAPK pathway, adenylate cyclase activation by the Ras2p was removed and *FLO11* dose–response curves were recalculated with respect to Gpa2p. Fig. 7c shows the dose–response curves of *FLO11* with respect to Gpa2p in the absence of Ras2p activation of adenylate cyclase (dotted lines) and in the presence of Ras2p activation of adenylate cyclase (solid lines), at different fractional activation of Ste12p-Tec1p complex (representing the MAPK pathway). In the absence of the crosstalk, the amount of Gpa2p required for the activation of adenylate cyclase remained invariant with varying Ste12-Tec1p complex concentrations. The response had an apparent Hills coefficient of 4 and $k_{0.5}$ of about 1 nM. Introduction of crosstalk between the two pathways resulted in amplification of the signal, which reduced the requirement of Gpa2p for activation of adenylate cyclase. This implied that $k_{0.5}$ for Gpa2p decreased in the presence of crosstalk from MAPK pathway. However, the sensitivity of the response was not altered due to the crosstalk. This analysis hence showed that the crosstalk of Ras2p did not contribute much to *FLO11* expression in wild type, but mainly amplified the Gpa2 signal. However, a hyperactive Ras2p can express *FLO11* even in the absence of Gpa2p indicating the dominant effect of crosstalk in such a mutant strain.

3.3. Positive feedback causes bistability in the MAPK module

A simplified MAPK pathway with the positive feedback through Ras2p is shown in Fig. 8a. The MAPK pathway activates the Ras2p through Cdc25p, which is further fed back to activate Cdc24p, which in turn activates Cdc42p, the upstream activator of MAPK pathway. Cdc42p along with Bmh1/2p associates with Ste20p and trigger the core MAPK pathway. Thus, Bmh1/2p acts as an external stimulus to the MAPK pathway. In this part of analysis, the role of the positive feedback with respect to Ste12p-Tec1p complex activation (MAPK pathway) and also on the *FLO11* expression was studied.

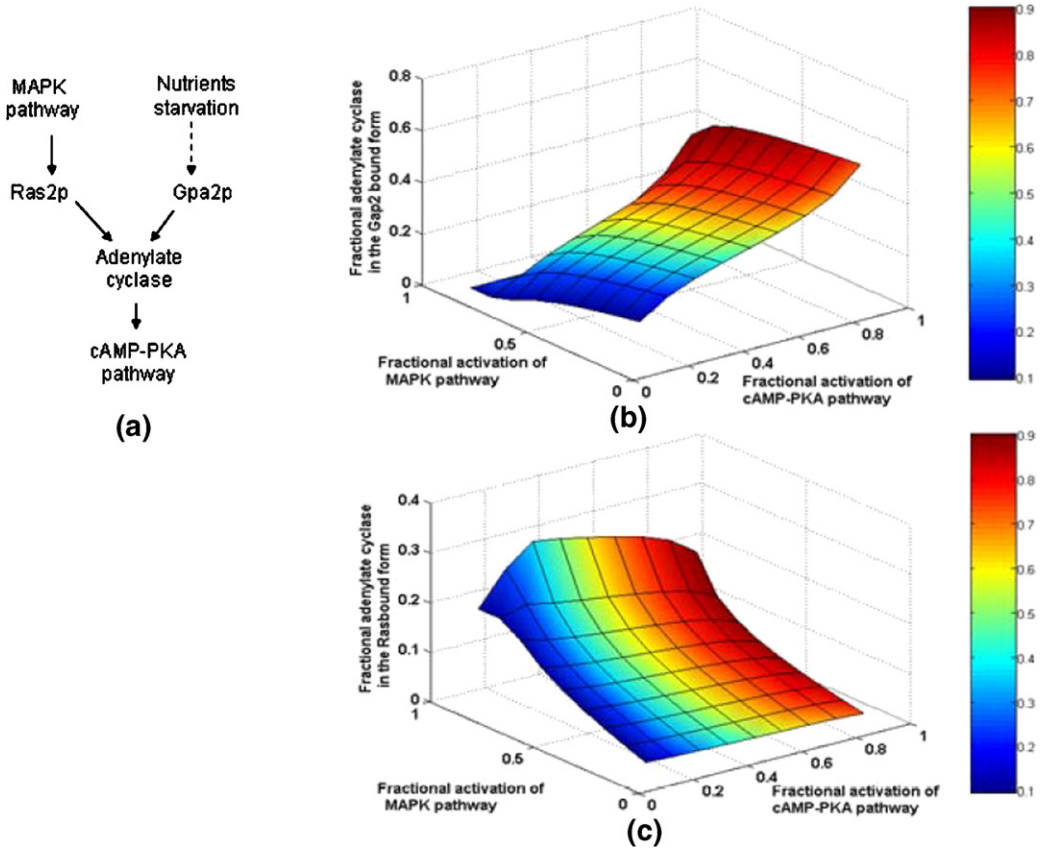

Fig. 6. Adenylate cyclase distribution during filamentous growth. (a) Simplified diagram showing activation of adenylate cyclase by Ras2p (activated by the MAP kinase pathway) and by Gpa2p coming from the GPCR system. (b) Fraction of the Adenylate cyclase in the Gpa2p bound form with varying cAMP-PKA and MAPK activation The fractional activation of cAMP-PKA pathway and MAPK pathway corresponds to fractional activation *FLO11* activators Flo8p (10–150 nM) and Ste12p-Tec1p (1–200 nM), respectively. The color coding represents *FLO11* expression and, hence, the filamentous growth, red being activation levels greater than 90%. (c) Fraction of the Adenylate cyclase in the Ras2p bound form over the same ranges of cAMP-PKA and MAPK pathway. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The simulated dose–response curves for fractional activation of Ste12p-Tec1p complex with varying Bmh1/2p concentration demonstrated a bistable response (curve a, Fig. 8b). At Bmh1/2p values greater than 0.75 nM, the fractional activation of Ste12p-Tec1p complex can be at 10% or 90% depending on the activated Ras2p concentration value. The bistability, as expected, was dependent on the feedback. Fig. 8b curve 'b' shows the case when Ras2p activation was minimal (less than 2% of the maximum Ras2p) and independent of the feedback. The response demonstrated that the higher concentration of Bmh1/2p (an increase of 9 fold) was required to activate the MAPK pathway. On increasing the Ras2p activation (greater than 90% of the maximum Ras2p) and removing the feedback, the response demonstrated that lower Bmh1/2p concentration was required (curve c, Fig. 8b). The feedback mechanism thus helped in providing bistability and also amplified the signal from Bmh1/2p.

However, the resulting response with respect to Bmh1/2p was a closed loop solution. This implied that the activation of MAPK pathway would reside either in the on state or in the off state. Further, once the pathway was switched off, the closed loop would ensure that it could never be activated. The closed loop pathway thus would not be reversible and was counter to reason. To overcome this problem an external source to directly activate Ras2p was considered in the analysis (Fig. 8c). This input was independent of the closed loop activation through Cdc25p. The external source can be an intracellular acidification or nutrient sensing [20]. The dose–response curve for fractional activation of Ste12p-Tec1p complex at different concentrations of Bmh1/2p with direct external activation of Ras2p included is shown in the Fig. 8d. The predicted dose–response curve indicates that there exist three steady states between 6 nM and 8 nM of Bmh1/2p for Ste12p-Tec1p complex activation. Out of these three steady states, Ste12p-Tec1p complex gets distributed between two discrete stable steady states, depending on the activated Ras2p concentration, thus showing a typical hysteresis response. Due to the hysteresis, the dose–response curve appears to be split, and therefore, we obtain two distinct half-maximal concentrations. This represents two threshold concentrations of Bmh1/2p required for switching on (6 nM) and switching off (8 nM). The observed hysteresis is characteristic of a bistable response obtained due to the positive feedback regulation of Ras2p on Cdc24p. The bistability is lost (supplementary Fig. S3a) and signal amplification is reduced by almost 4-fold on removal of the feedback loop. To

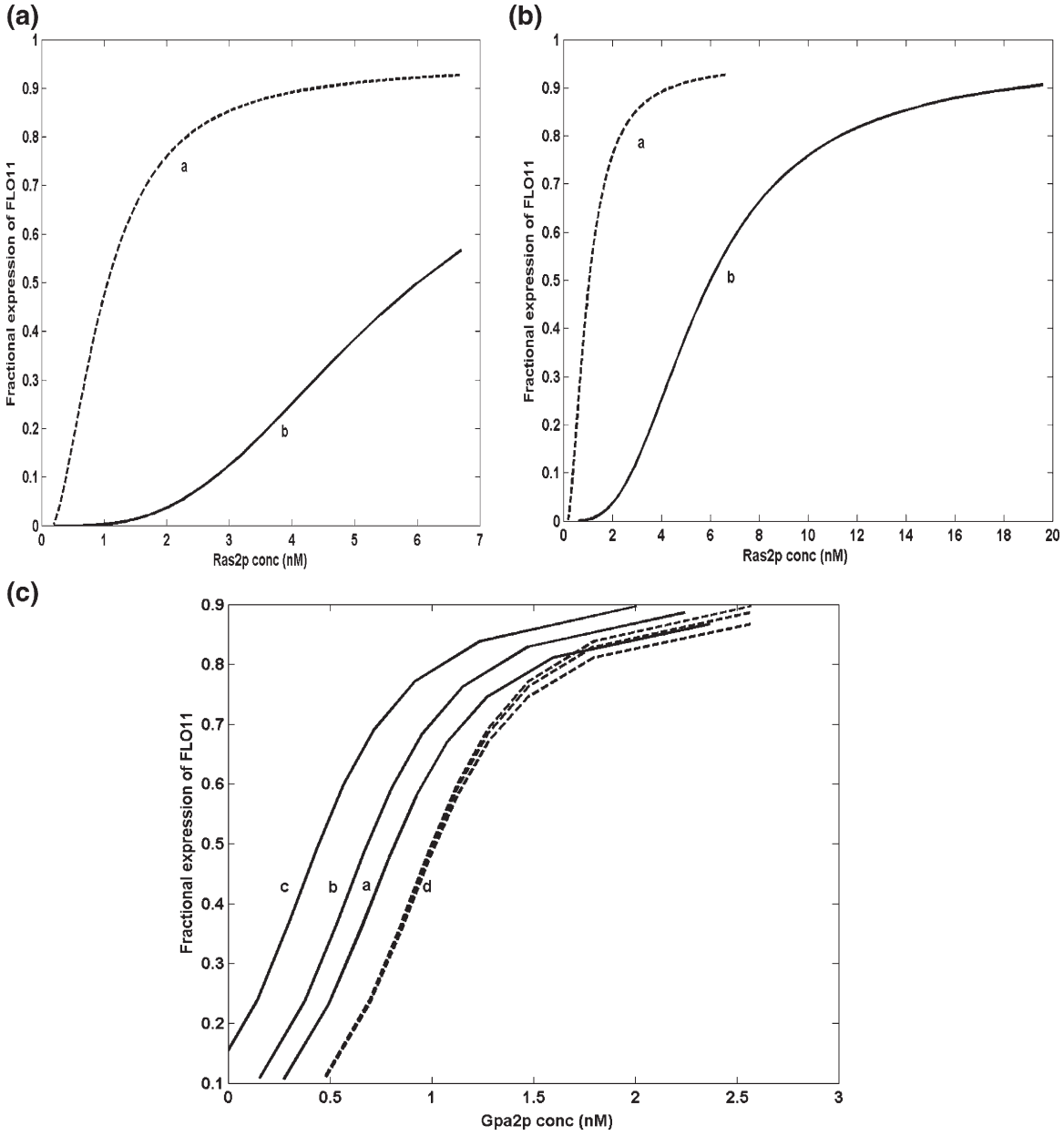

Fig. 7. Effect of Ras2p activation on *FLO11* expression. (a) *FLO11* expression with changing Ras2p activation (3 fold change). *Curve a*: The case for the wild type where *FLO11* expression can reach greater than 90% due to contribution from Gpa2 in Adenylate cyclase activation, *Curve b*: In an in silico *Δgpa2* mutant the *FLO11* expression is restricted to less than 60%. (b) *FLO11* expression with changing Ras2p activation. *Curve a*: The case for the wild type where due to presence of Gpa2p the *FLO11* expression can be greater than 90% *Curve b*: In an in silico *Δgpa2* dominant $RAS2^{Val9}$ strain the fold change in activation of Ras2p is 6 fold and it can overcome the *Δgpa2* defect to give *FLO11* expression greater than 90%. (c) The effect of the crosstalk on Gpa2p requirement: Increase in the activation of MAPK pathway decreased the Gpa2p ($k_{0.5}$) requirement for *FLO11* activation (solid lines) (a) at 40% MAPK activation, $k_{0.5}=0.81$ nM, (b) 60%, $k_{0.5}=0.69$ nM and (c) 80%, $k_{0.5}=0.45$ nM, (d) The dotted lines indicate the case when Adenylate cyclase activation by Ras2p was removed, which leads to fixed $k_{0.5}=1$ nM and amplification due to MAPK activation was lost.

investigate the effect of the feedback on bistable response, the parameters ηH and $k_{0.5}$ in the function representing Cdc24p and Ras2p interaction were varied. The response becomes highly bistable on increasing $k_{0.5}$ (supplementary Fig. S3b) and ηH (supplementary Fig. S3c). When feedback was made more switch-like, the bistability in the system increased very rapidly. Thus, the bistability behavior and the half saturation constant were dependent on the system parameters affecting the feedback loop.

To investigate the effect of this bistable behavior on the overall expression of *FLO11*, a dose–response surface of *FLO11* expression over the entire concentration range of the Bmh1/2p and adenylate cyclase (cAMP-PKA pathway) was generated (Fig. 9). It was clear from the surface plot that the *FLO11* expression primarily varies with the adenylate cyclase concentration (cAMP-PKA pathway) over the entire range (0–30 nM) and was less dependent on the Bmh1/2p (MAP kinase pathway). However, at lower values of Bmh1/2p (less than

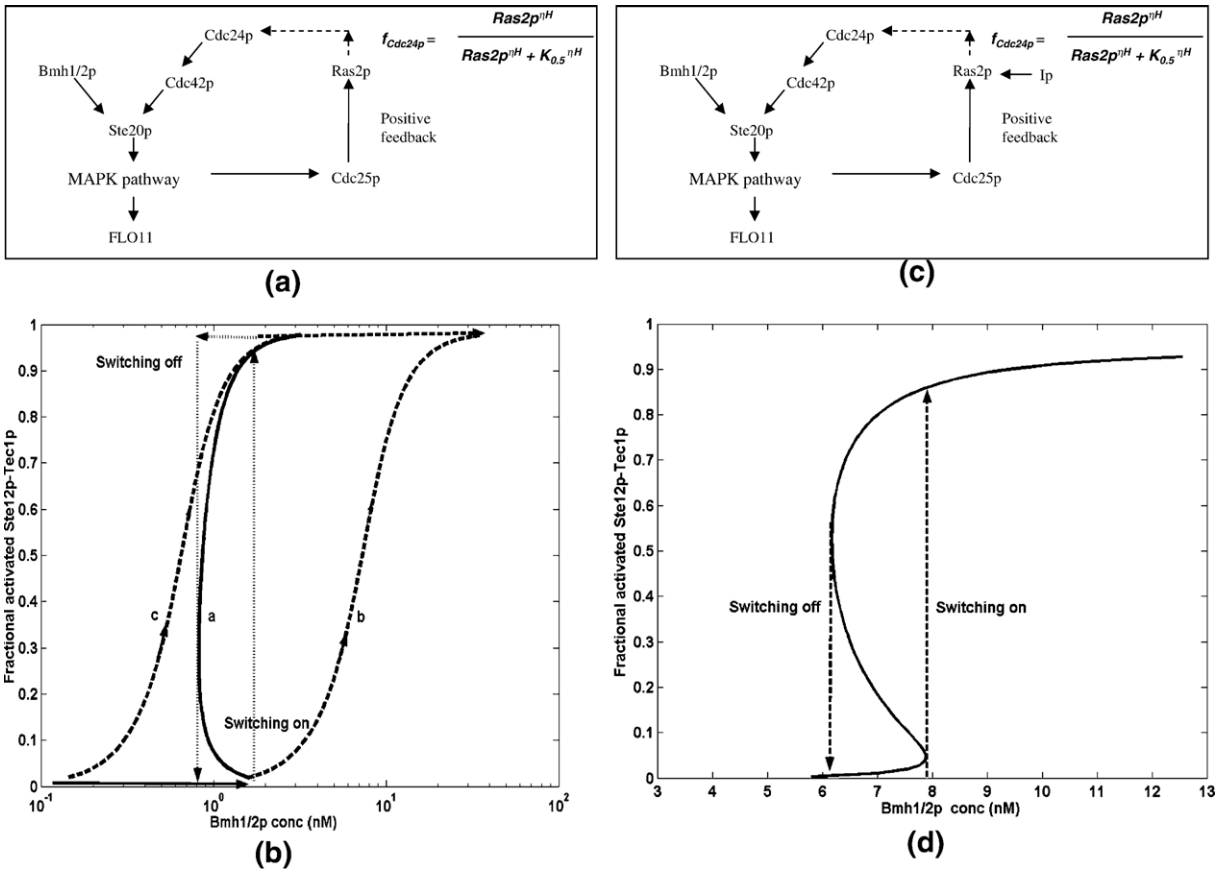

Fig. 8. Bistable response with respect to Bmh1/2p due to positive Ras2p feedback. (a) Simplified diagram showing the positive feedback on the MAP kinase pathway via Ras2p, the relationship between Cdc24p and Ras2p is assumed by a Hill function, η_h is the sensitivity and $k_{0.5}$ is the half saturation constant; Bmh1/2p is the independent signal that integrates with the Cdc42p signal to cause the Ste20p complex activation that will trigger the MAP kinase pathway. (b) The simulated dose–response curves for fractional activation of Ste12p-Tec1p transcription factor are shown with varying Bmh1/2p. *Curve a*: Ras2p with positive feedback. *Curve b*: Removing the feedback (Ras2p held at a very low value of 0.1 nM). *Curve c*: represents a case when the Ras2p is switched on (8 nM) and is made independent of the feedback. (c) Simplified diagram showing the positive feedback on the MAP kinase pathway via Ras2p including an external source for Ras2p activation. (d) Predicted dose–response curve for fractional activation of Ste12p-Tec1p at different concentrations of Bmh1/2p, including external activation of Ras2p, the predicted dose–response curve shows bistability with hysteresis, the switching on occurs at 8 nM whereas the switching off occurs at 6 nM.

2 nM), the expression gets restricted to 20–40% over the entire range of adenylate cyclase concentration. The bistability observed with respect to Bmh1/2p was not translated to the *FLO11* expression and a reduced degree of bistability was observed (Fig. 9).

3.4. Effect of system component concentrations and system parameters on FLO11 expression

To examine effect of variations in individual total protein concentrations on the fractional *FLO11* expression, the concentration of Flo8p, Ste12p-Tec1p complex, Cdc25p, Ras2p, Cdc42p and phosphatases, E2 were varied over a wide range. The dissociation constants were also varied to study the effect of parameters. In each case, the dose–response curve of fractional *FLO11* expression for different adenylate cyclase and Cdc42p concentrations was obtained and the response was quantified in terms of half saturation constant and the Hills coefficient. Table 1 gives the variation in the Hill coefficient with respect to total protein concentrations and parametric changes.

The sensitivity of the response, with respect to the upstream cAMP-PKA regulator adenylate cyclase, on varying the total concentration of Flo8p (over the range of 20–500 nM), was robust as indicated by the Hills coefficient which varied in the range of 3.5–4. However, on varying the total concentration of dephosphatase E2 which regulates the dephosphorylation of Flo8p, the effect was profound. For an increase in the total concentration of the E2 dephosphatase (E2t) from 10 to 70 nM, the increase in the Hill's coefficient with respect to adenylate cyclase was from 3 to 7 (Table 1). The response was also fairly robust to the changes in 'Kd5', a dissociation constant between Flo8p, Ste12-Tec1p complex and *FLO11* (Table 1).

The sensitivity of the response with respect to the upstream regulator of the MAP kinase pathway Cdc42p was found to be robust with respect to the changes in the parameters as 'Kd33' (the Ste12p-Tec1p complex, Kss1p and Dig1/2p complex dissociation constant) and also to the total concentration variations in Ras2p and Cdc42p. A parameter that was found to be crucial in regulating the fractional expression of *FLO11* was the relative total concentration of Ste12p-Tec1p complex (immediate upstream MAP kinase activator) and Dig1/2p

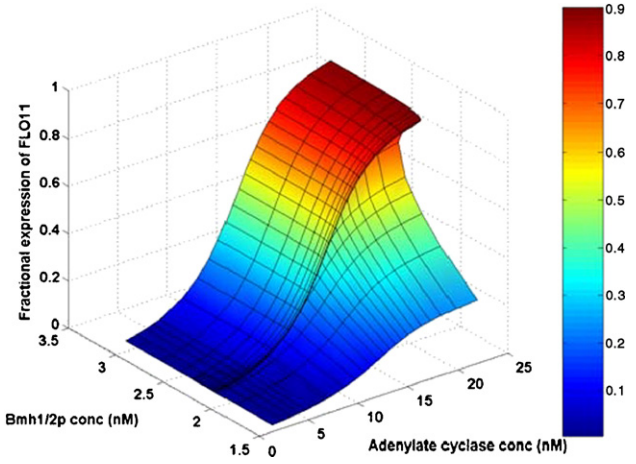

Fig. 9. Effect of bistability on *FLO11* activation. Simulated dose–response surface with respect to the concentrations of the upstream regulators Bmh1/2p (MAPK pathway) and Adenylate cyclase (cAMP-PKA) pathway to get the global response. The effect of bistability on overall activation of *FLO11* by Bmh1/2 is not as much as that compared to previous figure (Fig. 8d) of MAPK activation, probably due to the major control exercised by cAMP-PKA pathway on the *FLO11* expression. The red color represents *FLO11* expression greater than 90%, whereas the blue color represents expression levels lower than 10%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(immediate upstream MAPK pathway inhibitor). On increasing total Ste12p-Tec1p concentration while keeping the Dig1/2p concentration fixed at 200 nM, the Hill coefficient with respect to Cdc42p decreases (Table 1) from 4 (ultrasensitive) to 0.5 (subsensitive). However, on keeping the ratio of total concentration of Ste12p-Tec1p complex to Dig1/2 as 1, the sensitivity became fairly robust and varied between 1.1 and 1.5 for a Ste12p-Tec1p complex concentration variation over the range 20–500 nM.

4. Discussion

In the current analysis, we use steady state modeling to quantify the fractional expression of *FLO11* gene at different activation levels of cAMP-PKA and MAPK pathways. The analysis revealed that *FLO11* expression was more sensitive to cAMP-PKA pathway than to MAPK pathway. The analysis also demonstrated that the crosstalk through Ras2p between MAPK and cAMP pathways amplified the signal through the cAMP pathway. Adenylate cyclase was activated by a higher degree through Gpa2p as compared to Ras2p. The positive feedback present in the MAPK pathway can demonstrate bistability.

The higher sensitivity of cAMP-PKA pathway as compared to the MAPK pathway helps the cell to respond to changes in nitrogen availability, as nitrogen is sensed by the ammonium permease Mep2p, which is shown to be in the upstream of Gpa2p of cAMP-PKA pathway [9]. This high sensitivity can be directly related to inhibitor ultrasensitivity brought about by the Sfl1p-Tup1p-Ssn6p inhibitor complex. The apparent sensitivity of the response reduced from 4 to 1.6 on the removal of the inhibitor (result not shown). It should be noted that the mechanism of inhibition is linked through a phosphorylation cycle instead of a direct stochiometric inhibition. MAPK pathway also has an inhibitor, Dig1/2p; however, the sensitivity of the *FLO11* response was about 1. In MAPK pathway the interaction between the Dig1/2p and Ste12p-Tec1p complex forms a stochiometric inhibitor complex, and therefore, the ratio of the total concentration of Ste12p-Tec1p complex to Dig1/2p was assumed to be 1. This resulted in sensitivity being 1 which was robust to changes in the concentrations of activated Ste12p-Tec1p complex. Thus, the stochiometric inhibitor in MAPK pathway may offer an advantage of robustness but at the cost of a sensitive response.

In addition to higher sensitivity shown by cAMP-PKA pathway, our analysis also showed that the activation level of the cAMP-PKA pathway primarily controls the overall expression of the *FLO11* (Figs. 3 and 4). This was because the downstream component Ste12p-Tec1p complex in the MAPK pathway was invariant due to limitation in Flo8p concentration, allowing the cAMP pathway to exercise the major control to yield a sensitive response. However, our analysis also indicated that lower activation levels of MAPK pathway, the *FLO11* expression was restricted to lower than 20%. Thus, it should be noted that MAPK pathway was indispensable to *FLO11* expression.

Genetic studies [5] have demonstrated that MAP kinase pathway is, in part, dispensable for filamentous growth. It was reported that an overactive cAMP-PKA pathway caused by Δ*bcy1* mutation (the PKA kinase regulatory unit) suppressed the filamentous growth defect conferred by Δ*ste12tec1* mutations (the most downstream regulators of the MAP kinase pathway). However, in wild-type *BCY1*, cAMP pathway gets activated in spite of Δ*ste12tec1* mutation under nitrogen

Table 1
Parametric sensitivity analysis for the filamentous growth

Parameter name (standard value) (nM)	Varied range (nM)	nH apparent Adenylate cyclase (cAMP-PKA pathway)	nH apparent Cdc42p (MAPK pathway)
cAMP-PKA pathway			
Flo8t (150)	20–500	3.5–4	NA
E2 (10)	2–70	3–7.5	NA
Kd5 (0.3 [a])	0.05–0.6	2–4	0.5–1
MAPK pathway			
Kd33 (400/17)	10–400	NA	1.5–1
Cdc25pt (30)	10–90	NA	1–1.6
Ras2pt (200)	5–500	NA	1–1.5
Ste12p-Tec1pt (200)	20–250	NA	0.5–4.5
R = Ste12p-Tec1pt/Dig1/2pt and Dig1/2pt (200) Ste12p-Tec1pt (200)	20–250	NA	1.1–1.5

The term 'standard' indicates the parameter set used for simulation in this work and the value is indicated in parenthesis. These parameters were varied over a wide range to assess the sensitivity of the response. All the sensitivities are calculated with respect to the normalized *FLO11* that is *FLO11* values normalized to the maximal *FLO11* expression achievable for the given parameter. In all the parameter values the suffix 't' refers to the total concentration, see supplementary material for the details of the nomenclature of the parameters.

[a] nM^2.

starvation conditions and hence can be expected to activate *FLO11* expression. But they are not able to activate *FLO11* expression in the absence of Ste12p-Tec1p complex indicating that MAPK pathway is indeed indispensable for filamentous growth, in accordance with our simulation results. On over-activating the PKA by Δ*bcy1* mutation, *FLO11* gets saturated with the Flo8p activator contributing to *FLO11* expression. But in these studies, the exact quantification difference in *FLO11* expression with single and both the transcriptional activators (that is Flo8p from cAMP-PKA pathway and Ste12p-Tec1p complex from MAPK pathway) bound to *FLO11* is not clear. In our studies, we have quantified the *FLO11* expression with both activators bound to it, and hence, under this condition MAPK pathway was indispensable to *FLO11* expression. Thus, there seems to be a hierarchy in which cAMP pathway is the limiting of the two pathways. These results set the stage for further analysis of how different signaling pathways are coordinated to control gene expression.

We have analyzed the role of crosstalk brought about by Ras2p in controlling the activation of adenylate cyclase and MAPK pathway during filamentous growth. With both Gpa2p and Ras2p being activated during nitrogen starvation condition, the exact role played by these G-protein together during filamentous growth was not clear. Simulation showed that the distribution of activated adenylate cyclase in Gpa2 and Ras2p bound form was 60% and 20%, respectively (Fig. 6). This indicated Gpa2p to be the main activator of adenylate cyclase during filamentous growth. Under this condition, in silico Δ*gpa2* mutation affected the *FLO11* expression (Fig. 7a) and Ras2p activation of adenylate cyclase and also MAPK pathway was not sufficient to increase *FLO11* expression (Fig. 7a). This result corroborated with genetic study findings where Δ*gpa2* mutant strains demonstrated defects in pseudohyphal growth [21]. It was found that the Ras2p activation conveyed the state of activation of MAPK pathway to the cAMP-PKA pathway. As the activation of MAPK pathway increased the requirement for the Gpa2p decreased (Fig. 7c), which indicates that the crosstalk between these two pathways essentially elicits a cooperative behavior for the synergistic activation of *FLO11* gene.

However, in case of hyper-activated Ras2p, where a 6-fold change in Ras2p activation was observed, the *FLO11* expression increased and was unaffected by Δ*gpa2* mutation. This implied that a higher activated Ras2p concentration was able to activate adenylate cyclase by overcoming its higher 'Kd' value for adenylate cyclase activation and thereby negating the requirement of Gpa2p. However, the advantage of the crosstalk offering amplification to the cAMP-PKA pathway was lost (about 6-fold decrease in $k_{0.5}$ value, Fig. 7b). Thus, hyper-activated Ras2p regulates *FLO11* expression through both cAMP-PKA and MAPK pathways and, hence, overcome Δ*gpa2* mutation. This was also supported by the results of genetic study [25], where expression of the dominant $RAS2^{Val19}$ mutant suppresses the Δ*gpa2* pseudohyphal growth defects. This indicates that activation of cAMP-PKA pathway using only Ras2p or Gpa2p will result in defective pseudohyphal growth. Therefore, the crosstalk of Ras2p and the activation of adenylate cyclase by Gpa2p are both essential network structure to obtain *FLO11* expression.

The Ras2p activation controlled by Cdc25p is also a part of the positive feedback loop in the MAP kinase pathway (Fig. 2). The role of this positive feedback loop was two-fold: (1) it amplifies the MAPK pathway; and (2) it elicits a bistable response with respect to Bmh1/2p. The analysis of bistable response in the presence of feedback loops was done at varying concentrations of signaling components and system parameters in physiological range. It was found that the bistability and the half saturation constant are dependent on the system parameters affecting the feedback loop. As the feedback strength was increased bistability in the system also increased. However, this bistability was not dominantly observed in the *FLO11* expression (see Fig. 9), due to cAMP pathway playing a stronger role in the *FLO11* expression. Thus, there is no obvious answer to the question of the relevance of bistability in FLO11 expression, though it is known that bistability can maintain a biological response even when the input stimulus is brief and the high activity level is maintained only as long as the system requires.

Thus, the quantification of the signaling pathway used under nitrogen starvation in *S. cerevisiae* demonstrated that the system level properties such as amplification, inhibitor ultra-sensitivity, crosstalk and bistability play an important role in regulating *FLO11* expression. At the phenotypic level these properties might help the organism to respond to nitrogen starvation through pseudohyphal growth.

Although we have analyzed cAMP-PKA and MAPK pathways with respect to *FLO11* expression, the exact mechanism of how the most upstream signaling components of these pathways function with respect to nitrogen source availability is still not clear. Evidences show that Mep2p, an ammonium transporter, plays a key role of sensing, transporting and signaling [9]. The expression of *MEP2* is under the control of TOR pathway [27,28], which acts as a cytoplasmic sensor of nitrogen status. Also, there are studies showing the regulation of PKA by TOR which links TOR pathway with cAMP-PKA pathway [27]. Further studies including the crosstalk from TOR pathway is essential to unravel the systemic properties with respect to nutritional availability.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2006.06.012.

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