A Switch from a Gradient to a Threshold Mode in the Regulation of a Transcriptional Cascade Promotes Robust Execution of Meiosis in Budding Yeast

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Abstract

Tight regulation of developmental pathways is of critical importance to all organisms, and is achieved by a transcriptional cascade ensuring the coordinated expression of sets of genes. We aimed to explore whether a strong signal is required to enter and complete a developmental pathway, by using meiosis in budding yeast as a model. We demonstrate that meiosis in budding yeast is insensitive to drastic changes in the levels of its consecutive positive regulators (Ime1, Ime2, and Ndt80). Entry into DNA replication is not correlated with the time of transcription of the early genes that regulate this event. Entry into nuclear division is directly regulated by the time of transcription of the middle genes, as premature transcription of their activator NDT80, leads to a premature entry into the first meiotic division, and loss of coordination between DNA replication and nuclear division. We demonstrate that Cdk1/Cln3 functions as a negative regulator of Ime2, and that ectopic expression of Cln3 delays entry into nuclear division as well as NDT80 transcription. Because Ime2 functions as a positive regulator for premeiotic DNA replication and NDT80 transcription, as well as a negative regulator of Cdk/Cln, we suggest that a double negative feedback loop between Ime2 and Cdk1/Cln3 promotes a bistable switch from the cell cycle to meiosis. Moreover, our results suggest a regulatory mode switch that ensures robust meiosis as the transcription of the early meiosis-specific genes responds in a graded mode to Ime1 levels, whereas that of the middle and late genes as well as initiation of DNA replication, are regulated in a threshold mode.

Introduction

Precise and complex regulation is required for entering a developmental pathway at the correct time and in the appropriate cell type. Deviations from this regulation may lead to genome instability, causing either cell death or the formation of tumor cells [1]. Inducing the correct set of genes in a coordinated manner is a key for developmental pathway regulation and is often achieved through a transcriptional regulatory cascade [2,3,4]. The master activator initiating the cascade is usually controlled by multiple input signals, each with a small impact. It is the combinational nature of the induction of the master activator that ensures the correct spatial and temporal activity of the developmental pathway [2,3,4].

Transmission of a strong and short-lived signal by the master activator is assumed to be critical for the successful completion of a developmental pathway [1]. Studies in mice [5] and yeast meiosis [6] have demonstrated the importance of a short-lived signal for efficient entry into a developmental pathway. However, whether a strong signal is indeed essential for efficient entry into and completion of a developmental pathway and, if not, how cells cope with premature, delayed, reduced, or increased signals, remains a fundamental, unsolved question.

Our model system is meiosis in Saccharomyces cerevisiae, in which a transcriptional cascade governs the initiation and progression through meiosis (Figure 1 and [7,8]). This cascade consists of a master activator, Ime1, which is essential for the transcription of the early meiosis-specific genes. The transcription of the middle genes depends on Ime2, a kinase that belongs to the early genes, and Ndt80, a transcriptional activator that belongs to the early middle genes. The transcription of the late genes is indirectly dependent on Ime1, Ime2 and Ndt80 [9]. The early genes encode proteins involved in DNA replication, synopsis of homologs and meiotic recombination, whereas the middle genes encode proteins required for nuclear divisions and spore formation [10].

We envision two modes by which a transcriptional cascade is regulated. (i) Transcription of a network of genes (B) responds to a gradient (graded) effect produced by a master activator (A), i.e., the quantity of A directly affects the quantity of B, and (ii) transcription of the B genes requires a threshold level/activity of A. The first hypothesis is supported by a discrete computational model that faithfully describes the relations between a master transcriptional activator, IME1, in budding yeast, and the transcription of IME2, an early meiosis-specific gene representing the B genes [11]. The second hypothesis is supported by two observations. First, a study of the meiosis transcriptional cascade in S. cerevisiae using time-lapse fluorescence microscopy of single cells demonstrated high variability in the duration of the first stage in the cascade...
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We used qPCR to determine the pattern of expression of several representatives of genes induced in meiosis at specific times. IME1 was the first gene induced (Figure 2 and 3A), followed by the simultaneous induction of the early genes, IME2 and HOP1 (Figure 2 and 3A). The transcription of the early-middle gene NDT80 and the late gene DIT1 followed sequentially (Figure 2 and 3A). CLB5 was previously designated as a middle gene whose transcription depends on Ndt80 [7,14]. However, the use of the sensitive qPCR assay revealed that its transcription was also partially increased prior to the increase in the level of NDT80 RNA (Figure 3A). Moreover, its initial induction was also regulated by Ime1, as its level was correlated with that of Ime1 (Figure 3A). Thus, these results are in agreement with those of Raithatha and Stuart that demonstrated a meiosis-specific Ndt80-independent transcription of CLB5 in a deletion analysis of the CLB5 promoter [15]. Finally, our findings demonstrate that the representative meiosis-specific genes were induced in a sequential and transient manner.

Meiosis is robust to decreased or increased levels of IME1 RNA

We examined whether the pattern of IME1 expression is essential for efficient meiosis by studying isogenic strains carrying 1, 2, 3 or 5 copies of IME1. As expected, the increase in IME1 copy number resulted in a substantial increase in the level of IME1 RNA throughout the meiotic pathway (Figure 3A). The correlation between IME1 copy number and the maximal level of IME1 RNA was found to be non-linear (Figure 4A); Rather, it fitted a quadratic trend (ie, a polynomial of order 2) probably resulting from its positive autoregulation [9,11,16].

Modulation in IME1 copy number had no deleterious effect on sporulation efficiency, as determined by the similar viability and percentage of asc formation (Figure 4B). Our results indicate that meiosis is insensitive to changes in the transcript level of its master regulator, Ime1.

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Modulation in IME1 copy number had no deleterious effect on sporulation efficiency, as determined by the similar viability and percentage of asc formation (Figure 4B). Our results indicate that meiosis is insensitive to changes in the transcript level of its master regulator, Ime1.
Transcription of the early meiosis-specific genes responded to Ime1 levels in a graded mode, whereas transcription of the early-middle and late genes responded in a threshold mode. Two possible scenarios can explain why the level of IME1 RNA had no effect on meiosis efficiency: (i) the level of Ime1 protein might buffer the level of its RNA, creating a threshold effect, and (ii) the activity of an additional positive regulator might buffer Ime1 levels. The first hypothesis predicts that the expression levels of meiosis-specific genes will be insensitive to the level of IME1 RNA. Therefore, we measured the transcript levels of representatives of meiosis-specific genes (Figure 3A). We found that an increase in the level of IME1 RNA led to a subsequent increase in the levels of IME2, HOP1 (early genes), NDT80 (an early-middle gene), CLB5 (an early-middle gene) and DIT1 (an early-late gene), contrary to the first hypothesis. The second hypothesis predicts that only the early genes, which are directly regulated by Ime1, will respond in a graded mode to Ime1 levels, whereas the transcription of the middle genes will not. To examine this hypothesis, we determined how Ime1 affects the transcription time of these genes by plotting the level of transcription levels on a log scale (Figure 3B). The response of IME2 and HOP1 to the increasing levels of Ime1 was in a graded mode, responding to any level of Ime1 (i.e., increased levels of IME1 RNA due to an increased copy number of the IME1 gene, resulted in a gradual increase in the transcript levels of IME2 and HOP1, Figure 3B). Notably, the response of IME2 and HOP1 to Ime1 levels also showed a slight threshold effect. For example, after 6 hours in sporulation medium (SPM) the RNA level of IME1 in cells containing 5 copies of IME1 was 5.7-fold higher than that observed in the strain with 2 copies of IME1, whereas RNA levels of IME2 and HOP1, were 2.3- and 7.2-fold higher, respectively. We suggest that this “threshold” effect is due to the positive...
regulation of the early genes transcription by Ime2 (Fig. 1 and [9]).
In contrast, a true threshold response was observed for the late gene BDT1: a decrease in IME1 copy number resulted in its delayed transcription, whereas an increase in IME1 copy number resulted in its advanced transcription (Figure 3B). Furthermore, the early-middle genes, NDT80 and CLB5, showed a mixed response to the changes in Ime1 levels. At early meiotic times they showed a graded response, and at later meiotic times a threshold effect (delayed or advanced time of transcription). We suggest that this observation reflects a mixed mode of regulation for NTD80 and CLB5 by both Ime1 and Ndt80 (Figure 3A and [15,17]).

The abovementioned results suggest that an early meiosis-specific gene which functions as a positive regulator for the transcription of NDT80 and the middle genes acts as a buffer that switches a graded response to a threshold response.

Coordination between the transcription of meiosis-specific genes and meiotic events

We examined the effect of IME1 copy number on various meiotic events in order to elucidate how cells cope with drastic changes in the levels of positive regulators. Premiotic DNA replication, commitment to meiotic recombination, and nuclear divisions were delayed in cells carrying a single copy of IME1 (compared to cells with 2 copies of IME1, Figure 5), whereas the transcription of the early genes IME2 and HOP1 was not delayed (Figure 5). Moreover, these events were induced at an earlier time in cells carrying 3 or 5 copies of IME1 (Figure 5). These results indicate that initiation of early meiotic events (ie, DNA replication and commitment to meiotic recombination) are not correlated with the time of transcription of the early meiosis-specific genes that encode proteins required for these events. Conversely, entry into nuclear division is correlated with the time of transcription of NDT80.

Premature transcription of middle genes is deleterious to meiosis

We hypothesized that the delay in nuclear division observed in cells carrying a single copy of IME1 was due to the delay in the transcription of the middle genes that encode proteins required for nuclear division. This hypothesis predicts that premature transcription of the middle genes will cause premature entry into the first meiotic division (MI), loss of coordination with DNA synthesis and consequently a defect in meiosis. We achieved premature expression of the middle genes (NDT80, CLB5, and BDT1) by generating a mutant expressing Ndt80, their transcriptional activator, from the IME2 promoter (Figure 6A). Premature transcription of NDT80 resulted in reduced and constrected expression levels of IME1, IME2, and HOP1 (Figure 6A). These results imply that either Ndt80 has multiple roles, functioning both as a positive and a negative regulator, or that the negative effects of Ndt80 are mediated through its positive effect on the transcription of the middle meiosis-specific genes. Moreover, premature transcription of NDT80 induced the transcription of IME2 (Figure 6A), a result supported by studies demonstrating that Ndt80 is a positive transcriptional activator of IME2, and the existence of an Ndt80 consensus site within 600 bp upstream of IME2 [18].

Premature transcription of NDT80 advanced entry into meiotic S by one hour compared to the wild type (wt) strain (Figure 6B). We suggest that the advanced entry into meiotic S was due to the increased transcript levels of CLB5 (Figure 6A), which is required for initiation of premiotic DNA replication [19,20]. The major effect observed was advancing the time cells entered MI (MI was initiated at the same time cells initiated DNA replication, delayed transcription, whereas an increase in NDT80 copy number resulted in its advanced transcription (Figure 3B). Furthermore, the early-middle genes, NDT80 and CLB5, showed a mixed response to the changes in Ime1 levels. At early meiotic times they showed a graded response, and at later meiotic times a threshold effect (delayed or advanced time of transcription). We suggest that this observation reflects a mixed mode of regulation for NTD80 and CLB5 by both Ime1 and Ndt80 (Figure 3A and [15,17]).

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suggested that the activity levels of protein(s) controlling the time
that cells enter meiotic DNA replication may be regulated in a
threshold mode, similarly to the transcriptional regulation of
NDB80. We hypothesized that Ime2 may be the “threshold factor”
that buffers Ime1 levels, and whose activity coordinates DNA
replication, NDB80 transcription, and consequently nuclear
division, as Ime2 is required for timely entry into premeiotic
DNA replication and nuclear divisions, as well as for NDB80
transcription [9,21,22,23]. Since the predicted amino acid
sequence of Ime2 includes three potential Cdk1 phosphorylation
sites within its regulatory region (S/T-P-X-R/K at T302, and two
non-perfect S/T-P sites, at T202 and S252, [9]), and since Ime2
activity depends on phosphorylation of Y244 by Cak1 [24,25], we
hypothesized that Ime2 phosphorylation by Cdk1 on T302 (as well
as on T202 and S252) might hinder Cak1 ability to phosphorylate
Ime2, causing a threshold effect to Ime2 activity. This hypothesis
predicts that threonine to alanine mutations at these sites might
promote premature phosphorylation by Cak1, and consequently
premature activation of Ime2. Therefore, we constructed the ime2-
T202A,S252A,T302A allele (designated ime2-3SA) by site-directed
mutagenesis, and examined the ability of a diploid strain
expressing the Ime2-3SA allele to promote meiosis.

In wt diploids, nitrogen depletion caused accumulation of cells
in G1 (determined by an increase in unbudded cells with 2C DNA
content, Figure 7, A left panel and D), after which cells initiated
DNA replication (after approximately 5 hours in SPM, Figures 7,
A and D). Notably, bud emergence was not deleteriously affected
by the ime2-3SA allele; Conversely, arrest as unbudded cells was
faster and more efficient in cells carrying the ime2-3SA allele than
in wt cells (Figure 7A, and see discussion). However, cells carrying
the ime2-3SA allele initiated meiotic S prematurely; a third peak
with an intermediate level of DNA content appeared after only
1 hour in SPM (Figures 7, A and B). Furthermore, the level of cells
with 4C DNA content decreased in the first 6 hours in SPM,
implying that cells completed the mitotic cycle, whereas the level
of cells with 2C DNA content did not increase, implying that the

Figure 6. Premature transcription of NDB80 results in a premature entry into nuclear division, and consequently a defective
meiosis. Isogenic NDB80/NDB80 (Y1631, squares) and ndb80ΔC::IME2p-NDB80-TRP1/ndb80ΔC::IME2p-NDB80-TRP1 (Y1764, triangles) cells were shifted
to meiotic conditions (SPM), and at the indicated hours samples were taken to extract RNA (A), to process for FACS analysis and calculate the
percentage of cells with 4C DNA content (B), and to stain with DAPI to count the percentage of cells with 2 (open squares and triangles, dashed lines)
and 4 nuclei (filled squares and triangles) (C). Arrows mark initiation time for DNA replication and nuclear division.
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cells did not accumulate in G1, and immediately entered the premeiotic S phase (Figure 7B). These results suggest that the Ime2-3SA protein was activated prematurely and promoted initiation of DNA replication in the mutant strain; however, the lack of accumulation of cells with 4C DNA content (Figure 7B) suggests that an additional factor (perhaps a Cdk) is required for efficient progression through DNA replication, and its absence during the premature initiation of DNA replication prevents premature completion of DNA replication.

The ime2-3SA allele also affected meiotic recombination, as commitment to intragenic recombination at the ADE2 locus was delayed, and its maximal level was reduced compared to the wt strain (Figure 7C). Since NDT80 transcription depends on the recombination checkpoint [26], we expected that the delay and reduction in meiotic recombination would impact the time of NDT80 transcription in the mutant strain; indeed, NDT80 transcription in the mutant strain was delayed relative to the premature entry into S (initiated at the same time as that observed in wt, Figure 7D). Furthermore, NDT80 transcription was non-transient.

In agreement with the transcript analysis, DAPI staining revealed that MI was initiated at the same time in the wt and ime2-3SA/ime2-3SA strains (Figure 7D). However, in the mutant strain 27.4% of the cells remained arrested with a single nucleus, compared to only 11.2% in the wt strain. In summary, the multiple phenotypes observed suggest that ime2-3SA is a constitutive active allele.

The activity of Ime2 is negatively regulated by Cdk1/Cln3

The abovementioned results suggest that Cdk1 inhibits Ime2 activity. This hypothesis predicts that increased activity of Cdk1 at early meiotic times will delay Ime2 activity. Therefore, we increased the activity of Cdk1 at early meiotic times, by expressing the G1 cyclin CLN3 from the IME2 promoter. We assumed that ectopic expression of CLN3 will increase the activity of Cdk1, because Cdk1 level is constitutive throughout the meiotic pathway [27]. We chose IME2 and not IME1 as the promoter to bypass the negative effect of Cdk1/G1-cyclins on the transcription of IME1 [28]. MATa/MATα diploids carrying pIME2-CLN3 on a 2μ plasmid were shifted to meiotic conditions, and transcripts levels of IME1 and NDT80 as well as level of DNA replication were monitored over time. Ectopic expression of CLN3 caused a 2 hours delay in entering premeiotic DNA replication (Figure 8A), similar to the delay observed in cells carrying a single copy of IME1 (Figure 5A). This delay was not the result of reduced IME1 expression because conversely IME1 expression was substantially increased (Figure 8B). In addition, ectopic expression of CLN3 delayed NDT80 transcription compared to wt (Figure 8B). The observed delayed entry into premeiotic S and the effects on IME1 and NDT80 transcription support our hypothesis that Cdk1/Cln3 inhibits Ime2 activity, as Ime2 functions as a negative regulator of IME1 transcription and as a positive regulator of NDT80 transcription [9]. Finally, ectopic expression of CLN3 was deleterious, as the level of asci was 49% in comparison to about

Figure 7. The ime2-3SA mutation resulted in premature entry into premeiotic DNA replication. Isogenic IME2/IME2 (Y1631, squares) and ime2-3SA/ime2-3SA (Y1740, triangles or circles) cells were shifted to meiotic conditions (SPM). At the indicated hours samples were taken to process for FACS analysis, to count the number of unbudded cells, to plate on minus adenine and YEPD plates, to isolate RNA, and to stain with DAPI. A. FACS and budding index. The percentage of budded cells is given at the left side of each FACS. B. DNA replication. The percentage of ime2-3SA/IME2 cells with 2C DNA content (open circles, grey line), in S phase (triangle, dashed line), or with 4C DNA content (triangle, line) was calculated. The percentage of wt cells with 4C DNA content (square, line) was calculated. C. Commitment to intragenic recombination at the ADE2 locus. D. The percentage of wild type (square) and mutant cells with more than 2 nuclei (grey lines). The relative level of NDT80 RNA in comparison to RNA levels of ACT1 is shown (black line). The experiment was repeated 3 times and a representative result is shown. doi:10.1371/journal.pone.0011005.g007
Discussion

The role of positive autoregulation in establishing robust meiosis

Feedback regulation is one of the major characteristics of transcriptional activators that control entry into developmental pathways (for specific examples see [1]). In budding yeast, the transcription of the positive regulators of meiosis, IME1, IME2 and NDT80, is subject to positive autoregulation [Figure 1A and [9]]. It is usually assumed that transcript abundance of such regulators is tightly regulated, and essential for the correct response of their target genes [1]. Therefore, we hypothesized that meiosis would be sensitive to radical changes in the levels of such positive regulators. However, our findings do not support this hypothesis because drastic changes in IME1 levels had no deleterious effects on the efficiency of meiosis (Figures 3A, and 4B). The transcription of multiple positive regulators have been demonstrated to be negatively regulated eg, IME1 by Cdk1/Cln and Sok2 [16,20], IME2 by the ATP-dependent chromatin-remodeling Isw2 factor [29], as well as by deacetylation of histones H3 and H4 [30], and NDT80 by histone deacetylase complexes [7,22,31,32,33]. We suggest that positive autoregulation is required to overcome the initial negative regulation on the transcription and/or function of these regulators.

Negative feedback regulation ensures a transient transcription of IME1

The transcription of IME1 is transient in wt cells and non-transient in ime2Δ/IME2 cells [9] suggesting that Ime2 either directly or indirectly represses IME1 transcription. Moreover, phosphorylation of Ime1 by Ime2 tags it for degradation [34]. Our data support these findings as inhibition of Ime2 function by ectopic expression of the G1 cyclin, Cln3, resulted in a substantial increase in the level of IME1 RNA (Figure 8B). Premature transcription of NDT80 led to reduced and constricted levels of IME1 expression (Figure 6A), whereas its delayed transcription resulted in an increase in IME1 transcription (Figure 8B). These results are in agreement with a prior report showing that NDT80 deletion resulted in a non-transient transcription of IME1 [35]. As Ime2 is required for NDT80 transcription [36], our results suggest that the effect of Ime2 is also mediated through Ndt80. We propose that this negative feedback loop is mediated by Cdk1, as Ndt80 regulates the transcription of the B-type cyclins [7] that activate Cdk1 (Cdk1 level is constitutive in both the mitotic and meiotic cycles [27]). Phosphorylation of Ime1 by Cdk1/Cln3 sequesters it from the nucleus [28], thereby preventing positive autoregulation of Ime1. In addition, independent of Ime1, Cdk1 functions as a negative regulator for IME1 transcription (V. Gurevich and Y. Kassir unpublished data). We further propose that the reduced levels of IME1 caused by NDT80 premature expression led to a decrease in the transcription of the early genes HOPI1 and IME2 (Figure 6A).

Robust meiosis depends on the time of NDT80 transcription

Our findings demonstrate that meiosis in budding yeast is insensitive to a large range of IME1 transcript abundance (Figure 4C). Notably, meiosis in budding yeast has been previously reported to be sensitive to constitutive high expression levels of Ime1, leading to a decrease in the percentage of asci and an increase in both the level of non-disjunction and dyads [6]. Our results demonstrate that robust meiosis depends on the sequential transcription of the various clusters of genes (ie, early vs. middle meiosis-specific genes). Premature transcription of NDT80 resulted in premature transcription of the middle genes, as well as premature entry into nuclear division (Figures 6, A and C). Nuclear division is also regulated by the pachytene checkpoint which monitors defects in meiotic recombination and synopsis of homologs [26]. This checkpoint arrest is achieved by two parallel mechanisms, repressing NDT80 transcription and inhibition of Cdk1/B-type cyclins activity by Swe1 [37]. Premature transcription of NDT80, by itself, may not be sufficient to repress Cdk1 activity, as evidence by the fact that Ndt80 deletion resulted in an increase in NDT80 transcription (Figure 8B). Premature transcription of NDT80 resulted in a significant reduction in the level of cells that entered MII, (most cells accumulated with 2 nuclei, Figure 6C), we suggest that MII inhibition was due to inhibition of Cdk1 activity, most probably due to Swe1 activation.

Coordination between premeiotic DNA replication and nuclear division

We hypothesized that the coordination between meiotic S and nuclear division is accomplished by the cascade nature of the transcription of the early and middle meiosis-specific genes. The following observations support this hypothesis: (i) premature transcription of the middle meiosis-specific genes disrupts this coordination causing premature entry into MI (Figure 6), and (ii) the pachytene checkpoint, which is activated upon defects in meiotic recombination and synopsis of homologs, inhibits NDT80 transcription, and consequently the middle genes [7,35]. However, our hypothesis could not explain the observation that transcription of...
Ime2 serves as the buffer responsible for the switch from a graded to a threshold mode of response.

We propose that the switch from a graded to a threshold mode in regulation of premeiotic DNA replication and the transcription of NDT80 is mediated through Ime2. This hypothesis is based on the following observations: (i) deletion or a kinase-dead mutation in IME2 results in a delayed entry into meiotic S phase and nuclear division [21,27], (ii) in cells expressing the ime2-3SA allele meiotic S is induced prematurely (Figure 7A), and (iii) Ime2 is essential for the transcription of NDT80 [36]. The transcription of IME2 is regulated in a graded mode (Figure 3), but we propose that its activity is regulated in a threshold mode. Such a switch from a graded to a threshold mode is theoretically possible, based on mathematical analyses [2,38,39,40,41]. We suggest that the mechanism for the switch in Ime2 activity is a double-negative feedback loop between Cdk1/Cln3 and Ime2 (Figure 9). The following results support Cdk1 as an Ime2 inhibitor: (i) mutations in three putative Cdk phosphorylation residues in Ime2 (ime2-3SA) caused premature entry into premeiotic DNA replication (Figure 7A), and (ii) ectopic over expression of the G1 cyclin Cln3 caused an increase in the level of IME1 RNA as well as a delay in entering premeiotic S phase and the transcription of NDT80 (Figure 8). The following lines of evidence support Ime2 as a negative regulator of Cdk1/G1 cyclins function which is required for bud emergence [42]; (i) Ectopic overexpression of Ime2 is associated with accumulation of unbudded cells ([43] and M. Szwarewont-Cohen and Y. Kassir unpublished data), (ii) ectopic expression of either mouse Cdk1 or human Cdk2 prevented nitrogen-depletion induced G1 arrest only in ime2Δ diploids but not in wt strain [44], suggesting that Ime2 contributes to the G1 arrest, and (iiii) in cells carrying the constitutive active ime2-3SA allele, the level of budded cells is reduced in comparison to the wt strain, and upon nitrogen depletion the mutant cells arrest at G1 more efficiently (Figure 7). Taken together, the results suggest that these two kinases, Ime2 and Cdk1/Cln interact in a double-negative loop (Figure 9). Upon nitrogen depletion the activity of Cdk1 is reduced in a graded mode due to the absence of cyclins [45,46]. Nitrogen depletion also leads to a graded increase in the level of Ime1, and consequently Ime2. These two opposing effects may, at a certain level of Cdk1/Cyclin and Ime2, constitute a bistable switch between cells exhibiting Cdk and Ime2 activity. The mathematical model of James Ferrel [2] demonstrates that such a double negative feedback loop can switch a graded response to a threshold response.

In summary, our results explain how the meiosis developmental pathway is a robust process, initiated only under the right conditions, and the way by which two different regulation modes (i.e., graded and threshold) are used consecutively to induce the pathway at any level of Ime1, and to coordinate early and late meiotic events. Moreover, the proposed double negative feedback loop between the positive regulators of the cell cycle (Cdk) and meiosis (Ime2) explains the mechanism by which meiosis and the cell cycle exist as mutually exclusive developmental pathways, that is entering one pathway inhibits the concomitant entry into the second pathway.

Materials and Methods

Strains

Strains are listed in Table 1. Y1659 and Y1660 were constructed by transforming Y1065 with YIp2234 digested with PpuMII. qPCR analysis reveled that they carry 4 and 2 copies of IME1, respectively. Y1735 and Y1736 were constructed by transforming Y1064 and Y1065, respectively with YIp3045 cut BstEII. Y1760 and Y1761 were constructed by transforming Y1065 and Y1075, respectively, with YIp3078 digested with EcoRI. Because the EcoRI site is within the NDT80 ORF, transformation resulted in the formation of a ndt80Δ::IME2p-NDT80-TRP1 loci. Genotypes were confirmed by PCR.

Plasmids

YIp2234 carries IME1(−1439 to +1081)-ADH1 on a LEU2 vector. YIp3045 carries IME2-T202A,S252A,T303A (−23 to +223) on a URA3 vector. The plasmid was constructed by site-directed mutagenesis, and its sequence was verified. YIp3078 carries pIME2-ndt80Δ(1-1225) on a TRP1 vector. YEp3212 carries pIME2-CLN3 on a URA3 2μ vector. A detailed description on the constructions of these plasmids is available upon request.

Media and Genetic Techniques

Minimal acetate medium (PSP2) and sporulation medium (SPM) have been described previously [47]. Meiosis was induced as follows: cells were grown in PSP2 supplemented with the required amino acids to early exponential stage (0.8–1.2×10⁷ cells/ml), washed once with water, and resuspended in SPM. The number of cells that can form colonies (viability) was measured as described [47]. Staining with 4′,6-diamidino-2-phenylindole (DAPI) was performed as described [48]. Intragenic meiotic recombination within the ADE2 locus was performed as described [47]. As time of meiotic events can vary between experiments done at different days, the results described herein are from a single
experiment which was repeated ≥3 times (with all experiments showing the same behavior). Moreover, when different meiotic events are reported, they are all from the same experiment.

**FACS Analysis**

Cells were analyzed for DNA content by FACS analysis as described previously [21], using a FACScan analyzer (BD Biosciences, San Jose, CA). The percentage of cells with 2C, 4C and intermediate levels of DNA content was calculated using the WinMDI program.

**Quantitative analysis of RNA level**

RNA was extracted from 10⁶ cells by the hot acidic phenol method. Up to one microgram of total RNA was used for a reverse transcription reaction (total 20 µl) with random decamer primers and SuperScript reverse-iT™ transcriptase. The resulting cDNA was then used for real-time PCR (qPCR) analysis according to the manufacturer’s instructions (ABGene, Surrey, U.K.).

**Oligonucleotides**

The sequences of the oligonucleotides used in this study are available upon request.

**Author Contributions**

Conceived and designed the experiments: YK. Performed the experiments: VG. Analyzed the data: VG. Wrote the paper: VG YK.

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