Fission yeast rDNA-binding protein Reb1 regulates G1 phase under nutritional stress

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SUMMARY

Yeast Reb1 and its mammalian ortholog TTF1 are conserved Myb-type DNA-binding proteins that bind to specific sites near the 3'-end of rRNA genes (rDNA). Here, they participate in the termination of RNA polymerase I-driven transcription and block DNA replication forks approaching in the opposite direction. We found that *Schizosaccharomyces pombe* Reb1 also up-regulates transcription of the *ste9*$^{+}$ gene that is required for nitrogen starvation-induced growth arrest with a G1 DNA content and sexual differentiation. Ste9 activates the anaphase-promoting complex/cyclosome (APC/C) in G1 targeting B-cyclin for proteasome degradation in response to nutritional stress. Reb1 binds in vivo and in vitro to a specific DNA sequence at the promoter of *ste9*$^{+}$, similar to the sequence recognized in the rDNA, and this binding is required for *ste9*$^{+}$ transcriptional activation and G1 arrest. This suggests that Reb1 acts as a link between rDNA metabolism and cell cycle control in response to nutritional stress. In agreement to this new role of Reb1 in the regulation of G1/S transition, *reb1*Δ and *wee1*^{ts} mutations are synthetically lethal due to the deficiency of these cells to lengthen G1 before entering S phase. Similarly, *reb1*Δ *cdc10*^{ts} cells are unable to arrest in G1 and die at the semi-permissive temperature.

INTRODUCTION

The G1/S transition of the cell cycle is a critical stage for eukaryotic proliferating cells (Bahler, 2005). At late G1, cells make the decision to either commit to a new round of DNA replication and subsequent cell division, a process called Start in yeast, or to arrest and leave proliferative growing. In the fission yeast *Schizosaccharomyces pombe*, nutritional limitation such as nitrogen source deprivation, leads to growth arrest with a G1 DNA content (usually referred as G1 arrest) and cells undergo sexual differentiation by conjugation of two cells of different mating types. Meiosis of the resultant zygote produces four ascospores that will germinate and enter the mitotic cycle again if nutritional conditions permit. G1 arrest is required for conjugation and takes place by inactivation of Cdc2, the only cyclin-dependent kinase that drives G1/S transition in *S. pombe*. Cdc2 is inactivated in G1 under nitrogen starvation both by degradation of cyclin Cdc13 and by binding of the stoichiometric inhibitor Rum1 to Cdc2-Cdc13 complexes (Labib et al., 1995; Yamaguchi et al., 1997). Cdc13 is degraded at G1 upon nitrogen deprivation by APC/C-mediated ubiquitination and proteolysis by the proteasome. APC/C is inactive by itself and it is activated in G1 phase by the Fizzy-related protein Ste9/Srw1, which is therefore required for nitrogen starvation-induced G1 arrest and sexual differentiation. Ste9 is regulated by posttranslational modification (Blanco et al., 2000; Yamaguchi et al., 2000) and mRNA decay (Álvarez et al., 2006), but little is known about the regulation of *ste9*$^{+}$ transcription in response to nutritional stress.

Here we report that in *S. pombe* the Myb-type DNA binding protein Reb1 regulates transcription of *ste9*$^{+}$ under nitrogen starvation. *S. pombe* Reb1 was

originally found involved in the termination of rRNA transcription by binding to two identical 17-bp DNA sequences near the 3' end of the rDNA coding region (Zhao et al., 1997). More recently, it was shown that binding of Reb1 to these two sequences also blocks DNA replication, giving rise to two natural rDNA replication fork barriers (RFB) (Sánchez-Gorostiaga et al., 2004). These natural RFBs are highly conserved in eukaryotes and they function in a unidirectional manner stalling only replication forks moving in the direction opposite to transcription (López-Estraño et al., 1997; Mirkin and Mirkin, 2007). Therefore, these polar RFBs prevent head-on collision between transcription and replication machineries.

The orthologous Reb1 protein from budding yeast is also involved in rRNA transcription termination. In addition, *S. cerevisiae* Reb1 regulates transcription of several RNA polymerase II-transcribed genes by binding to their promoter regions (Angermayr et al., 2003 and references therein) and acts also as an enhancer for rRNA transcription (Morrow et al., 1993). In mammals, as in the fission yeast, the Reb1 ortholog protein TTF1 is one of the trans-acting factors for rRNA transcription termination (Jansa et al., 2001) and is also involved in rDNA replication fork arrest (Gerber et al., 1997; López-Estraño et al., 1998). TTF1 regulates rRNA transcription by binding to one cognate site placed immediately upstream the rDNA promoter (Langst et al., 1997; Strohner et al., 2004).

Here we showed that transcription of *S. pombe ste9*$^{+}$ is up-regulated upon nitrogen deprivation. Reb1 binds in vivo and in vitro to a short DNA sequence upstream *ste9*$^{+}$ and it is required for *ste9*$^{+}$ up-regulation and G1 arrest in response to nitrogen starvation. Consequently, mating efficiency of

cells lacking Reb1 is significantly reduced. Interestingly, Reb1 is binds *in vivo* to the promoter of *ste9*$^{+}$ (Pste9) both in cells growing exponentially in complete medium as well as after nitrogen deprivation. However, our results indicate that other proteins associate to the Reb1-Pste9 complex in exponentially growing cells, when *ste9*$^{+}$ expression is low. Upon nitrogen deprivation, these proteins dissociate from Reb1-Pste9 complex and *ste9*$^{+}$ is overexpressed.

RESULTS

Cells lacking Reb1 are defective in nitrogen starvation-induced G1 arrest and mating

In the course of genetic crosses carried out using *S. pombe reb1*Δ strains, we noticed that fertility of cells lacking Reb1 was significantly reduced. Mating efficiency of *reb1*Δ cells, expressed as the percentage of cells undergoing mating, was about four-fold lower compared to otherwise wild-type isogenic cells after 24 hours of mating, and about two-fold lower after 48 hours (Fig. 1A, black and grey columns).

The orthologous Reb1 protein in the budding yeast *S. cerevisiae* regulates the expression of several RNA polymerase II-transcribed genes. Therefore, we reasoned that *S. pombe* Reb1 could be involved in the regulation of some gene(s) required for efficient mating. In an attempt to identify candidate genes, we looked for putative Reb1 binding sites by BLAST-searching the nucleotide sequence recognized by Reb1 at the rDNA in other locations of the *S. pombe* genome. The highest score obtained corresponded to a 27-bp sequence located at the promoter region of gene *ste9*$^{+}$/*srw1*$^{+}$, covering

nucleotide positions -198 through -172 relative to the 5'-end of the coding sequence (Fig. 1B).

Ste9 is a WD-repeat Fizzy-related protein that activates APC, thereby promoting the ubiquitination and degradation of mitotic cyclins specifically during the end of mitosis and G1 (Blanco et al., 2000; Kitamura et al., 1998; Yamaguchi et al., 1997; Yamaguchi et al., 2000). Thus, Ste9 is required for an efficient G1 arrest of cells undergoing sexual differentiation in response to nutritional stress, such as nitrogen deprivation. The finding of a putative Reb1 binding site at the promoter of *ste9*$^{+}$ raised the hypothesis that Reb1 could be a positive regulator of the expression of Ste9, so that it would collaborate in the arrest of cells in G1, which is required for conjugation and, ultimately, sexual differentiation. To address this hypothesis, we used flow cytometry to analyze the response of *reb1*Δ cells to nitrogen starvation compared to those of *ste9*Δ and wild-type isogenic strains. In agreement with the hypothesis, *reb1*Δ cells were deficient in nitrogen deprivation-induced G1 arrest, similarly to *ste9*Δ cells (Fig. 1C, three left-hand columns). To study the relevance of the putative 27-bp Reb1-binding sequence at the *ste9*$^{+}$ promoter for the appropriate cellular response to nutritional stress, we constructed a new strain identical to the wild-type strain where these 27 nucleotides were substituted by unrelated ones (As by Cs, Gs by Ts and vice versa). As shown in Fig. 1C (right-hand column labeled mutPste9), this modification of the *ste9*$^{+}$ promoter also rendered cells deficient in G1 arrest. Moreover, mating efficiency of *mutPste9* cells was also reduced (Fig. 1A, white columns).

Expression of Ste9 under the control of the *nmt1* promoter reverts *reb1*Δ phenotype

The results described above suggested that the deficiency of reb1Δ cells to arrest in G1 could be due to a possible role of Reb1 in promoting Ste9 expression upon nitrogen starvation. If this hypothesis were correct, we would expect that expression of Ste9 under the *nmt1* promoter should facilitate cell arrest in G1 in *reb1*Δ cells. To verify the latter, we analyzed *reb1*Δ cells transformed with the pREP81X expression vector either empty or containing the *ste9*$^{+}$ gene (pREP81X-Ste9, kindly provided by S. Moreno). While the empty plasmid had no effect on the deficiency of *reb1*Δ cells to arrest in G1 (Fig. 1D, third histogram series), pREP81X-Ste9 efficiently reverted this defect and a percentage of cells similar to that observed in the wt strain, stalled with a 1C DNA content (Fig. 1D, right-hand histogram series).

reb1 and *wee1* mutations are synthetically lethal

Protein kinase Wee1 negatively regulates G2-to-M transition by inactivating Cdc2. Cells lacking Wee1 are viable, but mitosis takes place prematurely and consequently, about half-sized cells are generated after mitosis (Russell and Nurse, 1987). To survive, the G1 phase of these cells needs to be prolonged so that they can reach the minimum size required to pass Start. To examine if Reb1 plays a role in this G1 lengthening, we used the thermosensitive *wee1-50* allele in combination with *reb1*Δ mutation. While *reb1*Δ and *wee1-50* single mutants grew at all temperatures tested, these two mutations were synthetically lethal as growth temperature increased (Fig. 2A, upper panels). This means that Reb1 becomes essential in the absence of Wee1 function. Also, we analyzed the DNA content of cell cultures at different times after shifting them from the permissive temperature to 32ºC or 35ºC (Fig. 2B). A peak of 1C cells progressively appeared in the *wee1-50* single mutant and became more

prominent at 35ºC than at 32ºC (Fig. 2B, arrow heads). This indicates that in a number of cells, the G1 phase was lengthened enough for the cytokinesis to take place before they entered into the S phase. This 1C peak was absent in the double mutant and an additional wider peak corresponding to higher DNA content appeared. These results indicate that *wee1-50* mutant cells lacking Reb1 were unable to lengthen G1 and consequently entered prematurely into the S phase. As they complete replication, these cells enter mitosis also prematurely due to the *wee1-50* mutation. Reiteration of these premature G1-to-S and G2-to-M transitions leads to cell lethality. Consistently with this interpretation, double mutant cells cultured for 6 hours at the restrictive temperature showed up to four nuclei and multiple septa, indicating that cells undergo mitosis before cytokinesis was completed (Fig. 2C). Likely, completion of cytokinesis was compromised due to the small size and round shape of these *reb1*Δ *wee1-50* cells. As a consequence, cells with a DNA content higher that 2C were generated (Fig. 2B).

Finally, to test if premature DNA replication after mitosis was causing synthetic lethality of *reb1*Δ *wee1-50* cells, we used a low concentration of the DNA synthesis inhibitor hydroxyurea. This treatment allowed the growth of the double mutant at all temperatures tested (Fig. 2A, lower panels).

cdc10-129 **cells lacking Reb1 are defective in G1 arrest and are inviable at semipermissive temperature**

The expression of several genes coding for proteins required for initiation of DNA replication is regulated by the MBF transcription factor (Bahler, 2005). Cdc10 protein is an essential component of the MBF complex and, as a

consequence, its inactivation leads to cell arrest in G1. We found that *reb1*Δ cells bearing the thermosensitive *cdc10-129* allele were inviable at the semipermissive temperature of 32ºC, whereas single mutants formed colonies (Fig. 3A). At 30ºC the double mutant also exhibited reduced growth. As shown in the flow cytometry analysis of Fig. 3B (upper histograms), 4, 6 and 8 hours after the *cdc10-129* single mutant was shifted from 25ºC to 30ºC, a small percentage of cells with 1C DNA content was generated, indicating that at this temperature G1 phase lengthened enough for some cells to undergo cytokinesis before initiating S phase. The peak of 1C cells was absent in the *reb1*Δ *cdc10-129* double mutant. This effect was more evident when cells were shifted to a higher temperature. After 4 hours at 32ºC, a high percentage of *cdc10-129* cells showed a 1C DNA content, whereas only a very small 1C peak was visible in the double mutant at all times analyzed (Fig. 3B, middle histograms). This indicates that Reb1 is required for an efficient G1 arrest when the activity of Cdc10 is diminished, as previously observed for the *ste9*Δ *cdc10-129* mutant (Kitamura et al., 1998).

Cdc10-129 is a leaky allele and, therefore, *cdc10-129* cells escape from G1 arrest when they are maintained long enough at the shifted temperature. As a consequence, the 1C peak observed in *cdc10-129* cells 4 hours after the shift to 32ºC diminished at 6 and 8 hours, while the peak corresponding to 2C cells increased. This was not observed in the double mutant, as no significant accumulation of cells with a 1C DNA content took place in this strain. Our interpretation is that leaking of *cdc10-129* cells from G1 arrest is exacerbated in the absence of Reb1, which becomes deleterious. We have not observed a significant number of *cut* cells in this double mutant.

The DNA content profiles of *cdc10-129* and *reb1*Δ *cdc10-129* strains at 37ºC were also quite different (Fig. 3B, lower histograms). Two hours after the shift, only a low fraction of the double mutant cells arrested at G1 compared to *cdc10-129* cells. After 4 hours, the temperature shift time routinely used in cell synchronization experiments, most of the *cdc10-129* cells were in G1, while the profile observed in the double mutant consisted in either a wide peak of cells with a more heterogeneous DNA content or two almost fused peaks. As observed at 32ºC, leaking from G1 arrest was also detected when *cdc10-129* cells were maintained at 37ºC for longer than 4 hours. Double mutant cells, however, showed a heterogeneous distribution of DNA content after 8 hours at 37ºC.

Reb1 is required for ste9$^+$ up-regulation induced by nitrogen deprivation

The presence of a putative Reb1 binding sequence upstream *ste9*$^+$, together with the requirement of Reb1 for nitrogen deprivation-induced G1 arrest, suggested that Reb1 may regulate *ste9*+ expression under limiting nitrogen source. To test the latter, wild type and *reb1*Δ log-phase cells were transferred to nitrogen-free medium and the expression of *ste9*$^+$ was monitored at different times by Northern blotting.

Wild type cells growing exponentially in nitrogen-containing medium showed a low level of Ste9 mRNA (Fig. 4A, lane 1). The expression of Ste9, however, was rapidly up-regulated after nitrogen deprivation and the level of Ste9 mRNA reached a maximum after about 1 hour of nutritional stress (Fig. 4A, lanes 2-5). After 6 hours of starvation, Ste9 mRNA declined back to a level similar to that found in cells growing in complete medium (Fig. 4A, lane 6). This

wave of Ste9 overexpression induced by nitrogen starvation was not observed in *reb1*Δ cells (Fig. 4A, lanes 7-12), in agreement with their failure to arrest in G1. These results indicate that Reb1 is required for the overexpression of Ste9 leading to G1 arrest, a prerequisite for conjugation and sexual differentiation.

To study the relevance of the putative 27-bp Reb1 binding sequence present at the *ste9*$^+$ promoter for the overexpression of Ste9, the isogenic strain examined in Fig. 1A and C (mutPste9) was also analyzed by Northern (Fig. 4A, lanes 13-18). The rapid overexpression of Ste9 observed in wild-type cells upon starvation did not take place when this 27-bp sequence was mutated (lanes 13-16). Only after 4 hours of starvation Ste9 mRNA increased somewhat (lanes 17 and 18).

Reb1 binds to the promoter sequence of *ste9*$^+$ and causes replication fork stalling

As mentioned in the Introduction, Reb1 has two DNA binding sequences at the non-transcribed spacer of each rDNA repeat, close to the 3' of the coding region. We have previously shown that Reb1 binding to these two sites induces replication fork arrest in a polar manner, so that only forks moving in the anti-transcription direction are stalled (Sánchez-Gorostiaga et al., 2004). Due to the similarity of these two rDNA sequences and the 27-bp sequence present at the *ste9*$^+$ promoter, we expected that if Reb1 binds this sequence in vivo, it might also cause replication fork stalling upstream *ste9*$^+$. We used two-dimensional (2D) agarose gel electrophoresis to analyze whether the DNA upstream *ste9*$^+$ is able to arrest replication forks when inserted into an autonomously replicating plasmid and if this fork arrest is abolished in cells lacking Reb1. The use of this in vivo replication plasmid assay permitted us to verify if fork arrest occurs in a

polar manner as in the case of Reb1-induced rDNA fork barriers RFB2 and RFB3. We have previously demonstrated that these two rDNA barriers show identical behavior inserted into a replicating plasmid as in their chromosomal context (Sánchez-Gorostiaga et al., 2004).

As diagrammed in Fig. 4B, a 230-bp fragment containing the putative Reb1 binding site (Pste9) from the promoter of *ste9*$^{+}$ was inserted in both orientations next to the replication origin *ars1* of plasmid pIRT2. The resultant plasmids, pPste9(+) and pPste9(-) were introduced into wild type and *reb1*Δ strains, isolated from log-phase cells and digested with the restriction enzyme *Pvu*II. Finally, replication of the large *Pvu*II fragment containing the insert was analyzed in 2D gels. Plasmid pPste9(+) replicating in wild-type cells showed a strong hybridization signal at the expected position on the arc of Y-shaped replication intermediates (Fig. 4C, arrow), indicating that fork arrest at Pste9 took place. On the other hand, this signal corresponding to arrested forks completely vanished in *reb1*Δ cells (Fig. 4D), indicating that Reb1 binds to Pste9 sequence in vivo and induces replication fork stalling. Moreover, as in the rDNA, this activity was polar since we did not detect the signal corresponding to arrested forks when Pste9 was inserted in the (-) orientation in pPste9(-) (Fig. 4E).

Reb1 is bound to the promoter of *ste9*$^{+}$ in exponentially growing cells and remains bound upon nitrogen deprivation

We also analyzed binding of Reb1 to the promoter of *ste9*$^{+}$ in vivo by chromatin immunoprecipitation (ChIP) assays. We fused Reb1 to the tandem affinity purification (TAP) tag and used IgG-sepharose to immunoprecipitate chromatin

cross-linked to Reb1-TAP. Chromatin was prepared from three otherwise isogenic strains: a Reb1-TAP strain bearing wild-type *ste9*$^{+}$ gene, a Reb1-TAP strain bearing the mutPste9 gene described above and, as a control, a strain with the wild-type untagged *reb1*$^{+}$ and *ste9*$^{+}$ genes. Chromatin was prepared from exponentially growing cells and after 1 and 4 hours of nitrogen starvation. As diagrammed in Fig. 4F, amplification of the immunoprecipitated chromatin was carried out by duplex PCR using two pairs of oligonucleotides targeted to the promoter of *ste9*$^{+}$ and to a region downstream *ste9*$^{+}$. The co-amplified products, separated by 2.2 kb, were 230 (Pste9) and 174 bp (3'-ste9) long, respectively.

The promoter region of *ste9*$^{+}$ was significantly enriched (about 6-fold) in the immunoprecipitated chromatin from exponentially growing Reb1-TAP *ste9*$^{+}$ cells (Fig. 4G, left gel, 0 h in –N, quantified in Fig. 4H). Moreover, the enrichment of Pste9 was maintained after 1 and 4 hours of nitrogen starvation (Fig. 4G, left gels; Fig. 4H, dark gray rectangles). No enrichment was observed in the control Reb1-untagged *ste9*$^{+}$ strain (Fig. 4G, right gels; Fig. 4H, white rectangles). On the other hand, the *ste9*$^{+}$ promoter was not enriched when the 27-bp putative Reb1 binding sequence was mutated in the Reb1-TAP mutPste9 strain, neither before nor after nitrogen deprivation (Fig. 4G, middle gels; Fig. 4H, gray light rectangles). These results indicate that the Reb1 protein binds to the promoter of *ste9*$^{+}$ and that the 27-bp sequence upstream Ste9 is required for this binding. They also indicate that Reb1 binding to the promoter of *ste9*$^{+}$ was not triggered by nitrogen starvation, since Reb1 was bound to the promoter before transferring the cells to nitrogen-free medium.

It has been reported that *ste9*+ expression peaks in G1, in agreement with its role in the regulation of this phase of the cell cycle (Peng et al., 2005; Rustici et al., 2004; Tournier and Millar, 2000). Our observation that Reb1 is bound to the *ste9*+ promoter in exponentially growing cells raised the possibility that Reb1 could be involved in the periodic expression of *ste9*+ during a normal mitotic cell cycle. To address this issue, we used a cdc25-22 block at the restrictive temperature to synchronize wt and *reb1*Δ strains in late G2 and analyzed the *ste9*+ mRNA by retrotranscription real-time quantitative PCR (RT-qPCR) at different times after their release at the permissive temperature. The percentage of binucleate cells and the septation index was also measured by DAPI and calcofluor staining to monitor synchronization (Fig. 5A). As deduced from the time of appearance of binucleate cells after the release, *reb1*Δ cells showed a delay of about fifty minutes until they completed of mitosis (Fig. 5A, bottom panel), compared to wt cells (Fig. 5A, upper panel). As in wt cells, however, expression of *ste9*+ was up-regulated in *reb1*Δ cells while they entered G1 and declined later, just before the wave of septation. These results indicate that Reb1 is not required for the periodic expression of *ste9*+ in a normal cell cycle.

The effect of *reb1*+ overexpression was also studied. *reb1*$^{+}$*-HA* was cloned in the pREP24X expression vector under the control of the thiamine-repressible promoter Pnmt1 and the resultant plasmid pREP24X-Reb1 was used to transform MR003 strain (*reb1*Δ::*kanMX6 ura4-D18*). The highest expression level of *reb1*+ was observed 16 hours after cell's incubation in the absence of thiamine (data not shown). At the same time point, expression of *ste9*+ was 3.24-fold (SD=0.65) the level observed before overexpression of

reb1+, determined by RT-qPCR. As shown in the flow cytometry data of Fig. 5B, overexpression of *reb1*+ caused the progressive accumulation of cells with a 1C DNA content, which represented about 20% of the culture after 20 hours of activation. Significant diploidization resulting in cells with DNA contents higher than 2C was not observed.

Additional proteins interact with the Reb1p-Pste9 complex in a Reb1-dependent manner and dissociate upon nitrogen deprivation

Altogether, our results show that Reb1 is required for the overexpression of *ste9*+ that leads to G1 arrest under nitrogen starvation. For this up-regulation, Reb1 needs to be bound to the 27-bp upstream activating sequence at the promoter region of *ste9*$^+$. However, as shown above, Reb1 is already bound to the promoter of *ste9*$^+$ in exponentially growing cells. Therefore, binding of Reb1 to the promoter is required but not sufficient for up-regulation of Ste9. What does it ultimately make *ste9*$^+$ to be overexpressed upon starvation? We hypothesized that additional protein(s) might be involved in the trans-activation of *ste9*$^+$ by interacting with the Reb1-Pste9 complex. To detect these possible DNA-proteins complexes, we carried out electrophoretic mobility shift assays (EMSA) using as target the DNA sequence up-stream *ste9*$^+$ from position -198 through -160 containing the 27-bp Reb1-binding site. We first analyzed complexes formed using protein extracts from wild type and *reb1*Δ cells growing exponentially in rich medium (Fig. 5A, lanes 2-4). Using a wild type extract, five retarded complexes were visible (arrowheads). Interestingly, formation of all these complexes required Reb1, since none of them appeared when *reb1*Δ

extracts were used (Fig. 5A, lane 3). Next, we analyzed complexes formed using proteins from cells growing exponentially in minimal medium containing nitrogen source (Fig. 5A, lanes 6 and 8) or after 1 h of nitrogen starvation (Fig. 5A, lanes 7 and 9). As in rich medium, the same complexes described above were detected using wild-type protein extracts (Fig. 5A, lane 6). However, after 1 hour of starvation none of these complexes were formed except that showing the highest electrophoretic mobility (Fig. 5A, lane 7, empty arrowhead). Using *reb1*∆ extracts no complexes were detected regardless cells were grown with or without nitrogen source (Fig. 5A, lanes 8 and 9).

One interpretation of these results is that in exponentially growing cells, when expression of Ste9 is low (Fig. 4A, lane 1), other proteins interact with this region of the *ste9*$^{+}$ promoter (Fig. 6A, lanes 2 and 6) in a Reb1 dependent manner (Fig. 6A, lanes 3 and 8). After 1 hour of nitrogen starvation, when expression of Ste9 is high (Fig. 4A, lane 3), the affinity of these proteins for the promoter is lost and Reb1 is mostly the only protein that remains bound (Fig. 6A, lane 7). This suggests that Reb1 acts as a transcription factor that is capable to induce expression of Ste9. However, this activity would be inhibited by other negative trans-acting factors in exponentially growing cells in the absence of nutritional stress. Upon nitrogen deprivation, the affinity of these negative trans-acting factors is lost, and then Reb1 mediates Ste9 overexpression leading to G1 arrest.

This interpretation predicts that some of the DNA-protein complexes formed with *S. pombe* extracts should not form using Reb1 expressed in a heterologous system, since the *S. pombe* negative trans-acting factors would

be absent. Another prediction is that Reb1 should be present in all the DNA-protein complexes observed.

To verify the first prediction, *reb1*+ cDNA was obtained by RT-PCR and cloned into the *E. coli* expression vector pBAD24, under the control of the arabinose-inducible *araBAD* promoter (Mejía-Ramírez et al., 2005). EMSAs were performed using the same DNA probe as in Fig. 6A and proteins from the *E. coli* strain TOP10 transformed with either the empty vector pBAD24 (Fig. 6B, lanes 2 and 3) or the recombinant plasmid pBAD24-Reb1 (Fig. 6B, lanes 4-6). Only proteins from bacteria transformed with pBAD24-Reb1 and incubated in the presence of arabinose gave rise to the formation of retarded complexes. The specificity of the complexes was verified by the addition of an excess of unlabelled probe to the binding reaction (Fig. 6B, lane 6). The recombinant Reb1 protein expressed in *E. coli* formed one major complex and its electrophoretic mobility was similar to the *S. pombe* complex that showed the highest mobility (Fig. 6, lane 7, continuous line), indicating that Reb1 is the only protein present in this *S. pombe* complex. Two faint additional retarded bands showing a higher retardation were observed using Reb1 expressed in *E. coli*. They may correspond to multimeric forms of Reb1 bound to the probe, as it has been recently observed using as a probe the DNA sequence of the rDNA replication fork barrier RFB3 (Biswas and Bastia, 2008). The mobility of two of the complexes formed by the *S. pombe* extract coincided with these two faint bands (Fig. 6B, lane 6, dotted lines) and, therefore, they may also be generated by Reb1 multimers. However, other DNA-protein complexes that formed using the *S. pombe* extracts did not form using Reb1 expressed in *E. coli* (Fig. 6B,

lane 7, asterisks). This indicates that protein(s) different from Reb1 are present in these complexes.

To verify that Reb1 was present in all the DNA-protein complexes formed, supershift EMSAs were performed using extracts from a Reb1-HA *S. pombe* strain. Addition of anti-HA monoclonal antibody to the reaction mixture before (Fig. 6C, lane 5) or after (Fig. 6C, lane 6) the probe, supershifted all the DNA-protein complexes formed in the absence of the antibody (Fig. 6C, lane 4), while anti-FLAG monoclonal antibody, used as a control, had no effect (Fig. 6C, lanes 7 and 8).

DISCUSSION

Conjugation and sexual differentiation in fission yeast require cell arrest in the G1 phase, which can be induced by nutritional stress such as deprivation of nitrogen source in the medium. Ste9p is a G1-specific APC/C activator that promotes degradation of B-cyclin at this cell cycle phase, playing a crucial role in nutritional stress-induced G1 arrest. As a consequence, *ste9* mutant cells are unable to exit vegetative cell cycle from G1 under nitrogen deprivation and are deficient in conjugation and sexual differentiation. The activity of Ste9 is regulated at different levels. During S and G2, Ste9 is targeted for proteolysis by Cdc2-Cdc13-mediated phosphorylation (Blanco et al., 2000; Yamaguchi et al., 2000). Also, Ste9 mRNA decay has been recently shown to down-regulate Ste9 during G2, which permits accumulation of Cdc13 and the activation of Cdc2 for the timely entrance of cells into mitosis (Álvarez et al., 2006). Finally, in agreement with its role, *ste9*$^{+}$ is periodically expressed during G1 (Peng et al., 2005; Rustici et al., 2004; Tournier and Millar, 2000).

In the present study, we found that the sequence specific DNA-binding protein Reb1 is a transcriptional activator of Ste9 that binds in vivo to a specific sequence at its promoter. Null *reb1* mutant cells do not overexpress *ste9*$^+$ in response to nitrogen deprivation and are unable to arrest in G1. Expression of *ste9*$^+$ under the control of *nmt1* promoter reversed this G1 arrest deficiency of *reb1*Δ cells. Moreover, mutation of the nucleotide sequence recognized by Reb1 at the promoter of *ste9*$^+$ in otherwise wild type cells precludes its binding in vivo, significantly reduces transcriptional up-regulation of *ste9*$^+$ in the absence of nitrogen source, and renders cells deficient in G1 arrest and mating.

Interestingly, although binding of Reb1 to the *ste9*$^+$ promoter (Pste9) is required for the overexpression of Ste9 induced by nitrogen starvation, ChIP assays showed that Reb1 is already bound to the promoter before cells are deprived of nitrogen (Fig. 4G and H), when expression of *ste9*$^+$ is very low (Fig. 4A, lane 1). Thus, Reb1-Pste9 binding is not triggered by this nutritional stress, although this binding is required for the proper cell response to the stress. These findings made us to consider the possibility that other(s) protein factor(s) are also involved. EMSA experiments indicated that other proteins do associate to the Reb1-Pste9 complex in cells growing in nitrogen-containing medium, when expression of *ste9*$^+$ is low, while Reb1-Pste9 is nearly the only remaining complex after 1 hour of nitrogen deprivation (Fig. 6A), when overexpression of *ste9*$^+$ reaches the highest level (Fig. 4A, lane 3).

As shown in the model presented in Fig. 6D, these findings suggest that the associated proteins prevent Reb1-mediated transcriptional activation. Soon after nitrogen deprivation, the negative regulation proteins (NRPs) dissociate from the *ste9*$^+$ promoter and Reb1, which remains bound, is now able to

stimulate its overexpression. The interaction of the negative transcriptional regulation proteins with the *ste9*$^{+}$ promoter region must be mediated by Reb1, since in *reb1*∆ cells no Pste9-protein complexes formed regardless nitrogen is present or not in the medium (Fig. 6A), and Reb1 is present in all DNA-protein complexes formed (Fig. 6C). The quick release of negative transcriptional regulation proteins from the promoter upon nutritional stress, suggests that it might be prompted by post-translational modification of some of these proteins or Reb1 leading to loss of protein-protein interactions (Fig. 5B).

We observed that overexpression of *reb1*+ leads to an increase of *ste9*+ expression and to the accumulation of cells with a 1C DNA content. This is probably due to the sequestration of the inhibitory proteins by the overexpressed Reb1, leaving free Reb1 to promote transcriptional activation of *ste9*+.

It was recently shown that Reb1p expressed in *E. coli* binds in vitro as a dimer to the rDNA sequence corresponding to replication fork barrier RFB3 (Biswas and Bastia, 2008). Reb1 dimerization domain is located within the 146 N-terminal amino acids, while the two Myb/SANT DNA binding domains are located near the C-terminus. This raises the possibility that the negative transcriptional regulation proteins associate with the Reb1-Pste9 complex through the N-terminus of Reb1 under vegetative growing. Upon nitrogen starvation, modification of this Reb1 protein-protein interacting domain, as part of the cell response to nutritional stress, would lead to the dissociation of the repressing factors.

The mammalian ortholog of Reb1, TTF1, is involved in the regulation of rRNA transcription by binding to a short rDNA sequence adjacent to the

promoter. Interestingly, TTF1 also contains an N-terminal oligomerization domain that acts masking the DNA binding capacity of two Myb domains located near the C-terminus (Sander et al., 1996). Supporting the interpretation of our results in *S. pombe*, this N-terminal region of TTF1 directly interacts with the chromatin remodeling complex NoRC. This enables TTF1 to bind the rDNA promoter and to repress rRNA transcription (Nemeth et al., 2004; Strohner et al., 2004).

Ste9 is periodically expressed during the G1 phase in exponentially growing cells (Peng et al., 2005; Rustici et al., 2004; Tournier and Millar, 2000). It was suggested that the MBF complex (Mlu cell-cycle-box binding factor) controls this periodic expression in hydroxyurea-synchronized *S. pombe* cells (Tournier and Millar, 2000). MBF regulates expression of genes coding for proteins required for S phase (Bahler, 2005). Binding of MBF to the promoter of *ste9*$^{+}$ have not been proved yet, although two putative binding sequences are present around 670 bp upstream the ATG (Tournier and Millar, 2000) (475 bp upstream the Reb1-binding sequence). We found that up-regulation of *ste9*+ in G1 during a normal mitotic cell cycle occurs in the absence of Reb1. This indicates that Reb1 does not control periodic expression of *ste9*+ under normal growth, but it is responsible for the up-regulation of *ste9*+ under nutritional stress conditions, collaborating to sexual differentiation, and under conditions that require G1 lengthening for survival, such as in *wee1*-defficient cells.

We found that a sequence containing the Reb1 binding site from the promoter of *ste9*$^{+}$ caused replication fork arrest in an orientation- and Reb1-dependent manner (Fig. 4C-E). As mentioned before, binding of Reb1 to two sites present near the 3' end of the coding region of each rDNA repeat induces

stalling of replication forks moving in the direction opposite to rRNA transcription, while forks moving in the other direction pass unhindered (Sánchez-Gorostiaga et al., 2004). There is evidence indicating that these highly conserved natural RFBs prevent the deleterious effects of head-on collision between transcription and replication machineries (López-Estraño et al., 1998; Olavarrieta et al., 2002; Takeuchi et al., 2003). It is unlikely that the RFB at ste9+ plays a similar role, as it is located at the promoter region and the Reb1 binding sequence is oriented so that only replication forks moving in the same direction as transcription of *ste9*+ would be arrested. We speculate that direction of replication, determined by the Reb1-dependent *Pste9*-RFB, might be an important factor for the proper coordination between the transcriptional status of *ste9*+ and cell cycle. Experiments designed to address this hypothesis are underway. Interestingly, *S. pombe* RTS1, another natural RFB located in the mating-type locus, determines the appropiate direction of replication of this region required for an efficient mating-type switching (Dalgaard and Klar, 2001). Rtf1 is a DNA binding protein responsible for the function of RTS1 that shows a high similarity with Reb1 (Eydmann et al., 2008).

MATERIALS AND METHODS

Yeast strains and growth conditions. All *S. pombe* strains used in this work are listed in Table 1. Cells were grown in rich medium (YES) or in Edinburgh minimal medium (EMM) with supplements as required (Moreno et al., 1991). For nitrogen starvation, cells were grown in EMM to mid-log phase, washed and cultured in NH_4Cl-free EMM (EMM-N) at 25ºC for the indicated times. Activation or repression of *nmt1* promoter was achieved by growing cells in EMM or EMM

containing 15 µM thiamine, respectively. For mating efficiency determination, cells to be mated were separately grown to mid-log phase in YES medium and equal numbers of cells were pelleted, washed, mixed and spotted on sporulating MEA plates. After 24 and 48 h of growth at 25ºC, cells were examined under the microscope and mating efficiency was calculated as (2 X number of zygotes and asci formed) / [(2 X number of zygotes and asci formed) + number of non-mating cells], expressed in percentage.

Construction of strains. Mutation of the Reb1 DNA binding sequence at the promoter of *ste9*$^+$, named *mutPste9*, was achieved as follows. First, the complete *ste9*$^+$ ORF of strain S1335 (*h*$^-$ *ura4-d18*) was replaced by *ura4*$^+$ by one-step PCR-based substitution method, obtaining strain LR3. Next, *ste9*$^+$ gene was inserted back in strain LR3 by replacing *ura4*$^+$ with a PCR product containing *ste9*$^+$ and mutation *mutPste9* at its promoter. This PCR product was obtained using oligonucleotides Mut-ste9-Fw (5'-TTCGCTTAAAGTCCAACCCT CGCTACACGCTGTTCAGCCATTTAATTATGCCC**GGTACC**GCAAAGGAAATT GGGAGCAG TAGACAATGGTGATTGTGAAGGATGC-3') and Mut-ste9-Rv (5'-GATGATGTAGAAGTAGATGG-3') on DNA from a wild type *ste9*$^+$ strain. Oligonucleotide Mut-ste9-Fw contained a 27-bp mismatch (underlined) corresponding to *mutPste* mutation. Recombinant Ura$^-$ colonies were selected in EMM plates supplemented with uracil and containing 5-fluoro-orotic acid (5-FOA). The construction was confirmed by Southern blot, PCR and sequencing. Finally, the resultant strain, LR4, was made prototroph by crossing with *ura4*$^+$ strain 975 *h*$^+$, obtaining strains LR4Ph- and LR4Ph+.

C-terminal tagging of Reb1 with TAP was carried out by one-step PCR-based insertion method as previously described (Tasto et al., 2001). The PCR-

amplified sequence was obtained from plasmid pKLG1810 (pFA6a-kanMX6-CTAP2 , kindly provided by K. Gould) (Tasto et al., 2001), using 100-nucleotide-long primers TagRebFwd and TagRebRev (sequence available upon request). The same primers were used for C-terminal tagging of Reb1 with HA, using plasmid pFA6a-3HA-hphMX6 as substrate (kindly provided by T. Toda) (Sato et al., 2005). Strain selection was confirmed by PCR, sequencing and western blotting. Tagging did not affect Reb1 function, as cells responded to nitrogen starvation as wt cells and rDNA replication fork barriers were fully functional.

Overexpression of Reb1 was achived by cloning *reb1-HA* into pREP24X vector. The insert was obtained by PCR from strain MR020 and cloned at the *Xho*I site of pREP24X. The expressed Reb1-HA protein was functional as it reverted the *reb1*Δ phenotype of MR020 host strain.

Chromatin immunoprecipitation. ChIP assays were carried out as previously described (Pidoux et al., 2004), with few modifications. The immunoprecipitated (IP) and INPUT DNA were amplified by duplex PCR using two pairs of primers in the same reaction, one to amplify the promoter region of *ste9*+ (Pste9) (ChSte9Fw: 5'-CTTTCGCTTAAAGTCCAACC-3', and ChSte9Rev: 5'-GACTTCACCGATTAAATGTC-3') and the other to amplify a region 3' of *ste9*$^+$ (3'-ste9), used as internal control (CtrSteFw: 5'-GCTTCGTTATTCACGTAATC AA-3', and CtrSteRev: 5'-TTCAATTAACGTGCGTCCGT-3'). PCR of serial dilutions of DNA samples were performed to ensure analysis within the linear range of the PCR. Amplified products were separated by polyacrylamide gel electrophoresis and quantified using ImageJ software. Enrichment of Pste9

region in the immunoprecipitated chromatin (IP), indicative of Reb1 binding, was estimated as $(Pste9/3'\text{-}ste9)^{IP}/(Pste9/3'\text{-}ste9)^{INPUT}$.

RNA preparation and Northern blots. Total RNA was isolated from about $5x10^7$ cells using the RNeasy Mini Kit (Qiagen) following the supplier's instructions. RNA samples (8 μg) were electrophoresed through formaldehyde-agarose gel, transfered to membranes and hybridized with the *ste9*$^+$ ORF amplified by PCR.

Protein extracts. *S. pombe* and *E. coli* extracts were prepared as previously (Mejía-Ramírez et al., 2005), except that *S. pombe* extraction buffer contained 25 mM HEPES, pH 7.6, 150 mM KCl, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM urea, 25% glycerol, 0.2 mM PMSF and 1.5x protease inhibitors mix (Roche).

Electrophoretic mobility shift assay (EMSA). Binding reaction mixtures (20 μl) included 44 μg of *S. pombe* protein extract or 15 μg of proteins from *E. coli*, 25 mM HEPES, pH 7.6, 34 mM KCl, 10 mM EDTA, 0.5 mM DTT, 0.1 μg /μl poly(dI-dC), 0.1 μg/μl salmon sperm DNA and 10^4 cpm of radiolabeled probe. The probe was obtained by PCR from a pIRT2-derivative plasmid where oligonucleotides ste9a (5´-GATCCAAAGTGCATTACCCTTCCCGGTTTC TCAGTAGACAATGGG-3´) and ste9b (5'-GATCCCCATTGTCTACTGAG AAACCGGGAAGGGTAATGCACTTTG-3') were annealed and inserted at the *Bam*HI site. Primers used for PCR amplification flanked the insert. The PCR product was digested with *Bam*HI and the fragment containing the sequence from the promoter of *ste9*$^+$ was separated by electrophoresis and purified. Labeling was carried out by filling the 5' protruding ends with [α-^{32}P]dCTP and Klenow and purified through a G-25 Sephadex column (Roche). Binding

reaction mixtures were incubated for 20 minutes at room temperature and electrophoresed at 4ºC in 6% polyacrylamide-0.5x Tris-borate-EDTA gels at 10 V/cm for 2.5 hours. For supershift assays, 0.1 µg of anti-HA (3F10, Roche) or anti-FLAG M2 (F3165, Sigma) monoclonal antibody was added to the reaction mixture and incubated for 30 min at 4ºC before addition of the probe or after the DNA-protein binding reaction.

Retrotranscription real-time quantitative PCR (RT-qPCR). Total RNA was extracted using the RNeasy Mini Kit (Qiagen). On-column digestion with DNase was carried out using the RNase-Free DNase Set (Qiagen). cDNA was obtained using the Superscript II reversed transcriptase and random primers (Invitrogen). RT-qPCR reactions (20 µl) were performed in triplicate, containing IQ Sybr Green SuperMix (Bio-Rad), 1 µl of cDNA (at 1:1, 1:10 or 1:100 dilution) and 0.4 µM of each primer, with *cdc2* as internal control. The reactions were analysed using an IQ5 System (Bio-Rad). In each sample, the amount of *ste9*+ transcript relative to *cdc2*+ was calculated as $(1\text{-}E)^{\Delta Ct}$, where ΔC_T is the difference between the threshold cycle for the *cdc2*+ mRNA and for the *ste9*+ mRNA, and E is the efficiency for the pair of *ste9*+ primers. For each experiment, the data were normalized to the initial transcription level. The primers used were 5'-CCTACTTCATCGAATTCGTC-3' and 5'-GTAGAAGCA TCCCTAGAAGG-3' for *ste9*+, and 5'-ACCAGCTAGTGAACGGTGTA-3' and 5'-ACAATACTTCAGGAGCACGA-3' for *cdc2*+.

Two-dimensional agarose gel electrophoresis. DNA purification from asynchronous log-phase cultures and analyses of replication intermediates by 2D gel electrophoresis were performed as previously described (Sánchez-

Gorostiaga et al., 2004). Plasmids analyzed, pPste9(+) and pPste9(-), were constructed as follows. A 230-bp fragment containing the Reb1-binding sequence upstream *ste9*+ was obtained by PCR using oligonucleotides ChSte9Fw and ChSte9Rev (see above). This fragment was first inserted into the TA-cloning vector pGEM-T Easy (Promega), extracted by digestion of the resultant plasmid with *Bam*HI and cloned in both orientations at the *Bam*HI site of the *S. pombe* vector pIRT2. The probe used for hybridization of 2D gels was the *Pvu*II restriction fragment from pIRT2, containing *ars1* and *LEU2*.

Flow Cytometry. About 10^7 cells were spun down, fixed in 70% cold ethanol and washed in 50 mM sodium citrate. Cells were treated with 200 µg/ml RNaseA for 2 hours at 37ºC and stained with 1 µM Sytox Green (Invitrogen – Molecular Probes) in the same buffer. After sonication, a Becton-Dickinson FACS Vantage apparatus was used for flow cytometry.

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FIGURE LEGENDS

Fig. 1. Reb1 is required for efficient nitrogen starvation-induced G1 arrest and mating. **(A)** Wild type (117 h- and 118 h^+), *reb1*Δ (D7 h- and D9 h^+) and *mutPste9* (LR4Ph- and LR4Ph+) *S. pombe* strains were separately grown and equal numbers of cells were mixed and spotted on sporulating MEA plates. Wild type mating: 117 h- x 118 h^+, *reb1*Δ mating: D7 h- x D9 h^+, *mutPste9* mating: LR4Ph- x LR4Ph+. After 24 and 48 hours at 25ºC, mating efficiency was calculated as (2 X number of zygotes and asci formed) / [(2 X number of zygotes and asci formed) + number of non-mating cells], expressed in percentage. **(B)** The upper diagram represents the *S. pombe* rDNA non-transcribed spacer, containing replication fork barriers 1 through 3 (RFB1-3). Function of RFB2 and 3 (filled boxes) depend on Reb1 binding to two identical 17-bp sequences, while RFB1 (open box) is Reb1-independent and requires protein Sap1. Alignment of nucleotide sequence from RFB3 (the strongest of the two Reb1-dependent barriers) with the promoter region of *ste9*$^+$ gene is shown in the middle. Underlined nucleotides correspond to the 17-bp Reb1-binding sequence (Zhao et al., 1997), sufficient for replication fork arrest activity (Sánchez-Gorostiaga et al., 2004). In the lower diagram, the location of the sequence upstream *ste9*$^+$ similar to RFB3 is indicated. Numbers are relative to the translation start codon. **(C)** FACS analysis of cells cultured under nitrogen starvation. Wt (972), *reb1*Δ (D9P), *ste9*Δ (LR3) and mutPste9 (LR4Ph-) prototrophic strains were grown in complete minimal medium (EMM), washed, and cultured at 25ºC in EMM without nitrogen (EMM-N) for the indicated times. **(D)** Deficiency of *reb1*Δ cells to arrest in G1 upon nitrogen deprivation is

rescued by expression of *ste9*$^{+}$ under the *nmt1* promoter. Wild type (MR021) and *reb1*Δ (MR025) cells transformed with empty pREP81X expression vector or expressing *ste9*$^{+}$ (pREP81X-Ste9) were grown to midlog phase in complete EMM with thiamine. Then cells were cultured at 25ºC in EMM-N without thiamine for the indicated times and DNA content was determined by flow cytometry.

Fig. 2. *reb1*Δ and *wee1-50* mutations are synthetically lethal and deficient in G1 phase lengthening **(A)** Serial dilutions of exponential cultures of strains wt (972), *reb1*Δ (D9P), *wee1-50* (MR011) and *reb1*Δ *wee1-50* (MR014) growing at 25ºC were spotted on YES or YES+HU plates and grown at the indicated temperatures. **(B)** DNA content of the same strains used in (A) grown in YES at 25ºC to midlog phase and shifted to 32ºC or 35ºC for the indicated times. (**C**) Phenotype of *h*$^{-}$ *reb1*Δ *wee1-50* cells cultured at 35ºC for 6 hours and stained with DAPI and calcofluor. Wild-type cells are shown at the bottom (scale bar, 5 µm)

Fig. 3. *reb1*Δ *cdc10-129* double mutants are inviable at semipermissive temperature and defective in G1 arrest. **(A)** Serial dilutions of exponential cultures of strains wt (972), *reb1*Δ (D9P), *cdc10-129* (MR030) and *reb1*Δ *cdc10-129* (MR018) grown in YES at 25ºC were spotted on YES plates and grown at the indicated temperatures. **(B)** Flow cytometry analysis of the same strains used in (A) grown in YES to midlog phase at 25ºC and shifted to 30ºC, 32ºC or 37ºC for the indicated times.

Fig. 4. Reb1 is required for *ste9*$^{+}$ transcriptional activation induced by nitrogen deprivation and binds in vivo to the *ste9*$^{+}$ promoter. **(A)** Wt (972), *reb1*Δ (D9P)

and mutPste9 (LR4Ph-) prototrophic cells were grown in EMM to midlog phase, washed and cultured in EMM-N at 25ºC for the indicated times. RNA was extracted and Ste9 mRNA was analyzed by Northern blotting using a radiolabeled specific probe. A *ste9*Δ*::ura4* strain (LR3) grown for 1 hour without nitrogen was used as negative control (lane 19). 18S rRNA was used as loading control. **(B)** Binding of Reb1 to the promoter of *ste9*$^{+}$ arrests replication fork in a polar manner. Map of the autonomously replicating plasmids analyzed by 2D electrophoresis in C-E. A 230-bp fragment from the promoter of *ste9*$^{+}$ (indicated as Pste9 in F), was inserted in both orientations at the indicated location of vector pIRT2, resulting in plasmids pPste9(+) and pPste9(-). **(C-E)** Replication analyses of the large *Pvu*II restriction fragment of the plasmids shown in B. The arrow in panel B points to the signal corresponding to the specific Y-shaped replication intermediate accumulated as a consequence of fork stalling at the Pste9+ insert. Note that this fragment corresponding to the promoter of *ste9*$^{+}$ induced fork arrest in a wild-type strain (118) (panel C), but not in an isogenic *reb1*Δ strain (D9) (panel D). This Reb1-mediated fork arrest was unidirectional since replication fork did not suffer impediment when Pste9 was cloned in the (-) orientation (panel E). **(F)** Location of the primer pairs used for PCR amplification of immunoprecipitated chromatin. The size of each amplified product is indicated in parenthesis. **(G)** Strains ZG023 (*reb1-2xTAP:KanMX6 ste9*$^{+}$), ZGLR002 (*reb1-2xTAP:KanMX6 mutPste9*) and 972 (wt) were grown to midlog phase in complete EMM, washed and cultured in EMM-N at 25ºC. Before nitrogen deprivation (0 h) and after the indicated times of starvation, cells were cross-linked with formaldehyde, chromatin was sheared, a sample was taken as control (INPUT), and Reb1-TAP was

immunoprecipitated (IP) using IgG Sepharose resin. DNA from IP and INPUT chromatin were amplified by duplex PCR using both primer sets indicated in F to determine simultaneously the enrichment of the *ste9*$^{+}$ promoter (Pste9 product) and a region downstream *ste9*$^{+}$ (3'-ste9 product). (**H**) Quantification of the relative enrichment of *ste9*$^{+}$ promoter, calculated by dividing the relative intensity of Pste9 and 3'-ste9 PCR products from IP chromatin and from INPUT chromatin. Band intensity was determined using ImageJ software.

Fig. 5. (A) Periodic expression of *ste9*+ occurs in the absence of Reb1. Cells from *cdc25-22* and *cdc25-22 reb1*Δ (MR006) strains were synchronized at late G2 by incubation at 36ºC for 4 hours, and released from the blockage at 25ºC for the indicated times. The relative level of *ste9*+ mRNA was measured by RT-qPCR using *cdc2* mRNA as internal reference gene (●, right-hand ordinates). To monitor the release from the arrest, the percentage of binucleate cells (○, left-hand ordinates) and binucleate cells containing septa (△, left-hand ordinates) (septation index) were scored by DAPI and calcofluor staining. **(B)** Overexpression of *reb1*+ causes the accumulation of cells with 1C DNA content. Flow cytometry analysis of MR003 cells transformed with pREP24X or pREP24X-Reb1 and cultured for the indicated times in thiamine-free medium.

Fig. 6. Several Reb1-dependent DNA-protein complexes are formed at the promoter of *ste9*$^{+}$ and most of them dissociate upon nitrogen starvation. **(A)** Electrophoretic shift assays analysis (EMSA) using protein extracts from wt (117) and *reb1*Δ (D7) strains. The nucleotide sequence from -198 to -160 positions of the *ste9*$^{+}$ promoter was used as probe for all assays shown in this figure. Cells were grown exponentially in rich medium (lanes 1-4) or EMM

(lanes 5-10). The latter cultures were grown for 1 additional hour in the same medium (lanes 6, 8 and 10) or in the same medium without nitrogen source (lanes 7 and 9). In lanes 4 and 10, a 200-fold excess of unlabelled probe was added to the binding reaction as specific competitor and proteins from wild-type cells were used. **(B)** EMSA analysis of Reb1 expressed in *E. coli*. Proteins from TOP10 *E. coli* cells transformed with the empty expression vector pBAD24 (lanes 2 and 3) or pBAD24-Reb1 (lanes 4-6), containing *reb1*+ gene, were analyzed. To induce the *araBAD* promoter, cells were incubated for 2 hours in medium containing L-arabinose (lanes 2, 5 and 6). To verify the specificity of binding, an excess of unlabeled probe was added to the reaction in lane 6. To compare the mobility of the complexes, EMSA analysis of protein extracts from wt (lanes 7 and 8) and *reb1*Δ (lane 9) *S. pombe* cells were run in the same gel. In lane 8, two-fold excess of unlabeled probe was added to the binding reaction. **(C)** EMSA supershift using protein extracts from strain MR020 (*reb1-3HA*) (lanes 3-8). The reaction mixture was incubated with monoclonal antibody anti-HA (lanes 5 and 6) or anti-FLAG (lanes 7 and 8). This incubation was performed before (lanes 5 and 7) or after (lanes 6 and 8) addition of the probe. In lanes 3 and 4 binding reactions took place without antibody and in lane 3 an excess of unlabeled probe was present. **(D)** Model for up-regulation of *ste9*$^+$ transcription upon nitrogen starvation. See Discussion for details.

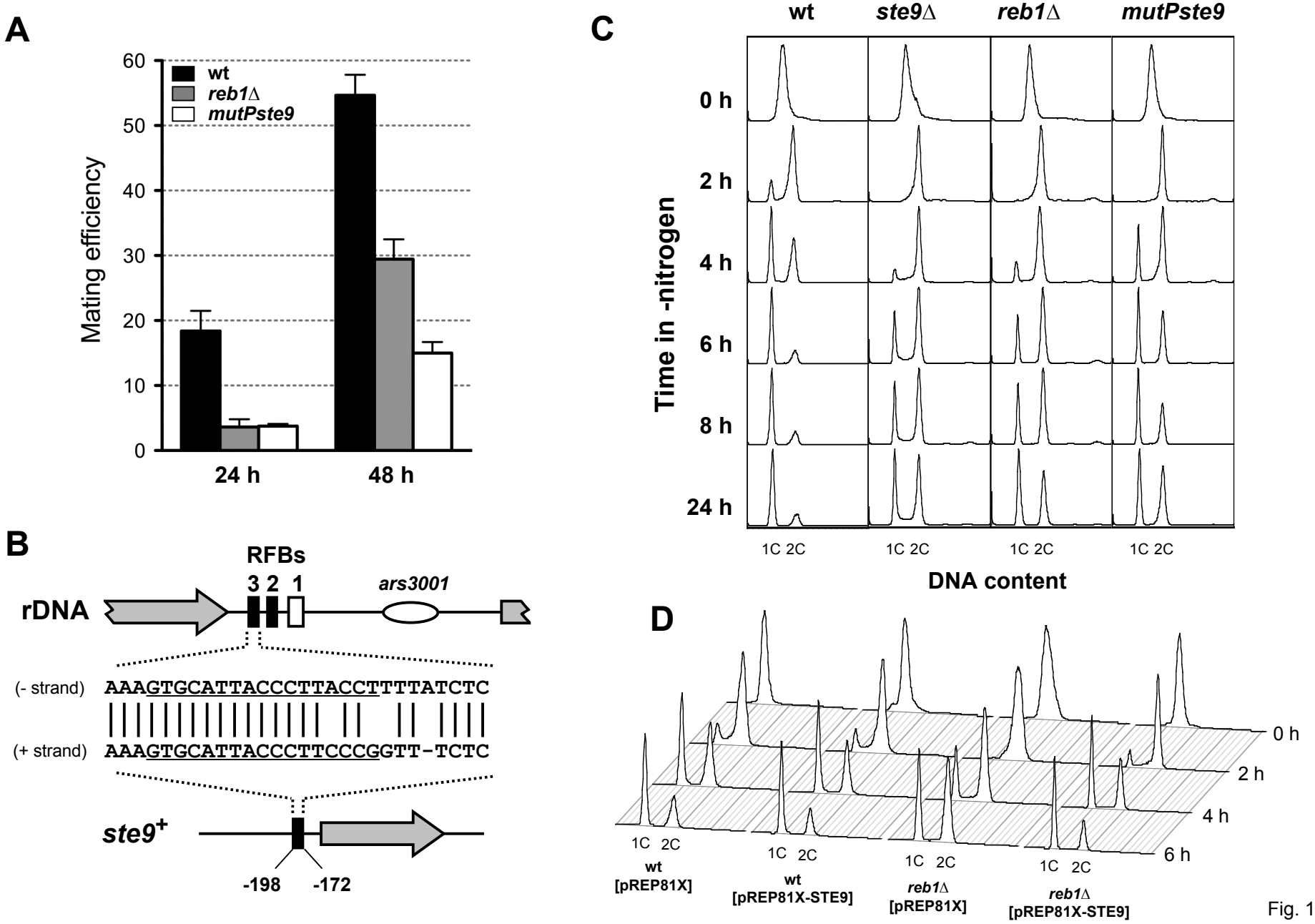

A
Mating efficiency
60
50
40
30
20
10
0
wt
reb1Δ
mutPste9
24 h
48 h
B
RFBs
3 2 1
ars3001
rDNA
(- strand) AAAGTGCATTACCCTTACCTTTTATCTC
(+ strand) AAAGTGCATTACCCTTCCCGGTT-TCTC
ste9+
-198
-172
C
wt
ste9Δ
reb1Δ
mutPste9
Time in -nitrogen
0 h
2 h
4 h
6 h
8 h
24 h
1C 2C
DNA content
D
0 h
2 h
4 h
6 h
1C 2C
wt
[pREP81X]
wt
[pREP81X-STE9]
reb1Δ
[pREP81X]
reb1Δ
[pREP81X-STE9]

Fig. 1

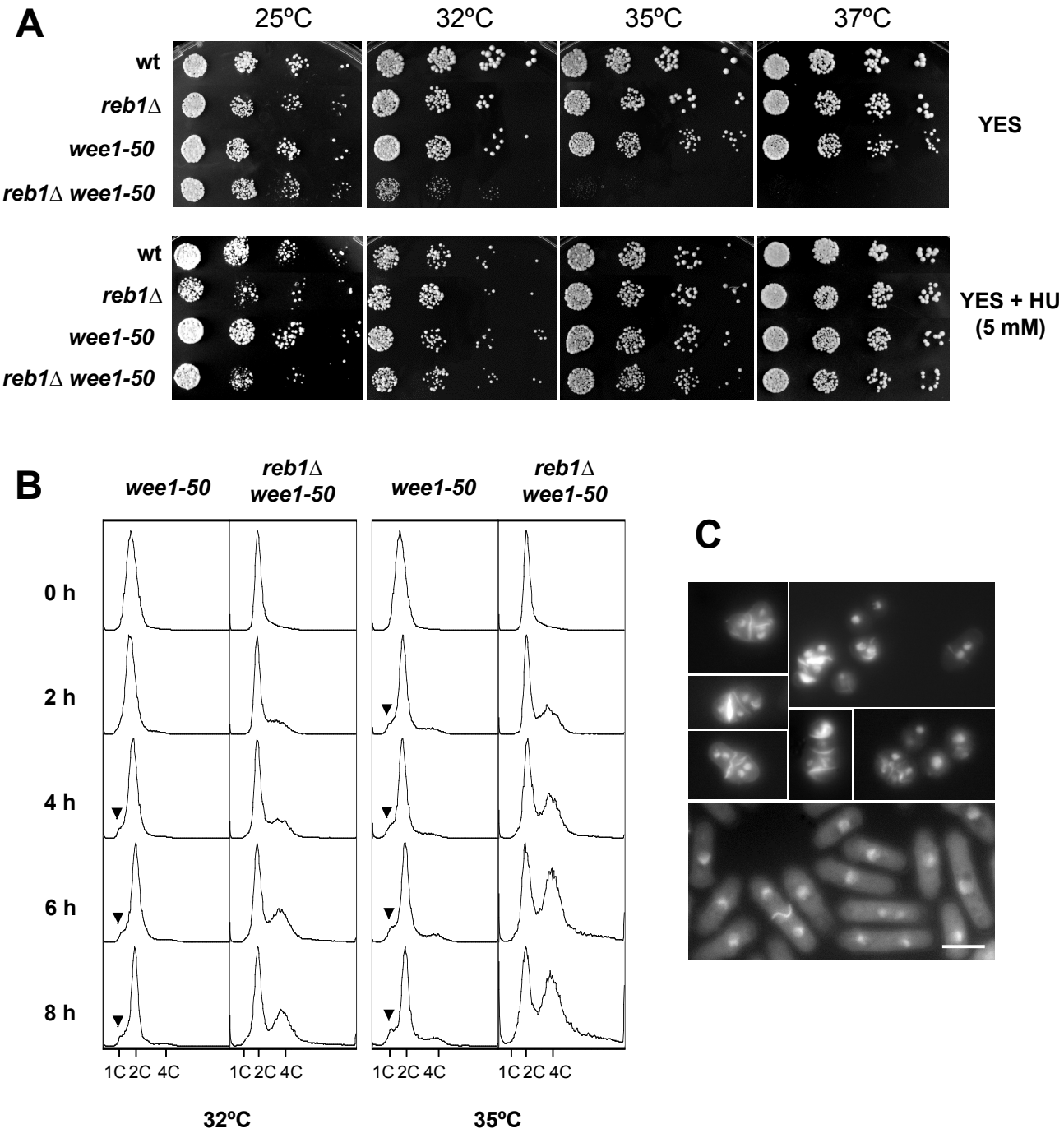

A
25ºC
32ºC
35ºC
37ºC
wt
reb1Δ
wee1-50
reb1Δ wee1-50
YES
YES + HU
(5 mM)
B
wee1-50
reb1Δ wee1-50
0 h
2 h
4 h
6 h
8 h
1C 2C 4C
32ºC
35ºC
C

Fig. 2

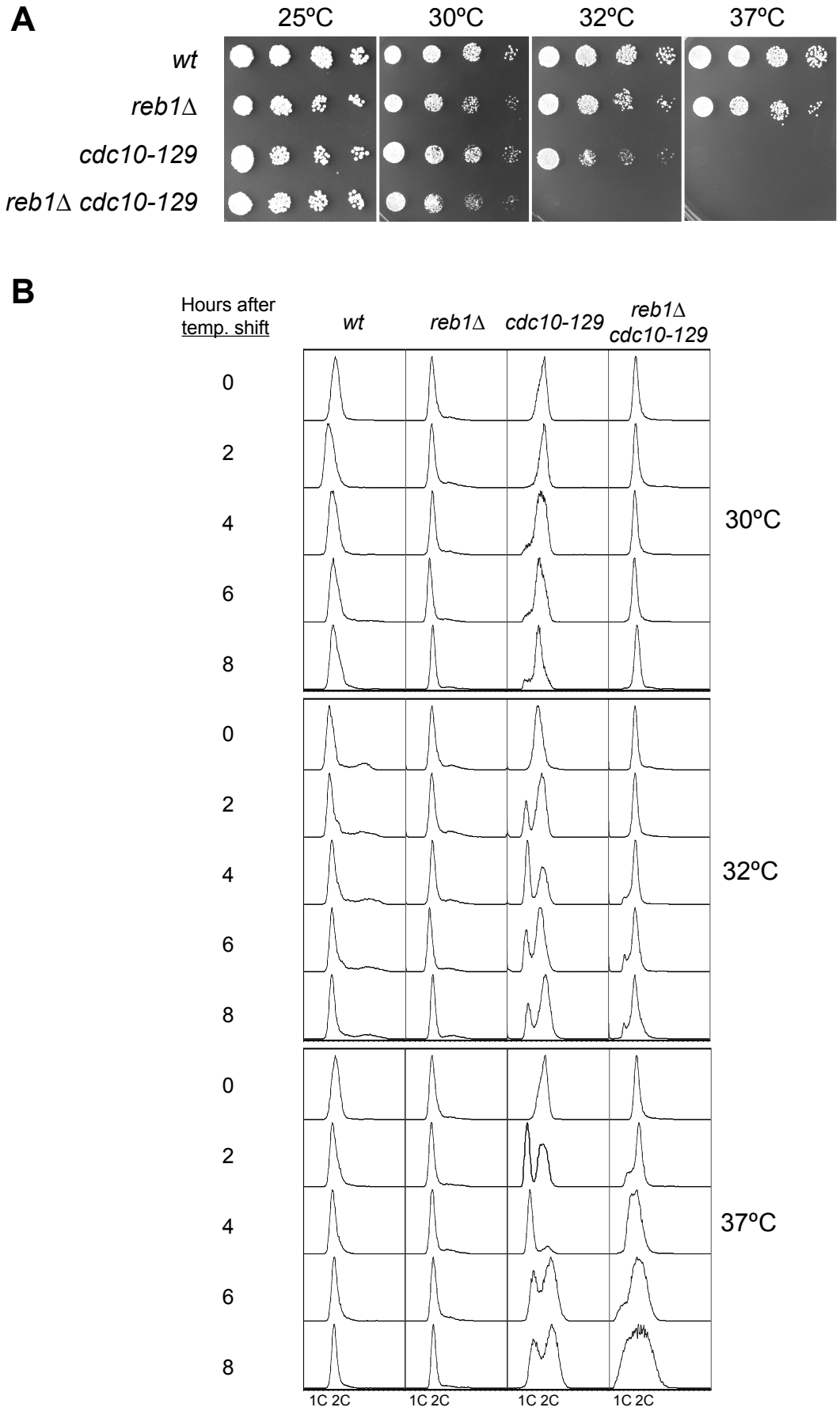

A
25ºC
30ºC
32ºC
37ºC
wt
reb1Δ
cdc10-129
reb1Δ cdc10-129
B
Hours after temp. shift
wt
reb1Δ
cdc10-129
reb1Δ cdc10-129
0
2
4
6
8
30ºC
32ºC
37ºC
1C 2C
1C 2C
1C 2C
1C 2C

Fig. 3

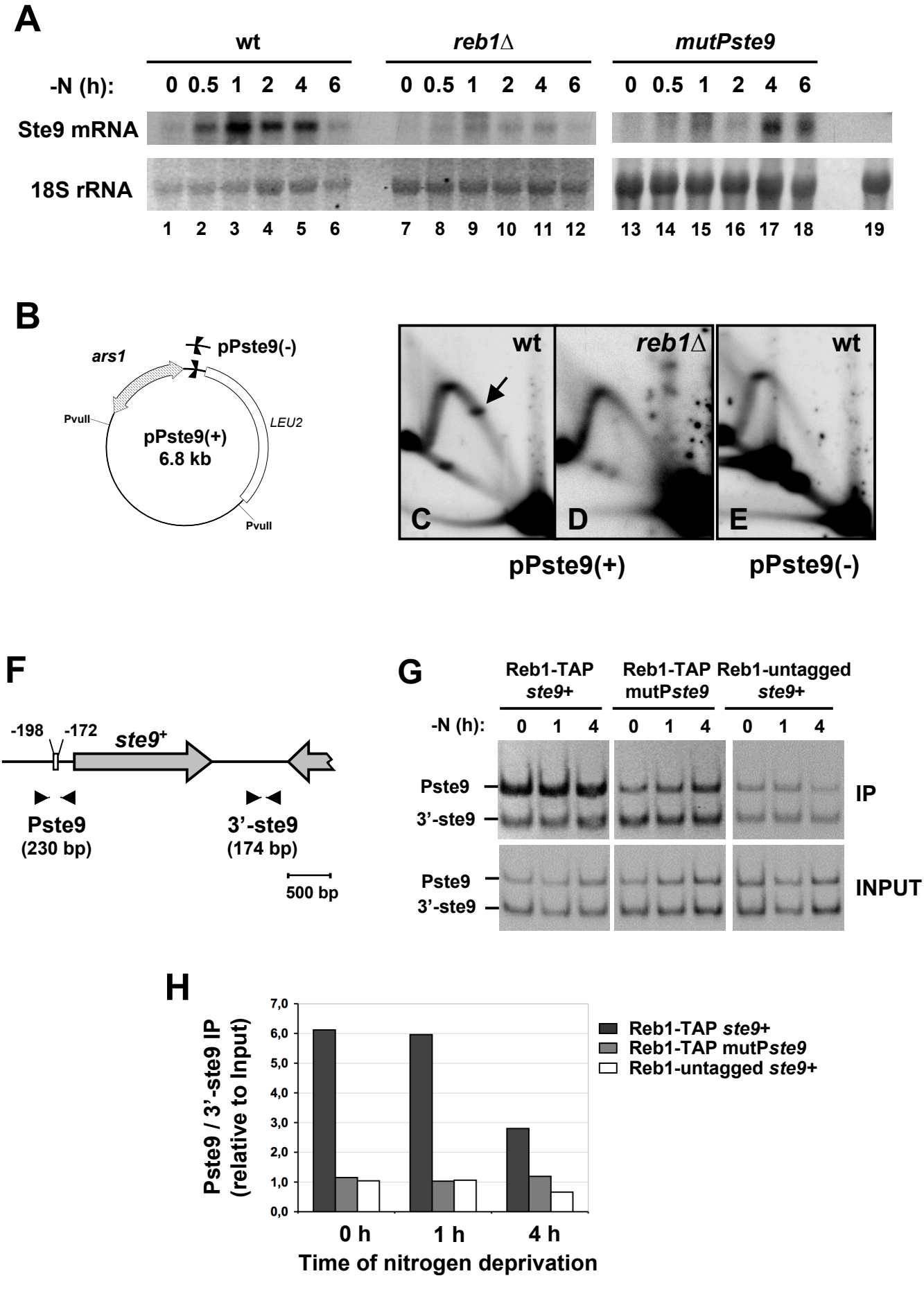
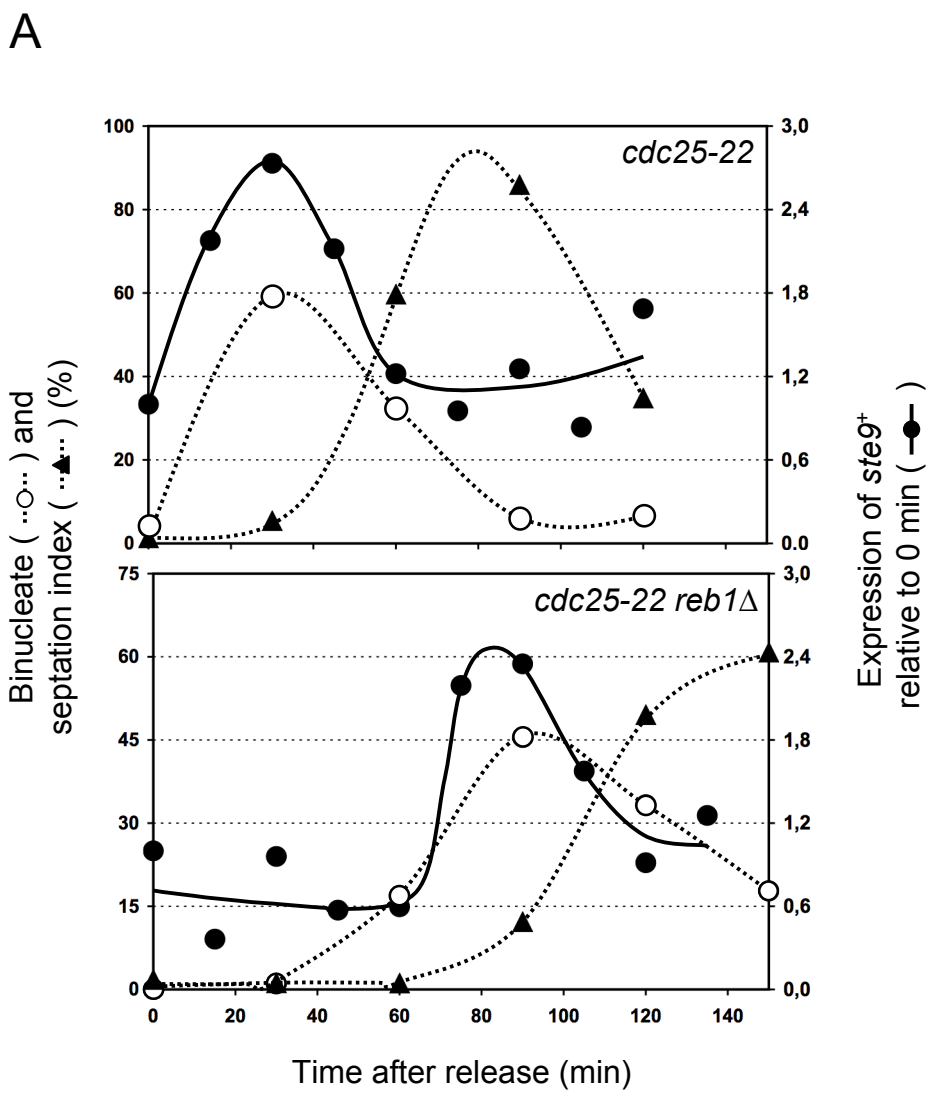

A
wt
reb1Δ
mutPste9
-N (h):
0 0.5 1 2 4 6
0 0.5 1 2 4 6
0 0.5 1 2 4 6
Ste9 mRNA
18S rRNA
1 2 3 4 5 6
7 8 9 10 11 12
13 14 15 16 17 18 19
B
ars1
pPste9(-)
PvuII
LEU2
pPste9(+)
6.8 kb
PvuII
wt
reb1Δ
wt
C
D
E
pPste9(+)
pPste9(-)
F
-198
-172
ste9+
Pste9
(230 bp)
3'-ste9
(174 bp)
500 bp
G
Reb1-TAP
ste9+
Reb1-TAP
mutPste9
Reb1-untagged
ste9+
-N (h):
0 1 4 0 1 4 0 1 4
Pste9
3'-ste9
IP
Pste9
3'-ste9
INPUT
H
Pste9 / 3'-ste9 IP
(relative to Input)
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0 h
1 h
4 h
Time of nitrogen deprivation
Reb1-TAP ste9+
Reb1-TAP mutPste9
Reb1-untagged ste9+

Fig. 4


A
cdc25-22
cdc25-22 reb1Δ
Binucleate (--○--) and
septation index (···▲···) (%)
Expression of ste9+
relative to 0 min (—●—)
Time after release (min)
100
80
60
40
20
0
75
60
45
30
15
0
0
20
40
60
80
100
120
140
3,0
2,4
1,8
1,2
0,6
0.0
3,0
2,4
1,8
1,2
0,6
0,0

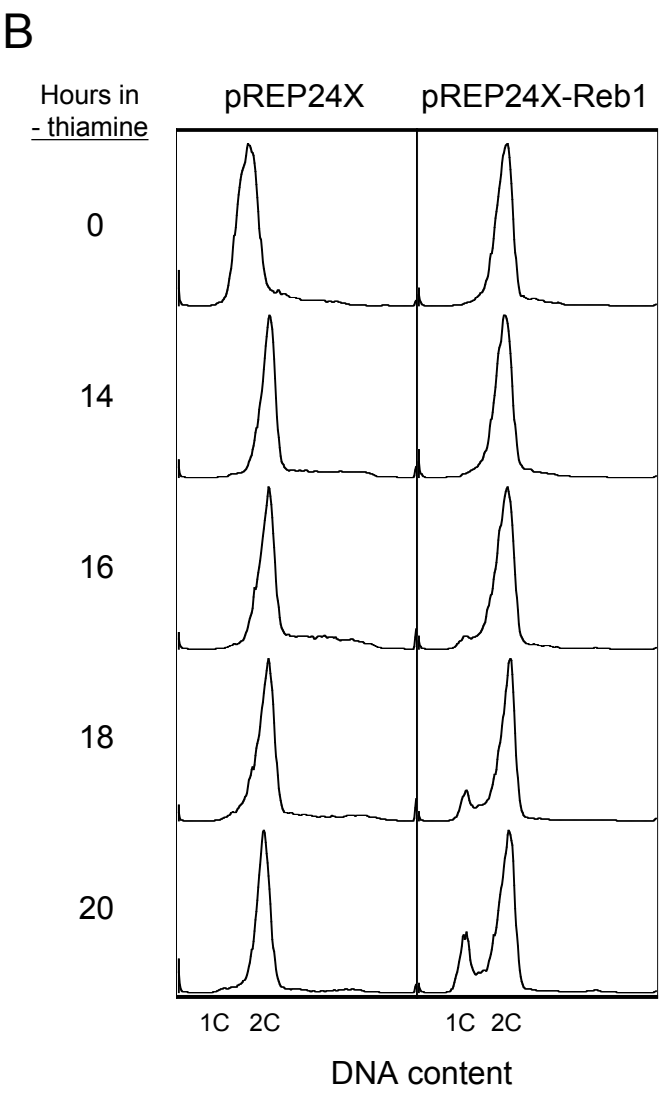

B
Hours in
- thiamine
pREP24X
pREP24X-Reb1
0
14
16
18
20
1C 2C
1C 2C
DNA content

Fig. 5

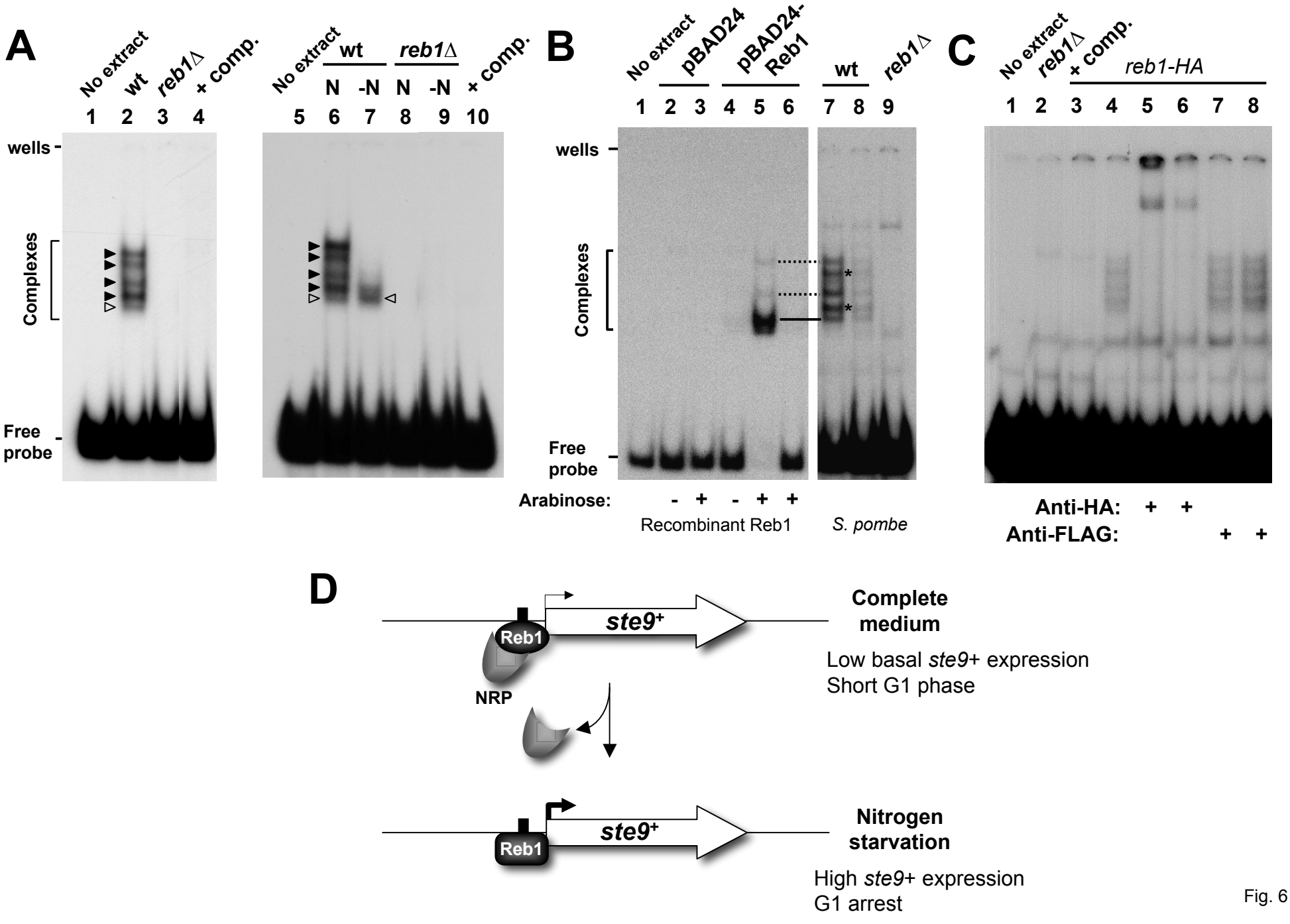

A
No extract
wt
reb1Δ
+ comp.
1 2 3 4
wells
Complexes
Free probe
No extract
wt
reb1Δ
+ comp.
N -N N -N
5 6 7 8 9 10
B
No extract
pBAD24
pBAD24-Reb1
wt
reb1Δ
1 2 3 4 5 6 7 8 9
wells
Complexes
Free probe
Arabinose: - + - + +
Recombinant Reb1
S. pombe
C
No extract
reb1Δ
+ comp.
reb1-HA
1 2 3 4 5 6 7 8
Anti-HA: + +
Anti-FLAG: + +
D
ste9+
Reb1
NRP
Complete medium
Low basal ste9+ expression
Short G1 phase
ste9+
Reb1
Nitrogen starvation
High ste9+ expression
G1 arrest

Fig. 6

Table 1. *S. pombe* strains used in this study

Strain	Genotype	Source
117	*h⁻ leu1-32 ura4-D18 ade6-M210*	S. Moreno
118	*h⁺ leu1-32 ura4-D18 ade6-M216*	S. Moreno
972	*h⁻* wt	-
975	*h⁺* wt	-
D7	*h⁻ leu1-32 ura4-D18 ade6-M210 reb1Δ::kanMX6*	Sánchez-Gorostiaga et al., 2004
D9	*h⁺ leu1-32 ura4-D18 ade6-M216 reb1Δ::kanMX6*	Sánchez-Gorostiaga et al., 2004
D9P	*h⁻ reb1Δ::kanMX6*	This study
LR3	*h⁻ ste9::ura4 ura4-D18*	This study
LR4	*h⁻ mutPste9 ura4-D18*	This study
LR4Ph-	*h⁻ mutPste9*	This study
LR4Ph+	*h⁺ mutPste9*	This study
MR003	*h⁻ ura4-D18 reb1Δ::kanMX6*	This study
MR006	*h⁻ cdc25-22 reb1Δ::kanMX6*	This study
MR011	*h⁻ wee1-50*	This study
MR014	*h⁻ reb1Δ::kanMX6 wee1-50*	This study
MR018	*h⁻ reb1Δ::kanMX6 cdc10-129 leu1-32 ura4-D18*	This study
MR020	*h⁻ reb1-3HA-hphMX6*	This study
MR021	*h⁻ leu1-32*	This study
MR025	*h⁻ leu1-32 reb1Δ::kanMX6*	This study
MR030	*h⁻ cdc10-129 leu1-32 ura4-D18*	A. Bueno
S1335	*h⁻ ura4-D18*	S. Moreno
ZG023	*h⁻ reb1-2xTAP:KanMX6*	This study
ZGLR002	*h⁻ reb1-2xTAP:KanMX6 mutPste9*	This study