Max1 links MBF-dependent transcription to completion of DNA synthesis in fission yeast

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A mi madre

SUMMARY

Summary

When DNA replication is challenged, cells activate a DNA synthesis checkpoint blocking cell cycle progression until they are able to overcome the replication defects. In fission yeast, Cds1 is the effector kinase of this checkpoint, inhibiting M phase entry, stabilizing stalled replication forks and triggering transcriptional activation of S-phase genes; the molecular basis of this last effect remains largely unknown. The MBF complex controls the transcription of S-phase genes. We have purified novel interactors of the MBF complex and among them we have identified the repressor Max1. When the DNA synthesis checkpoint is activated, Max1 is phosphorylated by Cds1 resulting in the abrogation of its binding to MBF. As a consequence, MBF-dependent transcription is maintained active until cells are able to overcome this challenge.

Resumen

Cuando la replicación del DNA se ve alterada, las células activan un mecanismo de control bloqueando la progresión del ciclo celular hasta que son capaces de superar el daño. En la levadura de fisión, Cds1 es la proteína kinasa efectora de dicha respuesta, mediante inhibición de la entrada en fase M, estabilización las horquillas de replicación bloqueadas, e inducción de la activación de la transcripción de los genes de fase S; siendo la base molecular de este último proceso poco conocida. El factor de transcripción MBF controla la transcripción de los genes de fase S. Hemos purificado proteínas que interaccionan con MBF, y entre ellas, hemos identificado al represor Max1. Cuando el checkpoint de síntesis de DNA es activado, Max1 es fosforilado por la kinasa Cds1, y esto se traduce en la disociación de Max1 del complejo MBF. Como consecuencia, la transcripción MBF-dependiente se mantiene activa hasta que las células son capaces de superar el daño.

INDEX

INTRODUCTION

RESULTS

DISCUSSION

INTRODUCTION

1. *Schizosaccharomyces pombe*

Schizosaccharomyces pombe is an eukaryotic unicellular organism widely used as a model organism due to its simple growth conditions in the laboratory, and specially its easy genetic manipulation. It has a small well characterized genome of 5036 genes, only three chromosomes, and it proliferates in a haploid state. Therefore it has one single copy of the genome, which facilitates simple gene function analysis working with mutations and deletions.

It has been particularly used as a model in cell cycle regulation research. The fundamental features of cell cycle regulation have been conserved for millions years of eukaryotic evolution, and *S. pombe* shares a great molecular similarity to higher eukaryotes regarding its mechanisms of cell cycle control.

This organism is also known as fission yeast because it divides by bipartition, forming a septum at a central position of the cell. This feature allows to easily identify by microscope observation the phase of the cell cycle in which cells are.

2. Mitotic Cell Cycle

Cell cycle control in eukaryotic cells depends on a precise regulatory machinery that ensures that the events of the cell cycle occur in the correct order. The main events to be regulated are the duplication of genetic content and the distribution of those components into two identical daughter cells.

Chromosome duplication and distribution are tightly regulated processes and occur in two phases of the cell cycle called S phase (DNA synthesis) and M phase (chromosome segregation). Between S and M phases, cells need time for growth, and these periods are known as gap phases: G1 occurs after M phase and G2 occurs after S phase. Gap phases are important for cell cycle regulation, to control the progression to the next phase.

Cell cycle control machinery ensures that:

- Chromosomes are duplicated once and only once every cell cycle.
- DNA synthesis is completed before entry into M phase.
- Chromosome segregation equally distributes chromosomes into the two daughter cells.

Also, cell growth must be regulated to maintain the proper cell size. All the steps of regulation take place at particular moments of the cell cycle named checkpoints. Any trouble in the accomplishment of one of the phases of the cycle is detected in a checkpoint control, and cell cycle arrests. Then, cell cycle progression is delayed until the problems are solved.

2.1. Cell cycle in fission yeast

Mitotic cell cycle of fission yeast consists of a long G2 phase where cells grow by length extension, followed by a rapid M phase where chromosomes are segregated, a short G1 phase, and a S phase where DNA is replicated. Mitosis is followed by formation of the septum at a central position in the cell, but it is a slow process that does not occur rapidly after M phase. In fact, septation takes place coinciding with S phase. Because of the delay between these two events, cariokinesis and citokinesis, *S. pombe* cells have a DNA content of 2C throughout the cycle. This makes asynchronous growing cultures to show a peculiar flow cytometry profile compared to other eukaryotes, with a single peak of 2C DNA content.

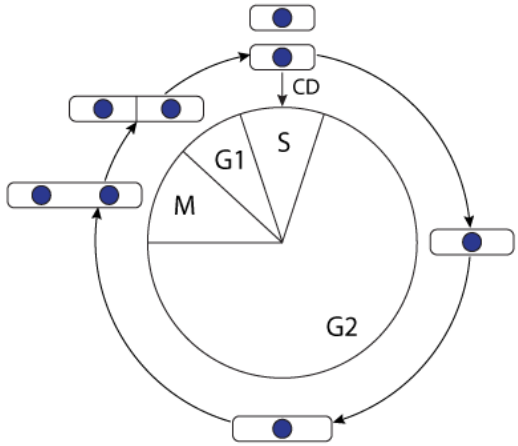


Figure 1. The fission yeast cell cycle (Image from The CellLMProject)

Cell growth by extension and nucleus division can be estimated by direct microscope observation. This feature allowed, in the 70s, to isolate mutant strains defective in cell cycle regulation. Many key regulators of mitotic cell cycle were identified, and the genes were named *cdc* genes (cell division cycle). Some of the strains

defective in cell cycle regulation showed an elongated phenotype, whereas other mutations caused a reduction in cell size (Fig. 2). Since most of these proteins are essential, the strains carrying such mutations were isolated as conditional mutants, and more precisely, as temperature sensitive (ts) mutants. Punctual mutations in these alleles allow cells to grow at permissive temperature (25ºC), but when shifted to restrictive temperature (36ºC), cells are not able to progress through cell cycle.

In *S. pombe*, there are several temperature sensitive strains that are used as a powerful tool to synchronize cultures. *cdc25-22* cells have an elongated shape due to a longer G2 phase, because cells are compromised to enter into M phase and get arrested in the G2/M transition, although they keep growing by length extension.

The opposite phenotype can be observed in the *wee* mutants, small cells because they enter rapidly into M phase shortening the growing period of G2. Because of this, cells divide at a smaller size. There is a cell size control at G1/S transition that ensures cells to proceed with DNA synthesis (S phase) only if they have the required critical mass. Mutant strains that are smaller when they enter mitosis extend their G1 phase until they achieve the threshold of size required to progress through cell cycle.

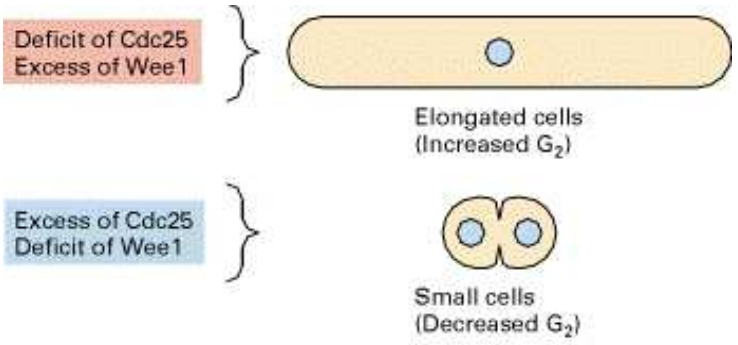


Figure 2. Schematic representation of the *cdc* and the *wee* phenotypes (From Molecular Cell Biology, Lodish, Darnell et al.).

2.2. CDK/Cyclin complexes

The mechanisms of cell cycle regulation mainly control the onset of M and S phases to ensure that these events occur in the correct order and that there is always alternancy between M and S phases. Such transitions are regulated by CDK/cyclin complexes, which belong to a highly conserved family of enzymes in eukaryotes.

CDKs (cyclin dependent kinases) are called so because their catalytic activity depends on their binding to the cyclins (regulatory subunits of the complex). They regulate the different phases of the cycle by their binding to different phase-specific cyclins.

Cyclin protein levels typically show a cell cycle periodicity, and they are regulated by several mechanisms to achieve the activation of the corresponding CDK/cyclin complex at the proper time. They are regulated at the level of gene expression, and also at the level of degradation. These two mechanisms allow the oscillations in the protein levels. On the contrary, protein levels of the kinases CDKs do no oscillate during the cycle. Their activity is regulated by the

cyclin concentration. Other layers of regulation modulate the kinase activity of the CDK complexes, like phosphorylations, dephosphorylations, or binding of CDK inhibitor proteins (CKIs).

CDKs phosphorylate multiple substrates with a role in the corresponding phase of the cell cycle. It is a robust network of phosphorylations that triggers the different events of mitotic cell cycle with the appropriate order and timing. The number of CDK complexes differs depending on the organism, but the mechanisms of cell cycle regulation have been highly conserved during the eukaryotic evolution.

Cell cycle regulation in fission yeast depends on a single CDK kinase, Cdc2, bound to different cyclins depending on the phase of the cell cycle (Hayles et al., 1994). Levels of Cdc2 protein are constant throughout the mitotic cycle, and the cell phase specific regulation is achieved by means of the binding to the different cyclins, which are Cdc13, Cig2, Cig1 and Puc1.

Cdc13 is a B type cyclin required for entry into mitosis (Booher et al., 1989; Moreno et al., 1989). Δ*cdc13* cells undergo multiple rounds of DNA replication without the subsequent mitosis (Hayles et al., 1994). Its transcription is not cell-cycle regulated, but protein levels fluctuate during the cell cycle, increasing during G2, and decreasing in anaphase due to the proteolytic degradation of the protein by the APC complex (Creanor and Mitchison, 1996).

Cig2 is also a B type cyclin. Although initially it was thought to have a role in mitosis (Bueno and Russell, 1993), its main function is in the onset of S phase (Connolly and Beach, 1994a; Mondesert

et al., 1996). Deletion of *cig2* does not have an effect on cell cycle or in cell viability, but Δ*cig2* cells show increased ability to enter the sexual cycle (Connolly and Beach, 1994b). Cig2 has a role in the regulation of the S phase, and among the substrates of the Cdc2/Cig2 CDK complex there are several proteins from the replication machinery, like Cdc18, that is inhibited when is phosphorylated by the complex (Lopez-Girona et al., 1998).

Cig1 (also a B type cyclin, although it lacks the destruction box) has a role in G1. Deletion of *cig1* does not cause mitotic defects, but a delay in initiation of S phase, and thus Δ*cig1* cells have a longer G1 phase (Bueno et al., 1991). However, there is functional redundancy between Cig1 and Cig2. None of them individually is required for S phase entry but deletion of both cyclins causes a delay in the progression through the G1 phase (Connolly and Beach, 1994b).

Puc1 has certain similarity to the G1 cyclins of *S. cerevisiae*. It was described to have a possible role in G1 (Forsburg and Nurse, 1994) but its function remains unclear. It was described to regulate the length of G1, coupling it to the achievement of a critical cell size (Martin-Castellanos et al., 2000).

Among all the cyclins, only Cdc13 is essential and it can substitute any other cyclin in the different phases of the cell cycle (Mondesert et al., 1996). The CDK/cyclin complexes in G1 and S phase phosphorylate high affinity substrates. Therefore, CDK activity of the complexes Cdc2/Cig2 and Cdc2/Cig1 is moderate, but enough to phosphorylate their substrates. On the contrary, substrates in G2/M are low affinity substrates, and they require a highly active

CDK complex to be phosphorylated, like Cdc2/Cdc13 (Broek et al., 1991; Fisher and Nurse, 1996).

2.3. G2/M transition regulation

Transition from G2 to mitosis depends on the activity of the G2 CDK complex. All the events required for mitotic entry are triggered when this complex reaches the highest kinase activity.

Studies in S. *pombe* allowed to identify the main regulators of this transition. It is a mechanism based on regulatory phosphorylations that are conserved in higher eukaryotes (Nurse, 1990).

In *S. pombe,* the complex Cdc2/Cdc13 accumulates as cells progress into G2, by an increase in the levels of the cyclin; however the complex accumulates in an inactive state, which is achieved by inhibitory phosphorylations at residue Tyr-15 of the CDK kinase Cdc2 (Gould and Nurse, 1989). The kinases responsible of the inactivating phosphorylations of Cdc2 are Wee1 and Mik1, with redundant activities. The active state of Cdc2/Cdc13 is reached by means of the dephosphorylation of tyrosine 15, which is done by the phosphatase Cdc25 (Millar et al., 1991; Russell and Nurse, 1986).

In higher eukaryotes this system is maintained, where there are at least two CDK complexes at G2, with two different B type cyclins involved, and being Wee1 and Myt1 the inactivating kinases and several isoforms of Cdc25 the activating phosphatases.

The proper order of these phosphorylation events is necessary for an activation of the complex at the required moment, and the system functions as a positive feedback loop, in which it is the CDK complex that triggers its own activation, by inactivation of the kinase Wee1, and activation of phosphatase Cdc25 through phosphorylations. When the balance between the two states of CDK, inactive and active, is switched to the active CDK state above a certain threshold, cells enter mitosis irreversibly.

Among the CDK substrates in mitosis, there are the proteins required for the early mitotic events. Phosphorylation of the APC (anaphase promoting complex), leads to destruction of securin (inhibitor of separation of sister chromatids) and of the mitotic cyclins (Cdc13 in fission yeast). Degradation of the cyclins ensures the irreversibility of the process: CDK complex is inactivated, and the subsequent dephosphorylation of its substrates avoids re-entry into early mitotic events, leading to the mitotic exit.

2.4. START

G1 is an important phase in eukaryotic cells. It includes the Start checkpoint (restriction point for mammalian cells), a decision point in late G1 in which cells decide between continue proliferation in the vegetative cycle or to remain in G1 phase and enter the sexual cycle or a quiescent state. After the passage through Start, cells are committed irreversibly to complete the subsequent mitotic cycle, completing chromosome replication in S phase.

Yeasts normally progress from one vegetative cell cycle to the next, and proliferation is limited at START only if external nutrient

levels are limited. In that case, they exit the vegetative cycle and enter the sexual cycle. In the case of mammalian cells, proliferation and passage through the restriction point depends on the appropriate extracellular signals (mitogens) and in many tissues cells may stay permanently in the G0 quiescent state. (Pardee, 1989).

The passage through Start requires two steps: (1) the activation of the G1 CDK and (2) the activation of the G1/S transcriptional program. In *S. pombe*, two regulators essential for the passage through Start have been described: the CDK Cdc2 (although its exact role in this passage is not clear, and Cdc10, which is part of the G1/S transcription factor MBF (see below) (Simanis et al., 1987). In *S. cerevisiae*, the key regulators of this decision point are the homologues to the ones in S. *pombe*: the CDK Cdc28, and the transcription factors SBF/ MBF (Epstein and Cross, 1992). Those transcription factors activate transcription of several genes required for the passage through Start (like G1 and S phase cyclins) and genes required in S phase for DNA synthesis.

Following the activation of CDK and MBF/SBF, many events in early cell cycle are triggered, like spindle pole body duplication, and DNA replication, and cells proceed with the cell cycle until its completion. Loose of control at the restriction point can lead to a misregulation in cell proliferation and is frequently associated to cancer (Pardee, 1989)**.**

2.5. DNA replication and S phase

Chromosome duplication occurs in the S phase of the cell cycle. Replication starts at specific regions of the chromosomes called replication origins, and then the replication machinery moves bidirectionally from them until chromosomes are completely duplicated.

However, the process starts earlier in the cell cycle. In early G1, *pre-replicative* complexes start assembling at origins in a process called origin licensing, preparing origins for future firing. Origin licensing is restricted to G1, to ensure that replication takes place only once per cycle (Blow and Hodgson, 2002). But it is not until S phase when the complexes become active, and pre-initiation complexes start recruiting the DNA synthesis machinery (Takeda and Dutta, 2005). The signal to activate the pre-loaded complexes and to start the DNA synthesis occurs in late G1, when cells are commited to enter a new cell cycle at Start, and CDK activity is required for this step.

DNA replication starts with the formation of pre-replicative protein complexes (pre-RC). The first step is the assembly of the ORC, (origin recognition complex) at the origins (Diffley, 1996). It is not well established how the ORC recognizes the origin sites at DNA, but it seems to depend on specific DNA sequences and on chromatin structure. These DNA sequences are well defined in *S. cerevisiae* (repetitive elements named ARS, autonomously replicating sequences) and less conserved in other eukaryotes (Antequera, 2004; Stillman, 1993). Then, other proteins of the pre-replicative complex are recruited (Cdc18 and Cdt1 in *S. pombe*).

The complex ORC-Cdc18-Cdt1 is required to recruit the DNA helicase, which is the Mcm complex, formed by 6 subunits (Mcm2-7) into the pre-RC. Helicase is necessary for the unwinding of DNA when replication starts, but it is preloaded in the pre-replicative complexes in G1 (Takeda and Dutta, 2005).

The rest of the replication machinery, pre-initiation complex and DNA polymerases, is recruited later onto the origins, originating the replication forks. The process of starting replication is called origin firing, and in eukaryotic organisms firing occurs at multiple sites in the chromosome to ensure that the duplication process occurs rapidly. Not all the origins fire at the same time, some of them fire earlier and others are late origins.

Once replication begins, it proceeds until its completion. Also, cells ensure that each chromosome duplicates only once per cycle, and once one origin has been activated, firing will not occur in the same origin until the next cell cycle. These two features of DNA replication are essential to maintain genome integrity and to avoid problems later in the cell cycle in chromosome segregation. CDK machinery is in charge to regulate the process, for example regulating the degradation of the components of the pre-RC once replication has been initiated, to avoid new origin recognition. (Diffley, 2004).

This process has to be absolutely accurate, and DNA integrity is maintained by the DNA damage response, that delays duplication until possible damage is repaired.

3. Transcriptional program in G1/S

3.1. *S. pombe:* MBF

MBF (Mlu1 cell-cycle-box binding factor) belongs to a family of transcription factors that plays an important role in cell cycle regulation because its activity contributes to the timely expression of genes required for early cell cycle progression, particularly genes regulating the G1 to S phase transition.

MBF is a heterodimeric transcription factor comprised of Cdc10, Res1, Res2, and other regulatory subunits. MBF mediates G1/S specific transcription of genes required for DNA synthesis and S phase. A group of about 20 genes is known to be under MBF control. Among them: *cdc22* (ribonucleotide reductase), *cig2* (S phase cyclin), *cdc18* and *cdt1* (both are part of the DNA replication machinery) (Hofmann and Beach, 1994; Nishitani and Nurse, 1997).

All these genes share a DNA motif in their promoters, the Mlu1 cell-cycle box (MCB), ACGCGT. MCB elements are present in several copies in the promoter, and the number, orientation and spacing of the motifs are crucial for the activation of transcription (Maqbool et al., 2003).

MBF is a high molecular weight complex identified by its binding activity to DNA motifs by gel retardation assay. Because its molecular weight of about 1 MDa, it is assumed to be a multisubunit transcription factor, although few components of the complex have been described so far. The major components

Cdc10, Res1 and Res2, associate with promoters throughout the cell cycle. However, the complex promotes the transcription of its target genes only during late M, G1 and S phases. It is still unclear how the complex is activated at M phase and inactivated at the end of S phase, and how it remains inactive during G2, but presumably MBF is regulated by posttranslational modifications or by other regulatory subunits.

Cdc10

Cdc10 is considered the activating component of the complex, since in *cdc10-* mutants transcription is reduced. Cdc10 does not bind to DNA directly; it binds DNA through its partners Res1 and Res2, thought to be the DNA binding subunits of the transcription factor.

The C-terminal part of the protein was shown to have an important role for the function of MBF, and seems to be critical for the formation of the complex ((Reymond and Simanis, 1993). It has a region with ankyrin repeats, motifs present in a large number of functionally diverse proteins and considered sites for protein-protein interaction. The ankyrin motifs are a conserved sequence of about 30 amino acids repeated four or more times, and it allows Cdc10 to interact with its MBF partners Res1 and Res2. However, ankyrin repeats seem to have a role in stabilizing the complex (maybe through interactions to other proteins) more than in direct interactions Cdc10/Res1/Res2 (Ayte et al., 1995; Ewaskow et al., 1998; Whitehall et al., 1999).

cdc10-C4 corresponds to a truncated form of Cdc10. A nonsense mutation in *cdc10* is responsible for a premature stop codon, and makes the gene to encode for a Cdc10 protein that lacks 61 amino acids of the C terminus. This protein has been widely used to understand regulation of MBF, since strains containing an MBF complex carrying the *cdc10*-C4 allele and growing at low temperatures have a highly induced transcription of MBF genes throughout cell cycle. Therefore, the C terminus of Cdc10 is important for the regulation of MBF function.(McInerny et al., 1995)

Overexpression of Cdc10 or Cdc10-C4 under a strong inducible promoter (pREP1) does not affect periodic transcription of MBF dependent genes (White et al., 2001). The fact that its regulation is maintained despite this overexpression reinforces the idea that other regulators, rather than the amount of protein, control the activity of Cdc10/MBF complex.

Res1 and Res2

Res1 and Res2 are the DNA binding subunits of the complex. They show high homology to each other and they bind DNA through a homologous N terminal domain. They also have ankyrin repeats domains in their C terminus part, although a clear function of these domains has not been established. Despite their common structural features, both proteins have different functions.

Res1 was isolated as a suppressor of *cdc10 (Tanaka et al., 1992)..* Overexpression of Res1 can rescue the lethal phenotype of strains bearing a temperature sensitive allele of *cdc10,* or even a complete deletion. Overexpression of only the N-terminal part, that contains

the DNA binding domain, is also sufficient to rescue this lethal phenotype (Ayte et al., 1995).

Overexpression of Res1 in a wild type context, however, induces growth arrest in G1. This arrest is not due to overexpression of MBF dependent genes, since overexpression of both proteins, Res1 and Cdc10, does not induce such an arrest. A possible explanation could be that an aberrant transactivation of genes that are not normally MBF dependent occurs, or maybe overexpression of Res1 might behave as a dominant negative mutant by sequestering other MBF components (Ayte et al., 1995).

On the other hand, Δ*res1* cells are unable to normally induce transcription of MBF-dependent genes, and they have a cold and heat-sensitive phenotype. This would indicate that Res1 plays a role, directly or indirectly, in the activation of transcription (Tanaka et al., 1992).

The main role of Res2 is in meiotic MBF (Ayte et al., 1997). Its expression is induced in premeiotic DNA synthesis, and Δ*res2* cells have severe defects in meiotic DNA synthesis (Miyamoto et al., 1994a). But Res2 also forms part of the mitotic MBF complex (Ayte et al., 1997; Miyamoto et al., 1994a; Whitehall et al., 1999), in which shows some different and overlapping functions with Res1. Overexpression of Res2 can rescue Δ*res1* defects (Miyamoto et al., 1994b).

Δ*res2* cells show the opposite pattern of transcription of MBF-dependent genes, when compared to Δ*res1* cells, i.e. there is a general derepression of MBF-dependent transcription (Baum et al.,

1997). It was thought that phenotype of the *cdc10*-C4 mutant was due to loss of interaction with Res2, but it is shown that was not the case.

The widely accepted roles of Res1 and Res2 as an activator and a repressor of MBF respectively are not so clear. There is no subunit switching from Res1 to Res2 to form an inactive MBF complex as it was thought for many years, since both components remain in the complex together with Cdc10 throughout the mitotic cycle (Whitehall et al., 1999). Also, microarray data recently published (Dutta et al., 2008) indicate that both, Res1 and Res2, can act as repressors and activators, but in different subset of genes. Δ*res2* cells show constitutive derepression of most MBF dependent genes, except for *yox1*, *cig2*, and *mik1*, which have wild type levels of expression. Δ*res1* cells have defects to induce transcription for a big subset of genes (including *cdc18*, *cdt1*, and *cig2*) but they also show constitutive derepression for a small subset of genes, like *cdc22.* These data taken together indicate that MBF regulation and the roles of Res1 and Res2 might be more complex than what has been considered until now.

Other comoponents of MBF

Other components/interactors of the MBF complex include Rep1, Rep2, Cig2 and Nrm1. Rep1 was first described as a component of the meiotic MBF, with no function in the control of mitotic transcription (Sugiyama et al., 1994). However, overexpression of Rep1 in mitotic cycle results in deregulation of MBF genes, which become constitutively transcribed throughout the cell cycle (White

et al., 2001). This is why Rep1 has been considered a possible activator of the complex.

Little is known about Rep2, but overexpression of Rep2 also leads to constitutive derepression of MBF genes (White et al., 2001). It is postulated to be a co-activator of the MBF complex during mitotic cycle (Tahara et al., 1998).

The mitotic cyclin Cig2 is the product of one of the MBF regulated genes. It has been described to have a role in MBF regulation by posttranslational modification: Cig2 binds MBF at the end of S phase and phosphorylates Res1 at residue S130. This phosphorylation inactivates the complex upon cells exit S phase (Fig. 3). Cig2 forms a negative feedback loop with MBF (Ayte et al., 2001) and this was the first evidence of a direct regulation of MBF transcription by CDKs in *S. pombe.*

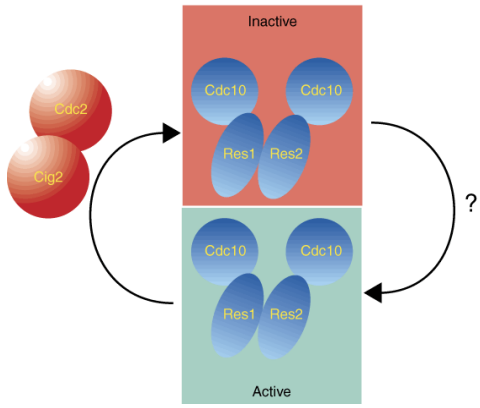


Figure 3. Negative regulation of MBF by Cdc2/Cig2 phosphorylation (Ayte et al., 2001)

Another negative regulator of MBF is the co-repressor Nrm1 (negative regulator of MBF targets). It is also encoded by a MBF

regulated gene, which is expressed during late G1. It binds MBF leading to transcriptional repression of MBF target genes in late S phase (de Bruin et al., 2006) in a negative feedback regulation loop. It was described that it requires the intact complex (Cdc10, Res1 and Res2) to bind DNA (de Bruin et al., 2008). This is a second mechanism of negative feedback regulation of MBF, independent to the one carried out by Cig2, indicating the robustness of the regulation of the complex by different mechanisms to ensure proper timing of transcription.

3.2. S. *cerevisiae*: MBF/SBF

In *S. cerevisiae*, the transcriptional program of genes necessary for entry into S phase depends on two different complexes, MBF and SBF.

MBF is comprised by at least two components, Swi6 and Mbp1. They are homologous to *S. pombe* proteins Cdc10 and Res1/Res2, respectively. This complex recognizes one specific DNA element, the MCB box (MluI cell cycle box, ACGCGTNA), present in the regulatory region of genes coding for proteins with a role in DNA synthesis (POL1, POL2) and also regulators of initiation of S phase, like the cyclins CLB5 and CLB6, and proteins with functions in DNA repair. The complex is necessary for the passage through S phase.

SBF is comprised by two homologous components of MBF, Swi6 and Swi4. It recognizes a different DNA element, called SCB box (Swi4-Swi6 cell cycle box (CACGAAAA)), present in genes expressed in late G1, like HO endonuclease, and G1 cyclins (CLN1

and CLN2). It is required for passage through START, activating transcription of genes required for spindle pole body duplication, budding and cell morphogenesis. It has been described to bind MCB boxes as well (Partridge et al., 1997).

The apparent distribution of genes in two different functional categories depending if they are SBF or MBF dependent is not strict, and each group includes genes that do not fit in the functional category. Actually, there is some overlap in the role of both transcription factors. Their sequence requirement to bind DNA is also not strict, and genome-wide analysis of the binding of both transcription factors to promoters show that overlapping of both transcription factors occurs (Iyer et al., 2001). Also, inactivation of SBF or MBF has little effect in G1 specific transcription, but deletion of both, Mbp1 and Swi4, is lethal (Koch et al., 1993), suggesting that just one transcription complex is sufficient for the transcriptional activation of the G1/S transition.

The three components Swi4, Swi6 and Mbp1 contain 4 ankyrin repeats (homologous to the ones in *S. pombe*), present in the C terminus of the proteins. The ankyrin repeats allow the interactions between the proteins. Like *S. pombe* Cdc10, Swi6 is not able to bind directly DNA and it does so through its interacting partners (Ewaskow et al., 1998). Swi6 is the transactivating component of the complexes (Dirick et al., 1992).

Both transcription factors MBF and SBF are the main regulators of START, activating transcription of more than 200 genes (Horak et al., 2002; Simon et al., 2001). However, there is a representative list of genes coding for proteins also necessary for passage

through START in budding yeast that are not directly under the control of SBF/MBF. This set of genes includes genes required for DNA replication, but also for bud growth initiation and spindle pole body duplication. There is a network of other transcription factors that bind promoters of those genes. Some of these transcription factors are themselves under SBF/MBF control (like HCM1, PLM2, POG1, TOS4, TOS8, TYE7, YAP5, YHP1 and YOX1), and they bind to promoters of other transcription factors (Horak et al., 2002).

Thus, there is a coordinated regulatory cascade of transcription factors that makes G1/S transcriptional program highly complex in *S. cerevisiae* in comparison to *S. pombe,* with periodic transcription having a key role in cell cycle control. On the contrary, in S. *pombe*, MBF is not activated by any transcription factor from a previous wave of transcription. It seems that *S. pombe* depends less on transcriptional control, and might be that post-transcriptional mechanisms are more important for the proper regulation in time of the transcription factors.

3.3. Metazoans: E2F/DP

E2F/DP is the functional homologous to MBF in metazoans and its activity is required for the expression of genes needed for early cell cycle progression, as well as other genes involved in apoptosis (DeGregori et al., 1997).

As in yeasts, it also works as an heterodimeric complex. In mammalians, this family is composed of at least eight E2F and two DP subunits. Together they work as E2F/DP heterodimers that regulate the E2F target genes (Trimarchi and Lees, 2002).

From the eight different E2F subunits described, only the first five subunits E2F1-E2F5 have a well characterized role in regulating the G1/S transcriptional program, and among them, two subfamilies can be distinguished (Fig. 4). Complexes that are activators of transcription, consisting in E2F/DP heterodimers that include E2F1, E2F2, and E2F3, and complexes that repress G1/S transcription, which are E2F/DP heterodimers composed by E2F4 and E2F5. Basically, E2F4 and E2F5 can bind DNA, but they lack of a transactivation domain and, thus, act as repressors (Attwooll et al., 2004).

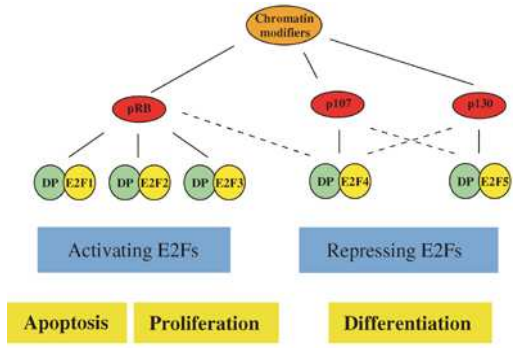


Figure 4. Schematic representation of the E2F transcription factor subgroups, their physiological roles and specific binding partners (Attwooll et al., 2004)

The two DP subunits described, DP1 and DP2, are essential for the DNA binding of the E2F subunits, and it is not clear if they also have a role in the selection of the DNA binding sites of E2F (Tao et al., 1997).

In Drosophila, there are only two E2F proteins and one DP, and they form two different complexes: one activator of G1/S transcription (containing E2F1) and one repressor (containing E2F2) (Frolov et al., 2001).

Transcriptional activation of G1/S genes depends therefore in the antagonistic activity of the two types of complexes. In non-proliferating quiescent cells, E2F promoters are occupied mainly by the E2F4 and E2F5, the repressor complexes, that maintain the transcription off. On the contrary, in response to mitogenic signals, cells can re-enter cell cyle by a switch in the composition of the transcription factors that occupy the promoters of the G1/S genes.

Overexpression of activator E2F complexes promotes entry into S phase, whereas their inhibition inhibits cell proliferation. The balance of the two activities is important for cell proliferation and for the control of differentiation processes. For instance, mutations in repressor E2Fs promotes cell proliferation and impairs the exit to the quiescent state needed for differentiation.

3.4. Regulation of G1/S gene expression

E2F, MBF and SBF dependent transcription is constrained to G1/S by inactivation of the transcription factors outside these phases of the mitotic cycle. The mechanism of regulation is highly conserved in yeast and metazoans. The fact that E2F and Rb show little homology to their functional equivalents in yeast is a beautiful example of convergent evolution and highlights the importance of this pathway.

In S*. pombe*, MBF dependent transcription is constrained to M, G1, and S phases by inactivation of the complex as cells exit S phase. However, little is known about the mechanisms activating

transcription activation at the beginning of each cell cycle, since the role of the co-activators Rep1 and Rep2 is not clear.

Nrm1 is a co-repressor that has a role in constraining the activity of MBF by repression in G2. The same happens with the CDK complex Cdc2/Cig2, that phosphorylates MBF to repress transcription as cells exit S phase, but does not have an effect in the activation of transcription.

The mechanism of activation is better understood in *S. cerevisiae*, especially for SBF. Activation of SBF and MBF transcription in budding yeast was known to depend on G1 CDK activity, being the complex Cln3/Cdc28 the primary activator and in cells with reduced levels of Cln3, G1/S transcription was delayed (Dirick et al., 1995; Trautmann et al., 2001). The target of the CDK complex to activate G1 transcription, however, remained unknown for a long time. In 2004, Whi5 was described in two independent works, pointing out Whi5 as the largely unknown regulator of SBF (Costanzo et al., 2004; de Bruin et al., 2004).

Whi5 was shown to be a repressor of SBF. It maintains SBF inactive until the initiation of the cell cycle, when it is required. Inactivation of Whi5 causes premature activation of G1 transcription and cells initiate cell cycle at a smaller size. Whi5 associates with SBF promoters in a SBF-dependent manner, and the release of Whi5 from SBF promoters correlates with an induction of transcription, suggesting the role as a repressor.

The mechanism of regulation of SBF by Whi5 is dependent on CDK activity. Whi5 is phosphorylated by the CDK complex

Cln3/Cdc28, and this phosphorylation promotes its dissociation from SBF (Costanzo et al., 2004; de Bruin et al., 2004). However, when phosphorylation mutants of Whi5 were tested, there was not any effect on transcription. Only in the work published by Wittenberg's lab, using a different strain background, a phenotype (an extension of G1 phase) for the Whi5 mutant (not phosphorylable by CDK) was showed.

Whether phosphorylation of Whi5 by CDK is or is not critical for SBF activation is not completely clear. There might be other CDK targets to activate SBF. One of them could be Swi6 itself. Only when eliminated the CDK phosphorylation sites of both proteins, Swi6 and Whi5, viability is lost (Costanzo et al., 2004). It is possible that the G1 CDK regulates the activation of SBF by several regulatory mechanisms to control cell cycle, not only through Whi5. Nevertheless, this direct activation of SBF by the G1 CDK complex is very similar to the one observed in higher eukaryotes. (see below and (Schaefer and Breeden, 2004).

Inactivation of SBF is also regulated by CDK, by dissociation of the transcription factor from promoters (Koch et al., 1993; Siegmund and Nasmyth, 1996). Swi4 and Swi6 dissociate in S phase, and Swi6 is exported to the cytoplasm. In this case, it is the S phase complexes CDK/Clb the ones that phosphorylate SBF. Thus, a cell cycle regulated phosphorylation of Swi6 by CDK occurs at the moment of maximum SBF/MBF activation of transcription, in late G1. From late G1 to M phase, Swi6 is localized mainly in the cytoplasm. At late M phase, Swi6 enters again in the nucleus, and this corresponds to a hypophosphorylated form of the protein. However, it was not found an effect of the nuclear export of Swi6

on SBF/MBF transcriptional regulation (Sidorova and Breeden, 1993).

Despite the overlapping in functions of both transcription factors in budding yeast, SBF and MBF, they are regulated by independent mechanisms, both in their activation at G1 phase and their inactivation. MBF activation is Cln3/CDK dependent, although the mechanism remains unknown. It is not regulated by Whi5 (de Bruin et al., 2004) and it is possible that besides Swi6, there are other components of MBF regulated by CDK. Regarding MBF inactivation as cells exit S phase, it seems that Clb/Cdc28 kinase complex is not required for the repression of MBF transcriptional activity in G2 (Siegmund and Nasmyth, 1996). MBF does not dissociate from its promoters as transcription is inactivated (as MBF in *S. pombe* does not, and contrary to SBF regulation).

Recently, a specific regulator for MBF was described: Nrm1 (Negative regulator of MBF). It is homologous to Nrm1 in *S. pombe* (de Bruin et al., 2006) and it is also a target of MBF. It has the same function in both organisms, constraining G1 specific transcription by inhibiting the complex at the end of S phase. The mechanism is the same as in fission yeast: a negative feedback loop in which Nrm1 protein starts accumulating as cells exit G1 and this accumulation correlates to its association to MBF promoters, thus repressing transcription. Deletion of NRM1 has little effect on cell size, indicating that de-repression of transcription observed in this strains does not affect cell cycle progression.

In mammals, to restrict the E2F/DP dependent transcription to G1/S phases, and to inhibit the expression in quiescent non-

proliferating cells, E2F activity is controlled through the association of regulatory proteins, known as pocket proteins, members of the familiy of the retinoblastoma protein (pRB). There are three pRB proteins in mammals: pRB, p107 and p130, and two in Drosophila: dRBF1 and dRBF2. This family of proteins adds a new layer of complexity to the regulation of transcription.

Retinoblastoma (Rb) is a transcriptional co-factor able to bind the different E2F transcription factors. pRB inhibits the activator E2F complexes, whereas p103 and p130 are co-repressors of the repressor E2Fs (Fig. 4). There are several studies suggesting that Rb may recruit multiple chromatin regulatory proteins to repress E2F, like HDACs (Trimarchi and Lees, 2002).

There is also a tight regulation of the activity of the E2F complexes at the level of phosphorylation, through cyclin-dependent kinases (CDKs), which can phosphorylate E2F regulators like Rb, and also E2F itself. The switch that allows cells to entry into cell cycle from quiescent state is the CDK activation in response to external signals. When CDK complexes are activated, pRB is phosphorylated and dissociated form E2F, and this enables G1/S transcription, which means entry into the cell cycle (Trimarchi and Lees, 2002).

Therefore, the family of the E2F and MBF transcription factors is regulated by their corresponding repressors. It is a conserved mechanism of regulation in eukaryotes: SBF/Whi5 in S. *cerevisiae*, and E2F/Rb in mammals. The common pattern of activation of the complexes in G1 is because of an inhibition of the repressors. This

occurs by phosphorylation, either in the transcription factor, either in the repressor (Schaefer and Breeden, 2004).

Implications of E2F/DP misregulation

Loss of E2Fs regulation leads to defects in cell proliferation and in differentiation (Frolov et al., 2001; Lukas et al., 1996). Retinoblastoma was the first tumour suppressor discovered. It is believed to have a role, directly or indirectly, in nearly all the human cancers (Burkhart and Sage, 2008). Why loss of RB function contributes to cancer is not clear (Classon and Harlow, 2002). The main role as a tumour suppressor is due to its ability to inhibit E2F transcription factors, which is an important mechanism to maintain cells in quiescent state in G1 (Kaelin, 1997). Cells can exit this quiescent state by inactivation of RB: in response to signals, G1 CDKs are activated, they hyperphosphorylate Rb, and as a result Rb dissociates from E2F. Then free E2F activates transcription, and initiation of cell cycle occurs. However, other functions of RB with a possible role in tumour initiation have been described, including differentiation processes, regulation of apoptosis, and preservation of chromosome stability (Hernando et al., 2004; Knudsen et al., 1996; van Deursen, 2007).

4. DNA DAMAGE AND DNA REPLICATION CHECKPOINTS

4.1. DNA damage

Genomic integrity is constantly threatened by many processes that occur at the DNA. Reactions like transcription and DNA replication, or the exposure to external damaging agents, suppose for the cell an increased risk of rearrangements in DNA or single nucleotide substitutions, defects that are a hallmark of cancer cells. In response to damaged DNA or unreplicated DNA, cell cycle must be arrested. DNA damage and DNA replication checkpoints regulate the cell cycle by preventing cells to undergo the cell cycle until the damage has been repaired.

Endogenous sources of DNA damage

During the processes of transcription, replication, and chromosome segregation, the cell machinery must face with several topological problems due to the unwinding of the DNA. Unwinding problems are solved by DNA topoisomerases. These enzymes introduce single strand breaks in DNA (type I topoisomerases) and double strand breaks (type II topoisomerases) and thus they produce a topological relaxation in DNA structure, which corresponds to an energetically more stable state of DNA. Despite the production of strand breaks, this is a safe mechanism for the cell, since they are transient breaks, protected by covalent binding to proteins, and do not generate DNA damage responses. Also, the DNA damage checkpoints monitor the proper activity of these enzymes to ensure a normal chromosome segregation and chromosome stability (Nitiss, 2009). However, although being a highly regulated

mechanism, the potential DNA damage that can be caused by Topo enzymes has been used as a powerful molecular tool in cancer chemotherapy and several anitcancer drugs directly target these enzymes.

Damage resulting from transcription has been termed as TAM (transcription associated mutagenesis). Also, when replication takes place, replication fork progression is paused or arrested at particular sites at the genome (like ribosomal DNA repeats, centromeres and telomeres). It is a moderate pausing, but many of these regions which prone to fork pausing, exhibit elevated levels of recombination (Azvolinsky et al., 2009). One specially threatening situation for genomic integrity is the collision of the replication machinery with the transcription machinery at highly transcribed genes (Hendriks et al.). In fact, the highest pausing of replication fork has been described to occur at the ORFs of highly transcribed genes (Azvolinsky et al., 2009).

Exogenous sources of DNA damage

Besides the DNA damage produced by normal cellular processes, cells can receive insults from exogenous sources. UV irradiation produces DNA damage by covalent binding of pyrimidines, causing damage in one strand of the DNA. These dimers of pyrimidines interfere with replication, provoking replication fork pausing. The mutagen MMS (methyl methanesulfonate) generates mutations by methylation of bases in the DNA, which causes mispair in DNA synthesis and therefore point mutations. Ionizing radiation or bleomycin produce double strand breaks, and hydroxyurea inhibits

the ribonucleotide reductase enzyme, causing a depletion of nucleotides that provokes replication fork stalling.

4.2. The DNA damage response

In order to maintain genomic integrity, eukaryotes have developed a highly conserved mechanism to detect, signal and repair damage in DNA, known as the DNA damage response. This regulatory mechanism allows cells to sense many types of damage and activate the proper response, which usually consists in the recruitment of repair proteins. When damage is severe there is a more complex response that includes cell cycle arrest (DNA damage checkpoint). In metazoans, on highly damaged cells, a permanent cell cycle arrest that leads to apoptosis is also triggered by the pathway; this apoptosis is mediated by p53 (Kuntz and O'Connell, 2009).

Protein function	Protein name	*S. pombe* gene	Human gene
Resecting Nuclease	ND*	ND*	ND*
ssDNA Binding Protein	RPA	*ssb1 (rad11)*	RPA1
		ssb2	RPA2
		ssb3	RPA3
Sensor Kinase	Rad3/ATR	*rad3*	ATR
	Rad26/ATRIP	*rad26*	ATRIP
9-1-1 Loader	Rad17-RFC	*rad17*	RAD17
		rfc2	RFC2
		rfc3	RFC3
		rfc4	RFC4
		rfc5	RFC5
9-1-1 Clamp	Rad9	*rad9*	RAD9A
	Hus1	*hus1*	HUS1
	Rad1	*rad1*	RAD1
Mediator Proteins	Cut5	*cut5*	TOPBP1
	Crb2	crb2	TP53BP1
	MDC1	-	MDC1
	Claspin	-	CLSPN
	BRCA1	-	BRCA1
Effector Kinase	Chk1	*chk1*	CHEK1
CDK Regulators	Wee1	*wee1*	WEE1
	Cdc25	*cdc25*	CDC25A
			CDC25B
			CDC25C

Table I. G2 DNA damage checkpoint genes in *S. pombe* and in humans (Kuntz and O'Connell, 2009)

The DNA damage response starts with the activation of the kinases ATM and ATR, which detect the damage and bind DNA in the specific site where the damage is produced. Then a cascade of phosphorylations is activated: the signal activates and recruits DNA repair protein at the damaged sites, and also activates the effector kinases Chk1 (Chk1 in *S. pombe*) and Chk2 (Cds1 in *S. pombe*). These kinases are responsible for the cell cycle arrest and the transcriptional response (Rhind et al., 2000).

The two upstream kinases, ATR (Rad3 in *S. pombe*) and ATM (Tel1 in *S.pombe*) have specialized functions. ATR is activated in response to many types of DNA damage, including stalled replication forks, and seems to detect damage in single-stranded DNA, whereas ATM is needed in the response to double-strand breaks (Shiloh, 2003) .

When damage in DNA is detected, chromatin that flanks this damage is marked by the checkpoint machinery. ATM and ATR phosphorylate histone H2AX (H2A in *S*. *pombe*). Phosphorylated H2AX (γH2A) signalling is the initial step of the checkpoint response. The phopshorylation acts as a scaffold for the recruitment of other proteins of the checkpoint cascade in the surroundings of the damaged sites (Williams et al.). Among the complexes recruited to the damage sites, there is the complex 9-1-1 (Rad9, Hus1,Rad1), which forms a ring surrounding the affected DNA, and then a series of adaptator proteins, that form a platform for the recruitment and activation of the effector kinases Chk1 and Chk2 (Kuntz and O'Connell, 2009).

The effector kinases of the response respond to opposite signals in the different organisms: Chk1 (S. *pombe*) and Chk2 (metazoans) respond to DNA damage, whereas Cds1 (*S. pombe)* and Chk1 (metazoans) respond upon the stalled replication forks (Dutta et al., 2008).

Cell cycle arrest upon DNA damage

Severe damage in DNA requires a block in cell cycle progression until cells are able to repair the damage. DNA damage may occur in any phase of the cell cycle but the responses are different depending on the organism. In mammalian cells, the major cell cycle arrest in response to DNA damage takes place in G1, and this response includes the activity of p53. The checkpoint response in G2, however, is conserved in all the eukaryotes, including yeasts (Kuntz and O'Connell, 2009). Damage detected in S and in G2 phases blocks entry into mitosis to avoid segregation of damaged chromosomes, but the mechanism is different depending on the organism.

In the case of metazoans and *S*. *pombe*, the arrest occurs at the G2/M transition. In *S*. *cerevisiae*, however, the arrest occurs at metaphase. In any case, the aim is to avoid sister chromatid separation. The fact that the effector kinases target different substrates in the different organisms, despite being a highly conserved pathway, indicates certain plasticity in the checkpoint response (Rhind et al., 1997; Rhind and Russell, 1998).

In *S. pombe* and metazoans the target of the checkpoint to block cells at the G2/M transition is the CDK kinase Cdc2 (Cdk1 in

metazoans). Cdc2 is maintained inactive during G2 by phosphorylation of Tyr-15. This is an inhibitory phosphorylation of Cdc2, which renders a Cdc2/Cdc13 CDK complex with an intermediate kinase activity that is not enough to trigger mitosis. The checkpoint role is to maintain Tyr-15 phosphorylated through several mechanisms (Rhind et al., 1997; Rhind and Russell, 1998).

The target of the checkpoint is the inhibitory phosphatase Cdc25. Thus, the checkpoint inhibits the dephosphorylation of Cdc2 in Tyr-15 by inhibiting Cdc25 (Lopez-Girona et al., 1998). In this pathway there are involved other targets: Rad24 and Rad25, two proteins with overlapping functions. They belong to the protein family 14-3-3, and they bind specific phosphorylated substrates. Rad24 and Rad25 control de cellular distribution of Cdc25. Thus, Cdc25 is exported to the cytoplasm when the checkpoint is activated. Not only the export, but also the inactivation of Cdc25 by direct phosphorylation of Chk1 is required to arrest the cycle. (Lopez-Girona et al., 1998). This mechanism is conserved in mammals, where the target of Chk1 is also Cdc25. (Peng et al., 1997; Sanchez et al., 1997).

It was also described, that, besides Cdc25, there are other targets of Chk1 kinase responsible of maintaining the Tyr-15 phosphorylation of Cdc2. Wee1 is an additional target (Kuntz and O'Connell, 2009), and Mik1 seems to have a role as well in the checkpoint response (Rhind and Russell, 2001).

In the case of replication stress or DNA-damage induced replication arrest, the mechanism to arrest cell cycle is the same as in the DNA damage response, since cells also need to prevent

entry into mitosis. The effector kinase in this case is Cds1 instead of Chk1, some of the proteins in the cascade are different, but the mechanism to inhibit Cdc2 is the same (Dutta et al., 2008). However this arrest in cell cycle is not so well understood. It was described that phosphorylation of Cdc25 is also required but the phosphorylation is accomplished by both kinases, Cds1 and Chk1, which function redundantly (Zeng et al., 1998). There are evidences that Chk1 plays a role in the DNA replication checkpoint and, in fact, Δ*cds1*Δ*chk1* cells are more sensitive to HU treatment than Δ*cds1* cells. Δ*cds1*Δ*chk1* cells undergo aberrant mitosis (observed as a "cut" phenotype). Also, overexpression of Chk1 can overcome sensitivity to HU of *cds1-* cells (Zeng et al., 1998).

4.3. DNA replication checkpoint:

The DNA replication checkpoint is the branch of the DNA damage response that is activated in response to replication fork stalling. During DNA synthesis, the replication machinery acts as a sensor of damage in DNA. When any obstacle for DNA replication is detected, a stalling of replication forks occurs and the DNA damage response pathway is activated. Some of the components of the response, like the adaptator protein Mrc1 that recruits Cds1, are already pre-loaded with the replisome during normal S phase.

The sensor kinase ATR (Rad3) is activated when the replication forks stall and, as a consequence, recruits the multiprotein complexes that are assembled at the stalled replication forks. It has been described that signalling through γH2A is also important for replication checkpoint. H2A phosphorylation is critical in both situations: replication-associated DNA damage (when replication

fork progression is paused or arrested at particular sites at the genome during replication) and external replication stress (like in responses to hydroxyurea, which stalls replication forks). Brc1 was described to be the major H2A binding protein in replication stress responses (Williams et al.), and Brc1 foci have been described to co-localize with the regions with replication fork stalling. This allows the subsequent cascade of phosphorylations that finish in the effector kinase Cds1 (Chk1 in mammals). The replication checkpoint response consists in:

1. Arrest of the cell cycle preventing mitosis, to ensure the damaged chromosomes will not be segregated. Signal is transmitted to the cell cycle machinery to inhibit entry into mitosis (see above).

2. Stabilization of stalled replication forks, to avoid lethal fork collapse. Stabilized forks are able to resume replication once the damaged is repaired. Replication forks have a role in both, sensing the damage and signalling it as effectors of the response. Checkpoint deffective mutants cause irreversible collapse of replication forks (Tercero et al., 2003).

3. Prevention of other replication origins to start firing. (Santocanale and Diffley, 1998; Santocanale et al., 1999). In *S. cerevisiae*, there is an inhibition of late origin firing when there is fork stalling in the early origins. It is an active process, Mec1/Rad53-dependent. Late origins are maintained in a pre-replicative state until they are necessary

for the completion of replication once the damage is repaired.

4. Activation of a transcriptional response (see below).

Stabilization of stalled replication forks seems to be essential for viability. On the contrary, cells with a defective checkpoint response regarding regulation of mitosis, gene expression or late origin firing do not have a notable defect in survival (Tercero et al., 2003).

4.4. Induction of transcription in the replication checkpoint response

Genotoxic stress induces the transcription of genes with a role in DNA repair and replication. The transcriptional response, despite being a necessary part of the surveillance mechanism, seems to be a less conserved mechanism than the other pathways of the response like the cell cycle arrest. What is the significance of this regulation for the survival of the cell? The role of transcriptional induction is to prepare cells to resume replication once the damage is repaired. In *S. cerevisiae*, in mutants with an impaired transcriptional induction new replication forks are not created in origins that did not fire. However, they can complete S phase, although slower, indicating that the transcriptional response is not essential for survival (Tercero et al., 2003).

Despite not being an essential part of the response, lethality of checkpoint essential genes like RAD53 and MEC1 (*S. cerevisiae*), can be rescued by increased expression of genes coding for the

RNR enzyme (Desany et al., 1998). This indicates that the transcriptional response has an important role in the recovery from DNA replicative stress. In *S. pombe*, transcriptional regulation also provides resistance to replication stress, although significantly less important than the one provided by the other responses, cell-cycle arrest and fork stabilization (Dutta and Rhind, 2009).

In budding yeast, there is a specific and well characterized transcriptional regulation under DNA replication and DNA damage checkpoints. It is a checkpoint-specific transcriptional program regulated by the phosphorylation of the kinase Dun1 (Zhou and Elledge, 1993) and inactivation of the repressors Crt1/Ssn6/Tup1 (Huang et al., 1998). Dun1-induced transcriptional activation is required for survival. *dun* mutants (DNA-damage uninducible) mutants are defective in the induction of the subunits of the ribonucleotide reductase: RNR1, RNR2, and RNR3, and they are sensitive to DNA damage. However, they are able to induce other genes, indicating the existence of a different transcriptional pathway (Hermand et al., 2001; Zhou and Elledge, 1993). Lethality of Δ*rad53* and Δ*mec1* can be recued partly by the activation of the RNR, and derepression of the Crt1 regulated genes also suppresses the lethality.

This seems to be a *S. cerevisiae* specific pathway, rather than a conserved mechanism in other eukaryotes, and is more reminiscent to the SOS response in prokaryotes. In prokaryotes, although the DNA repair mechanisms are different, there is also a transcriptional response (Davies et al., 2009). Hydroxyurea has been used to study replication fork arrest, and it induces several responses, including transcriptional responses such as:

1. Upregulation of Ribonucleotide reductase synthesis. An upregulation of all RNR genes is induced upon dNTP pool depletion.
2. Upregulation of primosome components, that would allow restart of replication after the HU treatment.
3. Upregulation of SOS response genes, which includes several genes involved in repair and other functions.

MBF and DNA replication checkpoint in *S.pombe*

All the MBF dependent genes are upregulated in response to the checkpoint activation (Dutta et al., 2008), although only some of them have a specific role in the checkpoint response. This points out MBF as the most likely direct and only effector of the transcriptional response.

Microarray data showed that MBF-dependent transcription is upregulated in a checkpoint-dependent manner. Δ*cds1* and Δ*rad3* mutants are not able to upregulate MBF-dependent transcription upon HU treatment. Also, these authors showed that the checkpoint response is affected in Δ*res1*, Δ*res2*, and *cdc10-C4* cells (Dutta et al., 2008).

How the signal goes from the checkpoint to MBF it is still unclear, since different components of the transcription factor might be phosphorylated by Cds1. It is possible that there are several independent mechanisms that end up with the same result, that is the activation of MBF transcription upon checkpoint activation.

One hypothesis points to a direct regulation through Cdc10. The work of Dutta et al. shows that Cdc10 has several consensus sites of phosphorylation by Cds1 in the C-terminus region, which is the region deleted in the Cdc10-C4 mutant and that plays a crucial role in Cdc10 regulation (Dutta et al., 2008). Although phosphorylation of those sites has been checked in vitro, the mutant *cdc10-8A* is however perfectly able to induce transcription upon HU treatment. This indicates that Cds1 phosphorylation on these sites is not important for the checkpoint response.

On the contrary, the authors also showed that mutations that mimic a checkpoint constitutive phosphorylation, have indeed a remarkable phenotype: *cdc10-2E* allele actually shows checkpoint-induced levels of transcription in untreated conditions, as if checkpoint response was constitutively activated. Consistent with this, *cdc10-2E* mutation conferes resistance to HU, and partly rescues the lethality of Δ*cds1* cells. This phenotype indicates that transcriptional response has a role in survival upon replicative stress in *S. pombe.*

Nrm1 was also described to play an important role in DNA replication checkpoint (de Bruin et al., 2008). It was the first direct mechanism described to regulate MBF dependent transcription in response to replication stress. Upon HU treatment, Nrm1 is phosphorylated and this phosphorylation corresponds to its dissociation from promoters. Nrm1 phosphorylation appears to be in part Cds1 dependent, although not totally. In Δ*cds1* mutants, Nrm1 is less phosphorylated, and therefore more bound to promoters, and transcription partially repressed. Cells deleted in *nrm1* are partly resistant to HU. This is because one of the

subunits of the ribonucleotide reductase (*cdc22*) is an MBF target. Therefore, in Δ*nrm1* cells, there is an enhanced expression of *cdc22*, what suppresses HU sensitivity.

Overexpression of the MBF co-activator Rep2 also suppresses the HU sensitivity of Δ*cds1* and Δ*rad3* mutants (Chu et al., 2009).

In budding yeast, the checkpoint promotes the persistent expression of G1-S genes. A transcriptional regulation Dun1-independent through transcription factors MBF and SBF, consisting in the upregulation of genes with a role in DNA replication and DNA repair, has not been clearly established (de Bruin and Wittenberg, 2009). It is likely that the mechanism is conserved. Δ*nrm1* budding yeast cells, as in *S. pombe*, are moderately resistant to toxic concentrations of HU (de Bruin et al., 2006). Also, Swi6 was reported to be a direct substrate of the Rad53 kinase in response to DNA damage (Sidorova and Breeden, 1997).

E2F/DP and DNA damage checkpoint

There are several evidences that the DNA damage checkpoint regulates E2F to achieve a transcriptional response. E2F directly links cell cycle progression with the coordinated expression of genes essential for both the synthesis of DNA as well as its surveillance, and among the E2F dependent genes there are also components of the DNA damage checkpoint and DNA repair pathways (Ren et al., 2002).

In response to DNA damage, E2F-1 is phosphorylated by Chk2, resulting in a transcriptional activation, and leading cells to E2F-1 dependent apoptosis. This supports the idea that E2F-1, besides its role in cell proliferation, has also a tumour suppressor activity (Stevens et al., 2003).

Regulators of E2F seem to be direct targets of the DNA damage checkpoint as well, like Rb, that was reported to be directly phosphorylated by Chk2 (Inoue et al., 2007) or DP subunits, described to interact with that 14-3-3 proteins (Milton et al., 2006).

However, so far there are not evidences of a regulation of E2F/DP by Chk1 (Cds1 in *S. pombe*) in response to replicative stress. The current model for the up-regulation of the G1/S transcription by the DNA replication checkpoint in the different organisms is based on the recent findings in *S. pombe* (de Bruin and Wittenberg, 2009) (Fig. 5).

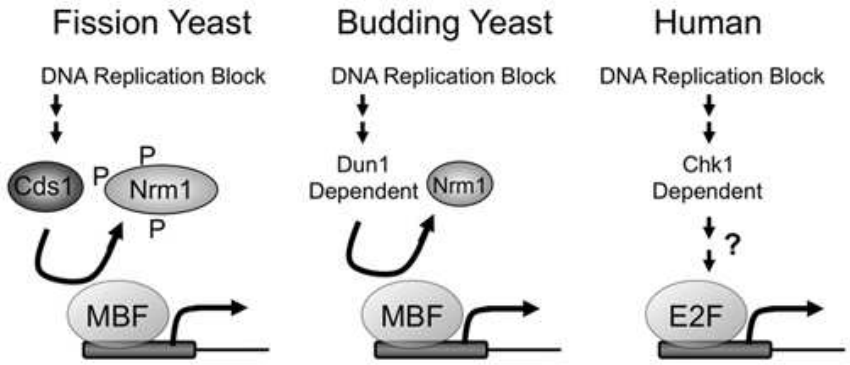


Figure 5. The DNA replication checkpoint promotes persistent expression of cell cycle regulated transcripts in eukaryotes (de Bruin and Wittenberg, 2009).

OBJECTIVES

We had two main objectives at the beginning of this project:

1. **To identify new MBF interacting proteins.**

2. **To better understand how periodicity of MBF-dependent transcription is achieved.**

RESULTS

Identification of MBF interactors

To isolate possible interactors of MBF, we used a proteomic approach that combines affinity purification and mass spectrometry (AP/MS) (Gingras et al., 2007). We wanted to immunoprecipitate MBF in one step of affinity purification. Our bait was going to be Cdc10, tagged with HA on its own locus, at the carboxi terminus. Purifying Cdc10 in a single step should be an efficient procedure of purification, more efficient than the usual protocols (like tandem affinity purification), and would increase the number of interactors identified. However, we knew we would have to deal with the inconvenience of an excess of unspecifically purified proteins.

At that particular moment, the requirement for a proper identification of putative interactors by mass spectometry was to purify a large quantity of Cdc10, large enough as to be detected on a silver stained SDS PAGE gel (Fig.6).

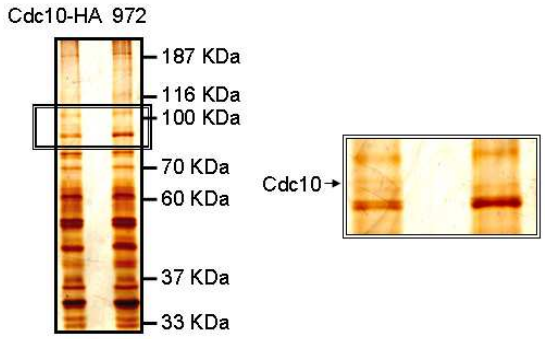


Figure 6. Silver staining of purified proteins. 972 (no tag) and Cdc10-HA strains were purified through an HA column. The purified proteins were silver stained. The band corresponding to Cdc10-HA was detected in the Cdc10HA lane and not in 972 (right panel). The identity of this band was corroborated by MS/MS.

Since Cdc10 is not an abundant protein in the cell, we decided to purify Cdc10 from extracts prepared from large scale cell cultures, to maximize the amount of purified Cc10.

The purified proteins (Cdc10 and the co-immunoprecipitated proteins) were analyzed by a method derived from mass spectrometry, a multiplexed tagging approach named iTRAQ. This technology makes use of amino-specific stable isotope reagents that bind covalently to every peptide in one complex sample. The use of these reagents as reporter ions allows to determine the relative abundance of each of the peptides in one sample. iTRAQ labelling also allows to analyze the data generated after the affinity purification in a quantitative way: iTRAQ reagents can label all peptides in several samples simultaneously and therefore we could label all the peptides in a control sample as an indicator of peptides purified not specifically when comparing to our sample of interest.

The first part of the protocol was the purification of Cdc10. We grew 30 litres of asynchronous cultures of two strains: one of the strains was carrying the HA tagged version of Cdc10. The other strain was a wild type (972) without any tagging, and it was our control strain for unspecific purification. We obtained native protein extracts from the cultures, and we quantified them by Bradford. An equal amount of whole cell protein extracts of both strains, 1.5 grams, was purified. There was an initial step of pre-clearing, in which extracts were incubated with a not specific resin (IgG sepharose crosslinked to α-Myc antibody). The aim of this extra step of purification was to get rid of proteins that could bind unspecifically to the matrix of the HA column. The pre-cleared extracts were then incubated with a column of protein-G-sepharose

crosslinked to α-HA antibody. The purification was performed as described in Materials and Methods.

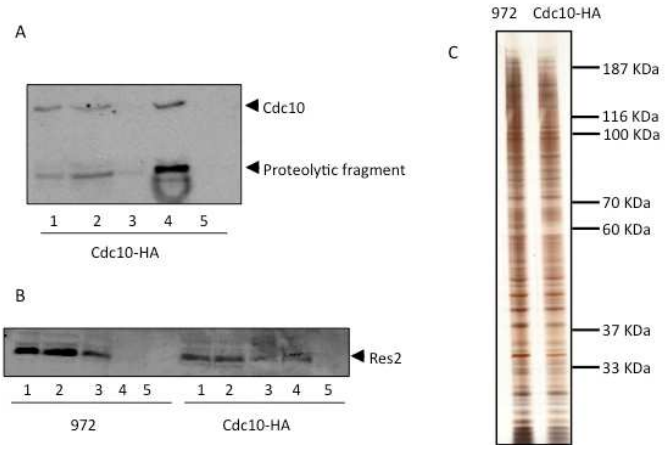


Figure 7. Purification of Cdc10-HA. A. Cdc10 purification**.** 1.5 g of native extracts were obtained from Cdc10-HA strain and 972 (no tag) strain, and were purified through an HA column. In a SDS PAGE were loaded 50μg of whole cell extracts (1), 50μg of precleared cell extracts (2), 50μg of extracts not bound to HA column (3), and the purified fractions (1/2000 of two eluted fractions) (4) and (5). Cdc10-HA was detected using monoclonal anti HA antibody. **B.** Co-immunoprecipitation of Res2 was analyzed in the same samples using α-Res2 monoclonal antibody. **C.** 1/10 of the purified fraction (lane 4 in panels A and B) was loaded in a SDS PAGE and silver stained. Equal loading of the two strains, 972 (no tag) and Cdc10-HA, was monitored.

The purification of Cdc10-HA was confirmed by western blot (Fig. 7A and 7B) and by silver staining (Fig. 7C). In the silver gel we loaded 1/10 of the purified fraction. Not any differential band was appreciated, not even for Cdc10, but the gel indicated us that both samples contained equal protein concentration.

The rest of the purified fraction (9/10) was labelled for the iTRAQ quantification. For this step we collaborated with the proteomics facility at the Universidad Complutense de Madrid, where the labelling and the subsequent identification of proteins was carried

out. All the peptides purified in the control strain (no tag, 972) were labelled with the iTRAQ isotope 115, and all the peptides purified in the strain Cdc10-HA were labelled with the iTRAQ isotope 114.

A total of 2046 peptides, were identified in both samples. Most of them were assigned an iTRAQ ratio of 1, which means that they were equally represented in both samples, and that therefore had been purified through the HA column not specifically (Fig. 8A). However, there were a few peptides overrepresented in our sample compared to the control sample. It is not possible to establish the threshold to consider any given peptide as clearly overrepresented in one sample, but the higher the iTRAQ ratio is, higher is the specificity of the purification.

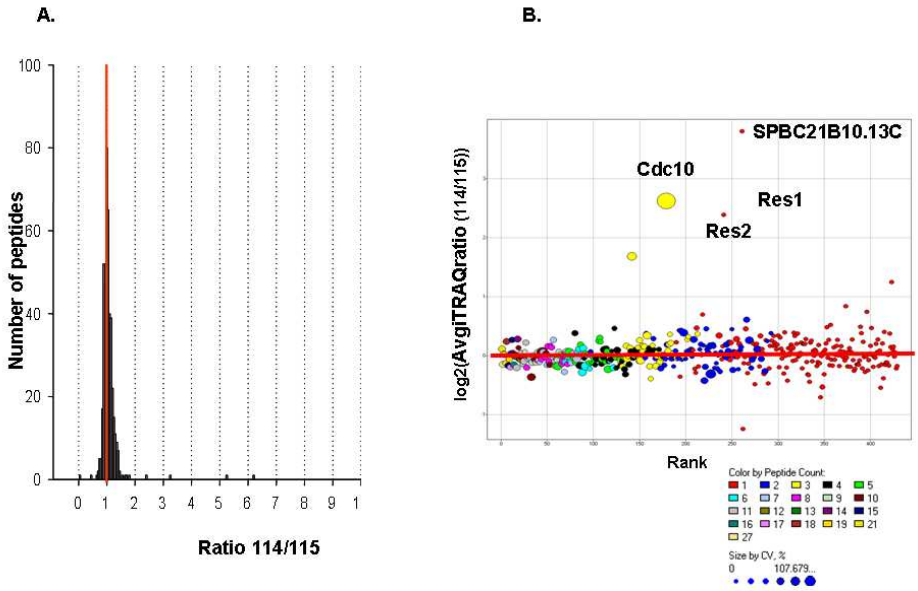


Figure 8. **A**. Metric plot representing the distribution of the iTRAQ ratio 114/115 in the 2064 analyzed peptides (Median=1.0035; SD=0,646)
B. $\log_2$ of the iTRAQ ratio of each protein was plotted. Rank indicates the position in which proteins were identified in the database search. Colours indicate the number of peptides identified for each protein.

Interestingly, none of the peptides was isolated exclusively in one of the samples. The presence of every peptide in both samples is an indicative of the complexity of the samples. It was also interesting to observe that there were peptides with an iTRAQ ratio below 1 (Fig. 8A). The reason why some proteins would be more abundant in the control sample than in our sample is something that we do not completely understand. But since it is a quantitative method, it indicates that the total amount of purified proteins was higher in the control sample.

As expected, Cdc10, the bait of our approach, was clearly overrepresented in our sample, with an iTRAQ ratio 114/115 of 6.18 (Fig. 8B, Table II). Also, other known MBF components were isolated with high iTRAQ ratios (Table II): Res1 (5.24), Res2 (3.21) and Cig2 (1.4). This was the confirmation that the co-immunoprecipitation had worked preserving the intact MBF complex. The other known component of mitotic MBF, Rep2, was not identified. Neither was Nrm1, although this protein had not been described at the beginning of this thesis (de Bruin et al., 2006).

Surprisingly, there was one protein more enriched than Cdc10. It was one peptide corresponding to a protein coded by the gene SPBC21B10.13c (Fig. 8B, Table II). There are several explanations for the fact that this peptide was more abundant than the ones corresponding to Cc10, but the most likely is that this peptide was more efficiently labelled by the iTRAQ reagents.

Name	Function	iTRAQ ratio
SPBC21B10.13c	Transcription factor, homeobox type (predicted)	14,01
Cdc10		6,19
Res1		5,24
Res2		3,22
Set5	Unknown function. Set domain implicated in lysine methylation of histones	2,39
SPAC22E12.02	Uncharacterized RNA-binding protein	1,79
SPAP27G11.09c	Probable GTP cyclohydrolase-2	1,68
SPBC3B8.09	U3 snoRNP-associated protein Utp3 (predicted)	1,63
His2	Histidinol dehydrogenase	1,53
Has1	RNA helicase : rRNA biogenesis	1,44
Ret2	Coatomer subunit delta. Secretory pathway vesicles	1,41
Cig2		1,40
Hsp9	Heat shock protein	1,39
Ribosomal protein		1,39
Prs1	Ribose-phosphate pyrophosphokinase 1	1,38
Prs4	Ribose-phosphate pyrophosphokinase 4	1,37
Glutaredoxin-1		1,37
Cdc22	Ribonucleoside-diphosphate reductase large chain	1,37
Ribosomal protein		1,35
Kes1	Ergosterol biosynthesis	1,34
Tti2	Tel two interacting protein 2	1,33
Sar1	Component of vesicles involved in endoplasmic reticulum to Golgi transport	1,32
Homocitrate synthetase	Mitochondrial precursor	1,31
SPBC28F2.11	DNA binding, HMG box (high mobility group box)	1,31
Prs3	Ribose-phosphate pyrophosphokinase 3	1,31
Vacuolar ATP synthase		1,31
Nhp6	Non histone chromosomal protein 6 (HMG protein).	1,31
Cct4	Component of Chaperonin-containing T complex	1,30
Translation initiation factor		1,30
But2	Uba3-binding protein but2 (Uba3: Ubiquitin activating family)	1,30
SPAC4G9.06c	Contains CHZ motiv (histone binding)	1,30
Htb1	Histone H2B-alpha	1,29
SPAC19B12.02c	Similarity to surface glycoprotein	1,27
Suc22	Ribonucleoside-diphosphate reductase small chain	1,27
FKBP	FK506-binding protein	1,27
Mitochondrial precursor	mitochondrial	1,27
Tom20	mitochondrial	1,26
Mas5	mitochondrial	1,26
60S ribosomal protein L29		1,25
Aminotransferase	mitochondrial	1,24

Table II. List of the 40 proteins purified with highest iTRAQ ratio 114/115.

The gene SPBC21B10.13c codes for a protein of 23 kDa, and it had been annotated as a putative transcription factor because it contains a homeobox domain. Interestingly, the gene transcript was known to peak at G1/S phase (Dutta et al., 2008), and therefore was likely to be an MBF dependent gene. We decided to start analyzing this protein as a putative interactor of MBF, and named it Max1, for MBF associated homeobox 1.

During the last year of this project, an article about the SPBC21B10.13C gene product was published (Aligianni et al., 2009). The group of C. Wittemberg had isolated and characterized the protein simultaneously as we did. They named it Yox1,

because its homology (in the homeobox region) to the Yox1 protein in *S. cerevisiae.*

This thesis is focused on the functional characterization of Max1 and its role in MBF regulation. The appendix of this thesis includes the manuscript of the article "Yox1 links MBF-dependent transcription to completion of DNA synthesis". In the article, we refer Max1 as Yox1 to avoid confusions with the nomenclature in the published literature. However, in the rest of the thesis, we maintain our initial name Max1 for the SPBC21B10.13c gene product.

Characterization of Max1

Max1 interacts with MBF

In order to determine whether Max1 indeed interacts with MBF, we tagged Max1 on its own locus with the Myc tag, at the carboxi terminus of the protein. We constructed a strain with both tagged proteins, Cdc10-HA and Max1-Myc, to perform co-immunoprecipitation experiments. We used native protein extracts, as described in methods, and antibodies against Myc or HA.

We verified the *in vivo* interaction of both proteins (Fig. 9), and we discarded unspecific binding to the antibodies using strains carrying only Max1-Myc or Cdc10-HA. Also, interaction was corroborated in both directions and when any of the two proteins was immunoprecipitated, the other was as well immunoprecipitated.

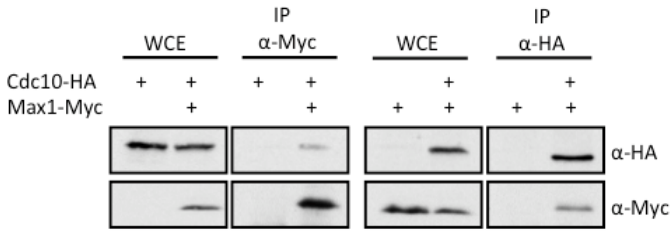


Figure 9. Max1 interacts with MBF complex. Extracts from strains expressing Max1-13Myc, Cdc10-HA, or both were immunoprecipitated (1 mg) with the indicated antibodies and proteins were detected by western blotting.

To further characterize the interaction of Max1 to the MBF complex, we analyzed the interaction of Max1 and Cdc10 in the absence of the other two constitutive components of the complex, Res1 and Res2. From Fig. 9 we could establish that Max1 needs

the intact core of MBF to interact with the complex, since deletion of Res1 or Res2 abrogates Max1 binding to MBF.

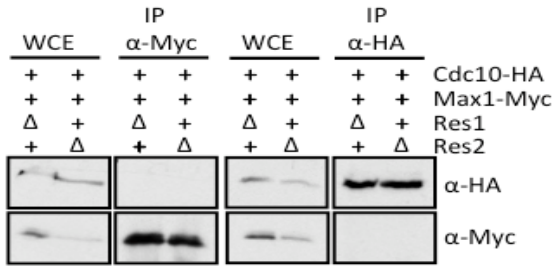


Figure 10. Max1 interacts with intact MBF complex. Extracts (1 mg) from Δ*res1* or Δ*res2* strains expressing Max1-13Myc and Cdc10-HA were immunoprecipitated with the indicated antibodies and proteins were detected by western blotting.

Max1 is a repressor of MBF dependent transcription

Once we knew that Max1 does interact with MBF, we wanted to determine the possible function of this protein regarding MBF regulation of transcription. To do so, we deleted *max1* using a ura4 cassette.

MBF transcripts levels are low in asynchronously growing cells, because of the special features of the *S. pombe* mitotic cycle. Cells spend most of their cycle in G2 phase, and therefore, in asynchronous cultures, 70% of the cellular population is in G2, where the activity of MBF transcription factor is low. This is why to study MBF dependent transcription is necessary to synchronize cells in other phases of mitotic cycle. In *S. pombe*, treatment with hydroxyurea (HU) is a commonly used tool to arrest cells in S phase. HU at toxic does (10mM) inhibits the enzyme

ribonucleotide reductase, encoded by the *cdc22* gene. Because of this, cells cannot progress into S phase where they are blocked. Cells collected at this phase accumulate high levels of MBF transcripts, because MBF is highly active at S phase.

We performed a northern blot to analyze MBF dependent transcription in a Δ*max1* strain compared to a wild type strain, in asynchronous cultures and in cultures synchronized with hydroxyurea. With the experiment represented in Fig. 11, we realized that Δ*max1* strain had very high levels of MBF transcripts. Such derepression pointed out Max1 as a repressor of MBF, with a possible role in repressing MBF outside the G1/S phases.

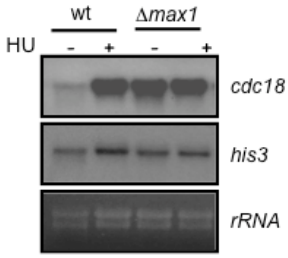


Figure 11. **Max1 regulates MBF-dependent transcription**. Total RNA was prepared from untreated (-) or hydroxyurea-treated (+) cultures (10 mM HU, 3 hours) of wild type (wt) and Δ*max1* cells, and analyzed by hybridization to the probes indicated on the right. *his3* probe was used as a loading control.

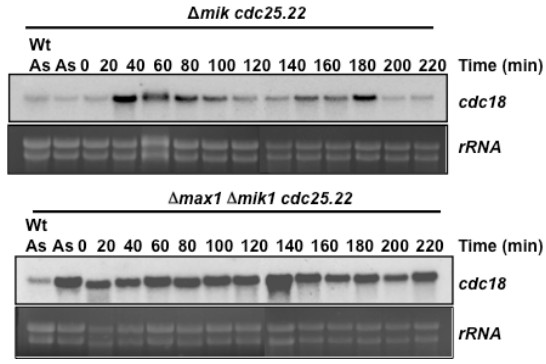
To further characterize the role of Max1 as a repressor, we performed northern blots to analyze MBF dependent transcription not in asynchronous cultures, but in the different phases of the mitotic cycle. Our approach was to construct a strain Δ*max1* in a temperature sensitive (ts) background *cdc25-22*. Strains carrying this conditional allele grow normally at 25ºC, but when shifted at 36ºC, they get blocked at the G2/M transition. After 4 hours of arrest at 36ºC, the whole population of cells in the culture is

synchronized at the end of G2 phase. When this synchronized culture is shifted back to 25ºC, cells progress synchronously into every stage of cell cycle.

However, when trying to construct the strain Δ*max1 cdc25-22*, we realized that cells were not viable at 25ºC. Only when growing at lower temperature (18ºC), cells survived. We thought that a possible explanation for this lethal phenotype of Δ*max1 cdc25-22* could be that this strain showed a derepression of all the MBF dependent genes, including *mik1.* Therefore, Δ*max1 cdc25-22* cells were facing a big deal: how to progress into cell cycle if they carried both alterations: the phosphatase Cdc25 mutated (mutation *cdc25-22*) and the kinase Mik1 upregulated. Both proteins are necessary to activate Cdc2 to progress into cell cycle, but with opposite roles: Cdc25 is an activator of this progression, whereas Mik1 is an inactivating kinase.

To solve this problem, we constructed the strain Δ*max1*Δ*mik1 cdc25-22*, which was viable. Mik1 is not an essential protein because it overlaps its function with the kinase Wee1, so deletion of *mik1* has no effects on cell cycle regulation. We analyzed by northern blot the levels of MBF transcripts in the strain Δ*max1*Δ*mik1 cdc25-22.* We performed a block and release experiment, and after releasing the cells to the permissive temperature, we obtained samples for RNA extraction every 20 minutes during two complete mitotic cycles. As a control, we used the strain Δ*mik1 cdc25-22,* that was expected to behave as a wild type *cdc25-22,* and indeed was wild type, in terms of both, timing of mitotic cycle (measured as septation index in Fig. 12B), and levels of expression of MBF dependent genes.

A.



B.

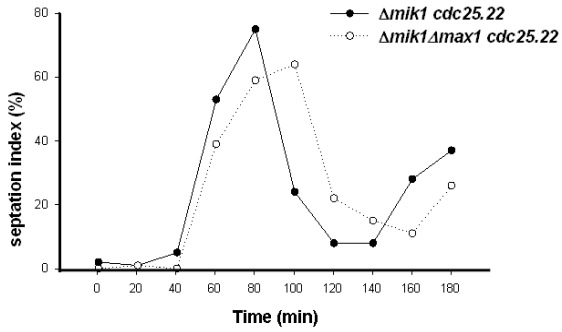


Figure 12. Max1 regulates MBF-dependent transcription. *cdc25-22* strains were synchronized by blocking at 36ºC for 4 hours and release at 25ºC. **A.** RNA from cdc25-22 Δ*mik1* (wt) and *cdc25-22* Δ*mik1*Δ*max1* synchronous cultures was probed for *cdc18* expression. **B.** Septation index of both strains was plotted to measure synchronicity.

From this experiment we confirmed the role of Max1 as a repressor of MBF; when *max1* is deleted, periodic transcription of MBF dependent genes is completely lost, and MBF is constitutively active. Although MBF is known to be tightly regulated by different mechanisms, deletion of this single regulator is sufficient to loose the control of transcription.

Δ*max1* cells have genomic instability

We wanted to further characterize the phenotype of Δ*max1* cells. Despite having this missregulation of MBF transcription, cells showed no apparent defects in cell cycle progression.

If Δ*max1* cells had some aberrant S phase regulation, a different way to detect it would be to analyze the possible consequences of this missregulation. So we tested for chromosomal instability of Δ*max1* strain. We constructed strains carrying an extra chromosome (minichromosome 16), that is an episomal plasmid that complements the *ade6*-M210 mutation in the *ade6* gene (required for the synthesis of adenine). This minichromosome was transformed in a wild type *ade6*-M210 strain and in a Δ*max1 ade6*-M210 strain. The transformed strains are able to grow in media without adenine unless they loose the extra chromosome. If chromosome loss occurs, cells growing in media without adenine become pink as a consequence of the accumulation of an intermediate product of the adenine biosynthetic pathway. Percentage of appearance of partially pink colonies (white colonies with pink sectors) is an index of chromosome loss and therefore indicates chromosomal instability (Fig. 13). Thus, Δ*max1* cells show increased genomic instability.

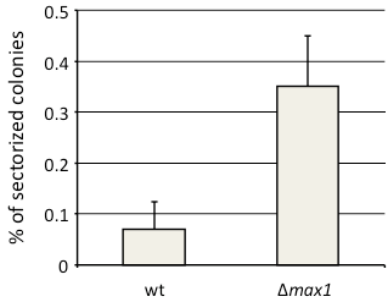


Figure 13. Δ*max1* strain shows genomic instability. Strains carrying the minichromosome 16, HM1109 (WT) and JA1003 (Δ*max1*), were grown in YE5S till midlog phase and 500 cells were spotted into MM plates. Number of sectorized (white and pink) colonies was measured as a percentage of chromosome loss.

max1 deletion renders resistance to HU

There are other situations, besides a normal S phase regulation, in which deletion of Max1 could be important for the cell. We tested if deletion of *max1* could be an advantage in situations of impaired MBF transcription. First, we used a *cdc10-129* mutant. This strain has a point mutation in *cdc10* that affects MBF transcription. Cells can grow at 25ºC, with low levels of MBF-dependent transcription, but at higher temperatures MBF is completely inactive, and MBF dependent transcription is impaired, leading to cell death. We deleted *max1* in a *cdc10-129* strain. Fig. 14 shows that the deletion does not rescue the lethality. This would mean that, even though *max1* deletion derepresses transcription, Cdc10 is necessary to reach the levels of transcription required for survival. (Fig.14A).

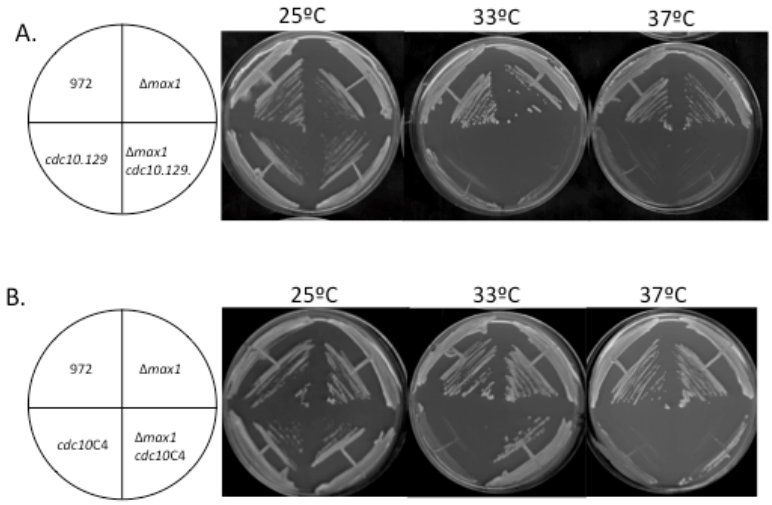


Figure 14. Growth of *cdc10* ts mutants in a Δ*max1* background. A. Wild type (972), Δ*max1*, *cdc10-129* and *Δmax1 cdc10-129* strains were grown at the indicated temperatures. **B.** Wild type (972), Δ*max1*, *cdc10-C4* and Δ*max1 cdc10-C4* strains were grown at the indicated temperatures.

We did the same experiment with a different Cdc10 temperature sensitive mutant. *cdc10-C4* cells have lost MBF regulation, and they show a peculiar pattern of MBF-dependent transcription. Cells can grow at 25ºC, with highly derepressed levels of MBF-dependent transcription, but at higher temperatures MBF dependent transcription is impaired, leading to cell death. When we deleted *max1* in a *cdc10*-C4 strain, the deletion partially rescued the lethality, since cells can grow at 33ºC.

A different situation in which deletion of *max1* could be beneficial for the cell was in response to hydroxyurea. The target of the drug is the enzyme ribonucleotide reducatase (Cdc22). Since the transcription of the gene that codes for Cdc22 is MBF dependent, there was the possibility that an excess of *cdc22* transcript supposed an advantage in front of HU. We tested this in survival

assays, plating serial dilutions of cells into plates with different concentrations of the drug (Fig.15A). With high concentrations of HU (10mM), Δ*max1* strain was partially resistant to HU.

Following with the same idea, we decided to check if survival upon HU might be more significant for Δ*max1* cells under more severe conditions. Δ*cds1* cells, which lack the effector kinase of the DNA synthesis checkpoint, are highly sensitive to HU. However, deletion of *max1* in Δ*cds1* cells partially rescued the lethality. Interestingly, survival upon HU requires the checkpoint response (Cds1), but activation of MBF transcription is sufficient to improve survival, despite the checkpoint response remains inactive.

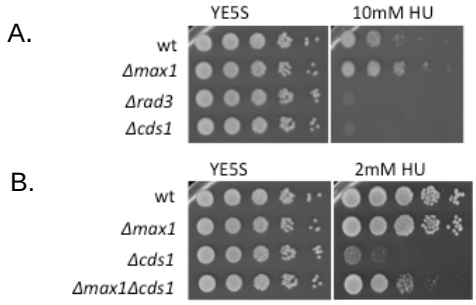


Figure15. Δ*max1* phenotype upon HU treatment. A. Sensitivity to HU of 972 (WT), Δ*max1*, Δ*rad3* and Δ*cds1* strains. **B**. Sensitivity to HU of 972 (WT), Δ*max1*, Δ*cds1* and the double delete Δ*max1*Δ*cds1*. Cells were grown in YE5S and were spotted from 10 to 10^5 in YE5S plates containing HU at the indicated concentrations and incubated at 30℃ for 3 to 4 days.

Next, and to ratify the effect of HU in Δ*max1* cells, we set out cell growth on liquid cultures. We used the strain *cdc10.129* as a tool to block cells in G1 and synchronize the cells. Once arrested at G1 (36ºC), cells were released at 25ºC, in the presence or absence of HU. In the flow cytometry profile of Fig. 16 is visible how cells shift from 1C of DNA content (cells blocked at START), to a peak of 2C

(100% of cells have completed S phase and have a DNA content of 2C after 2 hours). However, if cells are released into HU, they do not reach a peak of 2C because they cannot complete DNA synthesis. Δ*max1* cells, on the contrary (right panel, Fig. 16), can overcome the arrest, as if they were not affected by HU. This is one more evidence that Δ*max1* cells are partially resistant to HU.

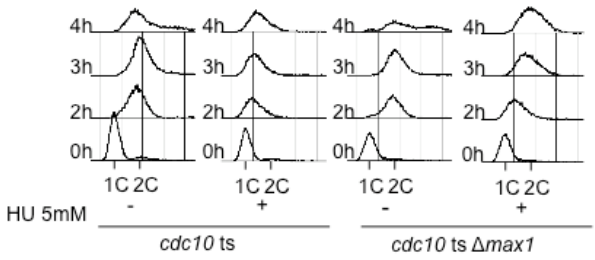


Figure 16. FACs profile of cells arrested at G1. Strains *cdc10.129* and Δ*max1cdc10.129* were arrested at G1 for 4 hours and then released at 25ºC. Time (hours) after the release is indicated. 5mM HU was added (+) or not (-) at the moment of the release at 25ºC.

Max1 protein levels

To find out if Max1 was present in the cell during the complete cell cycle, we performed a *cdc25-22* block and release experiment and obtained protein extracts every 20 minutes during 300 minutes after the release, which corresponded to two complete cell cycles. Max1 was tagged with Myc, and in Fig. 17 can be observed how protein levels remain constant.

There is less Max1 protein at time-points 0 min, 20 min and 40 min. The septation index corresponding to this time course (Fig. 17B) indicates that these time-points represent G2/M transition and M phase. However, looking at the second cycle of the experiment (time-points from 140 min to 300 min in Fig. 17B), protein levels

remain constant. The diminished amounts of protein at time-points 0 min, 20 min and 40 min are due to the fact that the cells were arrested for 4 hours at 36ºC. During the time of the arrest, MBF was inactive and Max1 transcription was completely switched off and therefore there is no protein at the beginning of the experiment.

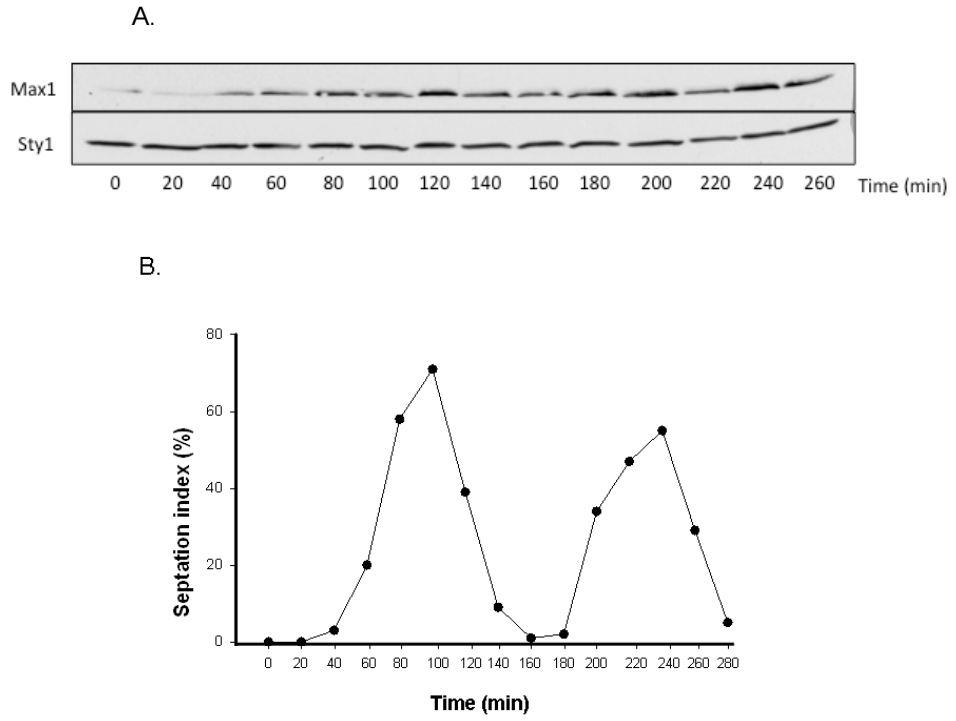


Figure 17. **Protein levels of Max1. A.** Cellular abundance of the fusion protein Max1-Myc was monitored in synchronous cultures. Time (minutes) after the release from a *cdc25* block is indicated. . The lower panel is a loading control for the western blot. **B.** Septation index was plotted to measure synchronicity.

The fact that Max1 protein levels did not fluctuate during the cell cycle, as it happens with other components of MBF (Cdc10, Res1 and Res2), made us wonder how could Max1 modulate MBF activity. There were different possibilities: it could be that there was a change in Max1 localization and it was not binding MBF

during the whole cycle. Another possibility was that it was part of MBF all the time, but with some posttranslational modifications modulating its activity. To find out we tried different approaches: we determined Max1 localization, and we determined when was Max1 binding MBF dependent promoters.

Max1 localization

When we tagged Max1 with GFP (green fluorescent protein), we could observe a nuclear localization in asynchronous cultures. In a time course experiment, using the strain Max1-GFP *cdc25-22*, we observed that Max1 was localized in the nucleus throughout the cell cycle (Fig. 18). This was important in order to understand Max1 function. We have shown that Max1 is a repressor of MBF during G2, and therefore we expected the protein to remain in the nucleus during G2, as it did. However, it remained nuclear during G1 and S phases, when MBF is active.

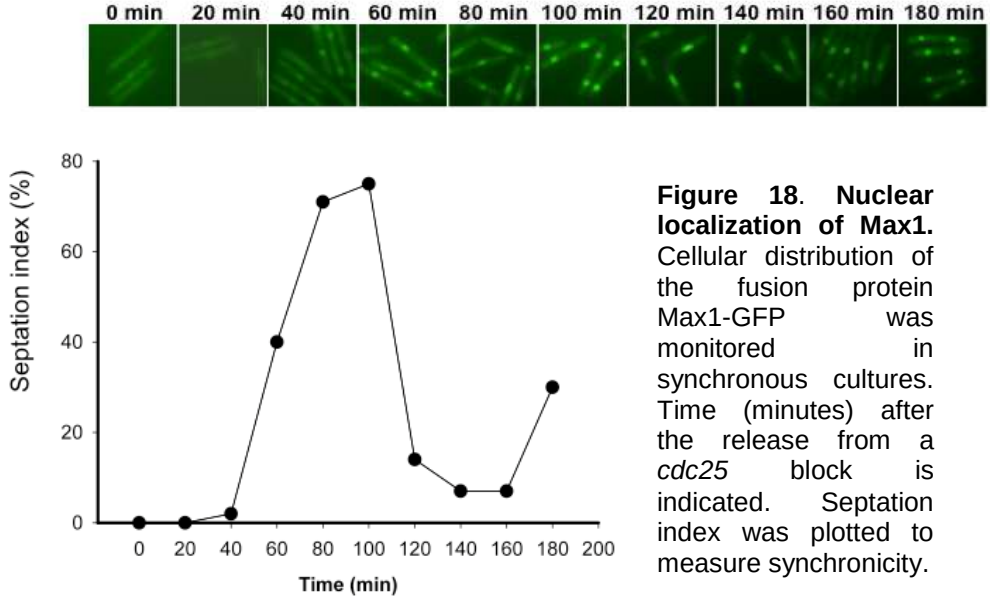


Figure 18. Nuclear localization of Max1. Cellular distribution of the fusion protein Max1-GFP was monitored in synchronous cultures. Time (minutes) after the release from a *cdc25* block is indicated. Septation index was plotted to measure synchronicity.

The accumulation of Max1 during G1 and S phases, detected as an increase in the intensity of fluorescence, was clear in this experiment. The increasing levels of Max1 can be explained by the fact that its transcription is MBF dependent, and the protein accumulates as the transcripts do.

For us it was especially important to understand what happens to the repressor in mitosis, when MBF switches from inactive to active form. For the other MBF repressor described, Nrm1, it was published that the protein is degraded at the end of G2 (de Bruin et al., 2006). In the case of Max1, degradation seemed not to occur.

To make sure that the localization and the levels of fluorescence were not affected by the *cdc25-22* background (which is not a physiological condition), we used the strain Max1-GFP to follow the localization of Max1 in every phase of the mitotic cycle by means of a time lapse experiment (Fig. 19). We followed the division of single cells during up to one hour. What we wanted to determine is what happened with the repressor in the G2/M transition, in M phase, and in G1 phase. Taking images every ten minutes allowed us to capture these phases of the mitotic cycle, which are very fast in *S. pombe*. Timings with this procedure were also more physiological than timings in a *cdc25-22* synchronization.

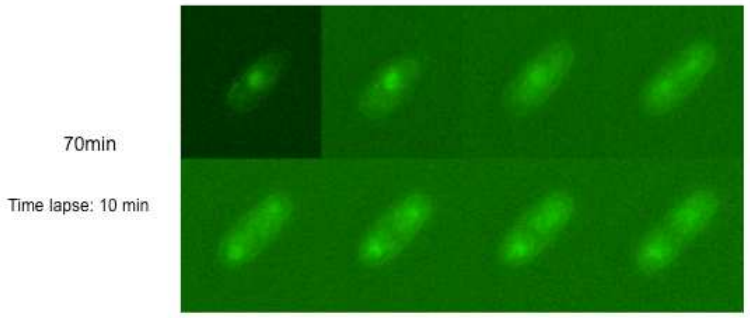


Figure 19. Max1 is nuclear throughout the cell cycle. Images were captured every 10 min during 70 min to detect localization of Max1-GFP in the G2/M transition and in mitosis.

We confirmed that Max1 is present in the cell during G2, M, and G1 phases, and it is not degraded. Localization keeps nuclear, although fluorescence intensity seemed to be lower.

Max1 binding to MBF

We knew that Max1 interacts with Cdc10 in asynchronous cultures (Fig. 9), which represent mainly cells in G2. MBF activity is very low in G2, and it made sense to detect a binding of the repressor to the complex during this phase. We next wanted to find out how was the interaction between Max1 and MBF during G1 and S, when MBF is highly active.

To further characterize this interaction, our aim was to immunoprecipitate Cdc10 in a time course experiment, and to check when was Max1 co-immunoprecipitating and when was not. The idea was to use the strain Cdc10-HA Max1-Myc *cdc25-22*. Disappointingly, we found that the strain was not able to progress into cell cycle after the release at the permissive temperature. The

reason might be that multiple taggings in MBF could be affecting somehow the interactions and the structure of the complex.

Our next approach was to synchronize cells in mitosis. To achieve this block in cell cycle, we used *cdc13*Δ90 mutants. Cells were transformed with an integrative plasmid that codes for a truncated version of cyclin Cdc13 that lacks 90 amino acids. This protein is expressed under the control of an inducible *nmt* promoter. Cells growing with thiamine do not express the truncated cyclin. When thiamine is washed from the medium, however, cells express the truncated Cdc13 protein. This protein is normally functional (it has associated kinase activity), but the truncation does not allow the protein to be degraded by the APC. Therefore cells proceed normally in the G2/M transition, but get arrested in anaphase, with a constitutively active Cdc2/Cdc13 complex. This condition is lethal, but it is a useful to tool to get synchronized cultures in anaphase. We analyzed the *in viv*o interaction between Max1 and Cdc10 performing co-immunoprecipitation experiments (Fig. 20) and we realized that the interaction between both proteins was lost in anaphase blocked cells. This loose of interaction between the repressor and MBF could explain why MBF is activated in anaphase.

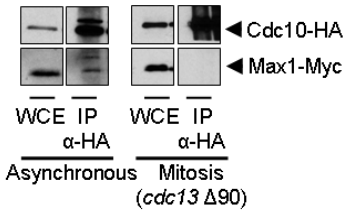


Figure 20. Max1 does not interact with MBF in mitosis. Extracts from strains expressing Max1-13Myc and Cdc10-HA were obtained from asynchronous cultures and from anaphase arrested cultures (*cdc13*Δ90 strains). Native extracts were immunoprecipitated (1 mg) with α-HA antibody and proteins were detected by western blotting.

Max1 binding to MBF promoters

To confirm the previous results in a more "in vivo" set up, we analyzed the binding of Max1 to MBF dependent promoters by chromatin immunoprecipitation (ChIP). Cross-linked cell extracts were immunoprecipitated with α-Max1 antibodies, and assayed for the presence at the *cdc18* promoter regions. As control, DNA was amplified from whole cell extracts (WCE) before immunoprecipitation.

We obtained synchronized cultures using the strain *cdc25-22*. Max1 is always binding promoters, but there are fluctuations in this binding during cell cycle (Fig. 21). The peaks with maximum loading of Max1 on *cdc18* promoters correspond to G2 phase (see septation index), and the valleys of minimum loading correspond to M, G1 and S phases. This means that, as expected, the repressor maximum binding corresponds to the periods of less transcriptional activity of MBF (in Fig. 21, upper panel, *cdc18* expression profile during cell cycle).

From those experiments we could not conclude if Max1 binds MBF promoters directly or it does so through the other components of the complex. What was important for us was the fact that there was a periodicity in Max1 binding to promoters, and this periodicity correlated with MBF activity. We wanted to find out how was this periodicity being achieved.

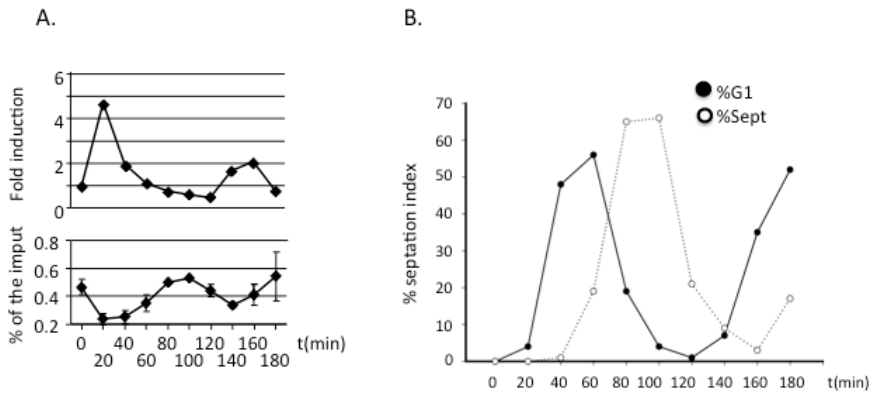


Figure 21. Max1 physically associates to promoters of MBF dependent genes. *cdc25-22* strain was synchronized by block at 36ºC for 4 hours and release at 25ºC. **A** RNA from synchronous cultures was probed for *cdc18* expression (upper panel) and representative ChIP data for Max1 occupancy on *cdc18* promoter was plotted (lower panel). **B**. Septation index was plotted to measure synchronicity.

If Max1 protein levels remain constant all over cell cycle, the question raised from the ChIP experiments was how entry and exit from promoters was regulated. Since CDKs are the main regulators of almost all the events important for cell cycle progression, we hypothesized that CDKs might be regulating Max1. Also, there was previous evidence that CDKs in fact regulate MBF activity, at least switching off transcription as cells exit S phase (Ayte et al., 2001; Stern and Nurse, 1997).

Max1 is a phosphoprotein

To investigate further the possible regulation of Max1 by CDKs, we analyzed the mobility of Max1 protein by western blot, searching for possible phosphorylations.

We had already performed the experiment shown in Fig. 17, in which we had obtained protein extracts during a complete time

course experiment, using a Max1-Myc *cdc25-22* strain. No obvious shift corresponding to a change of mobility of Max1 at any phase of the cell cycle had been observed. Thus, we synchronized cells in S phase with hydroxyurea. The aim was to have an accumulation of Max1 protein (as it happens with other proteins encoded by MBF dependent genes). We hypothesized that a phosphorylation shift might be easily detectable by western blot if we increased the amount of Max1 protein. We used a Myc-tagged strain which allowed us to detected Max1 in native extracts using α-Myc antibody.

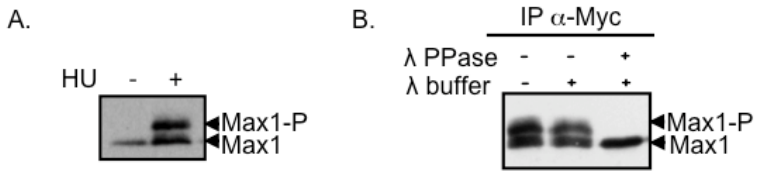


Figure 22. Max1 is a phosphoprotein. A. Max1 is phosphorylated after HU treatment. Max1-Myc cultures were treated with (+) or without (-) 10mM HU, and pellets were collected for native extracts after 3h of treatment. **B**. Native extracts from Max1-Myc strain were prepared and incubated with (+) or without (-) lambda phosphatase, as indicated. Max1 was detected after SDS/PAGE followed by Western blot using monoclonal anti-Myc antibody.

As expected, there was an accumulation of Max1 protein in the HU treated cells (Fig. 22A). But the surprise was to detect a very noticeable slower migrating band in the SDS/PAGE. To check if such a shift corresponded to a phosphorylated form of the protein, we treated the protein extracts with lambda phosphatase (Fig. 22B). The shift disappeared, which was indicating us that Max1 was indeed a phosphoprotein.

There were two possible explanations for Max1 being phosphorylated upon HU treatment: either Max1 was phosphorylated in the S phase of cell cycle, or the phosphorylation was a consequence of the HU treatment itself.

Max1 regulation by the DNA synthesis checkpoint

Despite our initial interest in possible CDK dependent phosphorylations, we became intrigued as well in a possible phosphorylation of Max1 by the replication checkpoint kinase Cds1.

Both kinases, CDK and Cds1 have highly conserved consensus sites of phosphorylation. CDK sites are S/T-P-X-K/R (SP or TP sites followed by a basic aminoacid two residues after the serine or the threonine), and Cds1 sites are LXRXXS/T (Seo et al., 2003; Xu and Kelly, 2009). We analyzed Max1 protein sequence to search for them.

MSLSD**SP**SKSGNTGKDLISNNEAKNHEDEETHQKKRRRRTTDAEATLLEQ
YFLK**TP**KPSLIERQELSKKLKSSM**TP**RELQIWFQNKRQSLRRSNCLSRNR
LEGTGENSLL**RRKST**LTLCETSTGQAELFFQSWPLHSQSVEMIHHEQDDY
NKENKQQKVVDTTKDISRGSNGNEDSAAHQELEECARSLVELQQQCNDH

Figure 23. Phosphorylation sites in Max1 protein sequence. CDK and Cds1 kinases consensus phosphorylation sites are marked in bold.

Max1 has only three consensus sites of phosphorylation by CDK, and two by Cds1. The next step was to determine if the change of mobility observed in Fig. 23 was due to the HU treatment and therefore it was checkpoint dependent. To answer this question, we checked if the phosphorylation shift band disappeared in cells deleted for the kinases of the replication checkpoint pathway. We tested both, Rad3, the upstream kinase, and Cds1, the effector kinase. We analyzed by western blot the mobility of Max1-Myc in Δ*rad3* and in Δ*cds1* strains (Fig. 24). The phosophorylation shift was completely abrogated in both strains carrying the deletions.

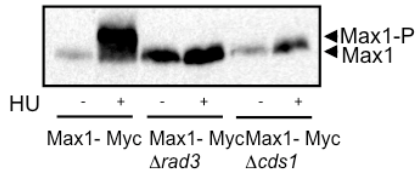


Figure 24. Max1 is a substrate of the DNA replication checkpoint. Native extracts prepared from untreated (-) or 10mM hydroxyurea-treated (+) cultures of wild type, Δ*rad3* and Δ*cds1* strains expressing Max1-Myc were analyzed to detect changes in the electrophoretic mobility of Max1-Myc.

The fact that the replication checkpoint was regulating Max1 was very outstanding. At that particular time of our work, there was no evidence of a specific checkpoint-dependent regulation of MBF. The two articles describing the activation of MBF by the replication checkpoint in *S. pombe* (de Bruin et al., 2008; Dutta et al., 2008) had not been published yet. Neither there was an evidence of such a regulation in *S. cerevisiae*. Hence our discovery that replication checkpoint might be directly regulating MBF through Max1 was very promising. Consequently, we wanted to understand the mechanism of MBF regulation by the replication checkpoint. We immunopreciptated Cdc10 and Max1, after treating cells with 10mM HU. We used the strain carrying Cdc10-HA and Max1-Myc, and performed the immunoprecipitations using the α-HA or the α-Myc antibodies. As it can be observed in Fig. 25, there was no co-immunoprecipitation of both proteins in extracts prepared under the stress conditions.

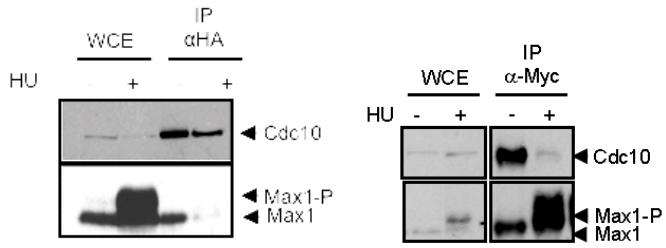


Figure 25. Interaction between MBF and Max1 is lost upon HU treatment. Extracts from the Cdc10-HA Max1-Myc strain were immunoprecipitated using α-HA or α-Myc antibody and proteins were detected by western blotting.

However, there was still some interaction in the HU treated cells, probably due to the fact that there was a pool of Max1 protein not phosphorylated. We were immunoprecipitating both forms of Max1, phosphorylated (upper band) and not phosphorylated (lower band, probably corresponding to newly synthesized Max1). To get rid of the not phosphorylated form of the protein, we added cycloheximide to the cultures. This drug is an inhibitor of translation, and as can be observed in Fig. 26, using the drug we detected an accumulation of the phosphorylated Max1 in the whole cell extracts. This time, interaction with Cdc10 was completely lost.

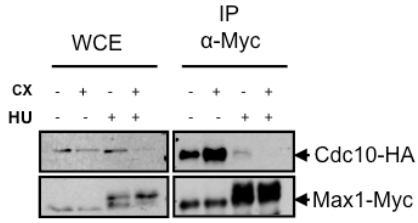


Figure 26. Interaction between MBF and Max1 is lost upon HU treatment. Extracts from the Cdc10-HA Max1-Myc strain were immunoprecipitated using α-Myc antibody and proteins were detected by western blotting. Translation was inhibited by adding 200 mg/ml cycloheximide (CX) into MM cultures at mid-log phase, and pellets were collected after 30 min of CX treatment.

Accordingly with the results obtained in the co-immunoprecipitation experiments with HU, we hypothesized that upon HU treatment, Cds1 phosphorylated Max1, and this phosphorylation abrogated Max1 binding to MBF. To ratify this idea, we analyzed how was loading into promoters of Max1 when cells were treated with HU (Fig. 27). Indeed, Max1 was released from MBF promoters in the presence of HU.

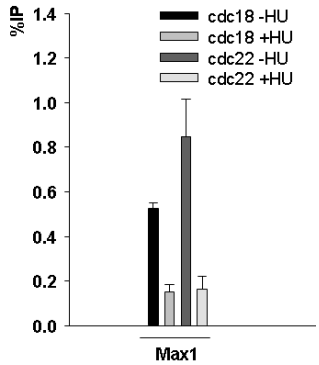


Figure 27. Max1 is released from MBF dependent promoters upon HU treatment. Loading of Max1 on *cdc18* and *cdc22* promoters was measured in untreated or hydroxyurea-treated cultures.

If phosphorylation of Max1 by Cds1 under replicative stress abrogates binding to MBF and releases Max1 from MBF promoters, there was a possibility that the phosphorylation caused a change of localization of Max1 (as it has been described for some checkpoint substrates). To test if there was an export of Max1 to cytoplasm, we analyzed the localization of Max1-GFP by fluorescent microscopy (Fig. 28) and observed that there was not a change of localization of Max1. Thus, we could discard that the Cds1-dependent phosphorylation was exporting Max1 from the nucleus.

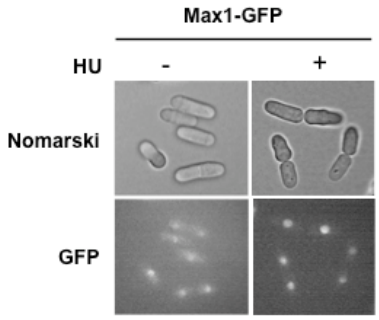


Figure 28. Nuclear localization of Max1 after HU treatment. Max1-GFP cellular distribution was determined by fluorescence microscopy in cultures treated (+) or not (-) with 10mM HU (GFP; lower panels). The same cells under differential interference contrast (Nomarski) optics are shown in the upper panels.

Cds1-phosphorylation mutants

Since Cds1 phosphorylation sites are highly conserved in *S. pombe* (Fig. 23), we mutagenized the sites to mimic unphosphorilable forms of the protein. Our first approach was to mutagenize the amino acids 114 (replacing a serine by an alanine), 115 (replacing a threonine by an alanine), and also we obtained the double mutant 114.115 (Fig. 29A). We generated punctual mutants of Max1 to obtain the mutant Max1 forms in their own locus. Mutants S144A, T115A and S114A-T115A will be referred from now as SA, TA, and SATA, to simplify the nomenclature.

Next we examined the behaviour of the Max1 mutants we had generated, comparing them to wild type Max1. We treated cells with hydroxyurea 10mM and analyzed by western blot the electrophoretic mobility of Max1 mutants (Fig. 29B). The slower migrating band corresponding to the phosphorylated form of the protein was reduced to some extent in mutants Max1-SA and

Max1-TA, and clearly reduced in the double mutant Max1-SATA. There are two interesting observations to be noticed from Fig. 29B: there was still a minor change of mobility when comparing the SATA without/with hydroxyurea (see below). This could mean, that there was still some phosphorylation. The second observation is that in the SATA mutant, not only the major phosphorylation shift disappeared, but also protein levels of Max1 decreased noticeably. This was giving us a hint that this mutant might had problems in the induction of transcription upon HU treatment.

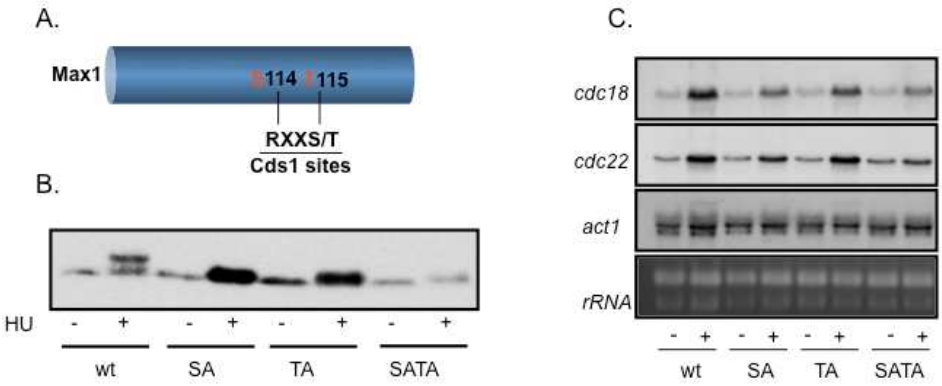


Figure 29. Max1 Cds1-mutants under replicative stress. A. Schematic representation of Max1 Cds1-phosphorylation sites. **B.** Max1 mutants electrophoretic mobility. Strains Wt (Max1-Myc), and the mutants SA, TA, and SATA were treated with 10mM HU for 3 hours. Native extracts were analyzed by Western blot with anti-Myc (9E11) to detect Max1-Myc protein. **C.** Total RNA was prepared from untreated (-) or hydroxyurea-treated (+) cultures (3 hours at 30ºC) of wild type (wt) and SA, TA, SATA mutants, and analyzed by hybridization to the probes indicated on the left. *act1* probe was used as a loading control.

To test this point, we first analyzed how the mutants were behaving regarding MBF dependent transcription (Fig. 29C). We extracted RNA from both, untreated and hydroxyurea-treated cells in all the mutants. Transcription was only barely induced in the SATA mutant, what indicated us that if checkpoint cannot phosphorylate

Max1 (SATA mutant), then MBF dependent transcription is not induced.

We wanted to make sure that the double mutation SATA was not interfering somehow with the normal MBF regulation. We checked that transcription under normal conditions (not under replicative stress) was wild type in the SATA mutant. To do so, we analyzed transcription of *cdc18* during a complete mitotic cycle using a Max1SATA *cdc25-22* temperature sensitive strain (Fig. 30). The differences were minor and the SATA mutant was perfectly able to periodically induce transcription like wild type Max1.

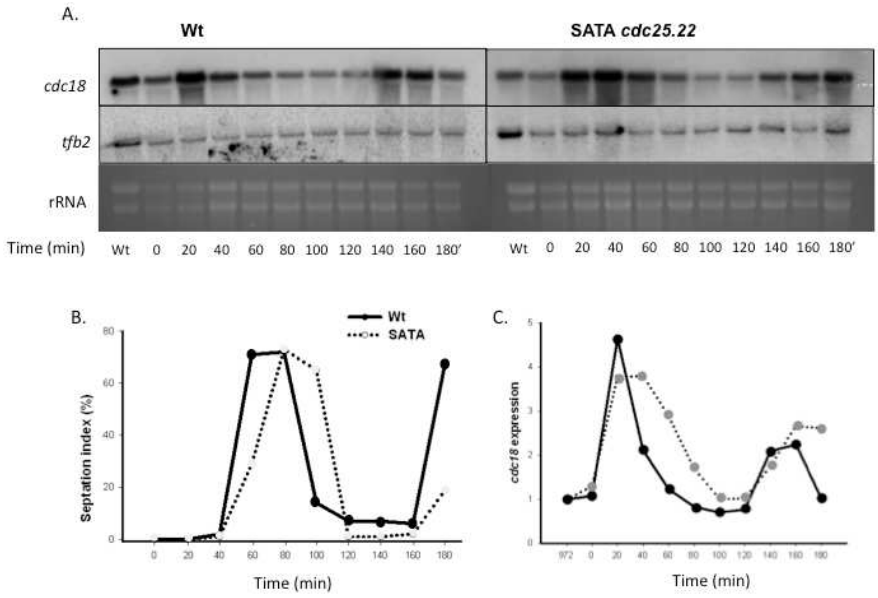


Figure 30. Max1-SATA is wild type regarding MBF transcription regulation in a normal cell cycle. Max1-SATA *cdc25-22* strain was synchronized by block at 36ºC for 4 hours and release at 25ºC. **A.** RNA from synchronous cultures was probed for *cdc18* expression and *tfb2* expression as a loading control **B.** Septation index of both strains was plotted to measure synchronicity. **C**. *cdc18* mRNA levels were quantified after normalization with the probe *tfb2*.

The conclusion of figures 26, 27 and 29 was that, upon HU treatment, replication checkpoint is activated and Max1 is phosphorylated. Phosphorylation of the repressor abrogates its binding to MBF, what allows the induction of transcription. If this phosphorylation is impaired (SATA mutant), transcription is not induced.

To confirm this model of regulation, we analyzed what happened with mutant Max1-SATA upon HU treatment regarding its interaction with MBF. We performed immunoprecipitation experiments, using the strains Cdc10-HA Max1-Myc and Cdc10-HA Max1SATA-Myc. We immunoprecipitated Max1 using α-Myc antibody. In Fig. 31 can be observed how Cdc10 and Max1 interacted in untreated conditions (-), but upon HU treatment (+), Cdc10 co-immunoprecipitation was impaired in wild type strain (Max1-Myc). On the contrary, when Max1-SATA was immunoprecipitated, the co-immunoprecipitation with Cdc10 was preserved even in the presence of HU.

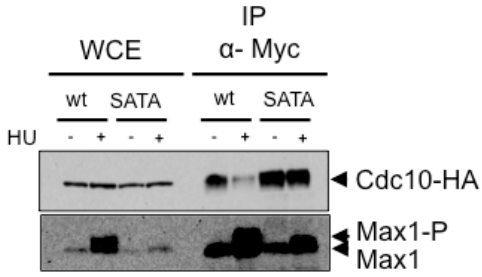


Figure 31. Interaction between MBF and Max1-SATA upon HU treatment. Extracts from the Cdc10-HA Max1-Myc strain (wt) and the Cdc10-HA Max1SATA-Myc strain (SATA) were immunoprecipitated using α-Myc antibody and proteins were detected by western blotting.

Then we performed chromatin immunoprecipitation experiments to further validate the model. We knew from the ChIP experiments in Fig. 27 that Max1 is released from MBF promoters upon HU treatment, and we had corroborated that this was due to a checkpoint dependent phosphorylation. We decided to analyze for the presence of Max1 in MBF promoters in Δ*rad3* and Δ*cds1* strains, where checkpoint response is impaired. As shown in Fig. 32A, Max1 was still binding *cdc18* and *cdc22* promoters in the in the absence of Rad3. However, in the Δ*cds1* strain Max1 was partially released from promoters. This can be explained by the fact that in a Δ*cds1* strain there is still some checkpoint activity, because the kinase Chk1 can compensate the lack of Cds1 (Zeng et al., 1998). Only in the Δ*rad3* strain the checkpoint response is completely impaired.

We also tested the occupancy in promoters of the mutant Max1-SATA (Fig. 32B). Unexpectedly, it was partially released from promoters after HU treatment, although we had previously proved that this mutant was not phosphorylated upon HU treatment. This experiment did not fit with our previous experiments (Fig. 29 and Fig. 32), where we had verified that Cdc10 and Max1-SATA were still interacting upon HU treatment, and also that transcription was not induced in the Max1-SATA strain upon HU.

If Max1-SATA was released from promoters after the treatment, then the only explanation for the null induction of transcription was another change in the MBF activity. We performed ChIP experiments to test for the presence of Cdc10 on promoters after HU (Fig. 32B). Cdc10 was being partly released from promoters in

the SATA mutant and this slight decrease would be enough to explain the decreased transcription. Why in the SATA mutant background Cdc10 was being evicted from promoters is something we do not completely understand. It is possible that a conformational change in the mutant Max1 increases its affinity for Cdc10.

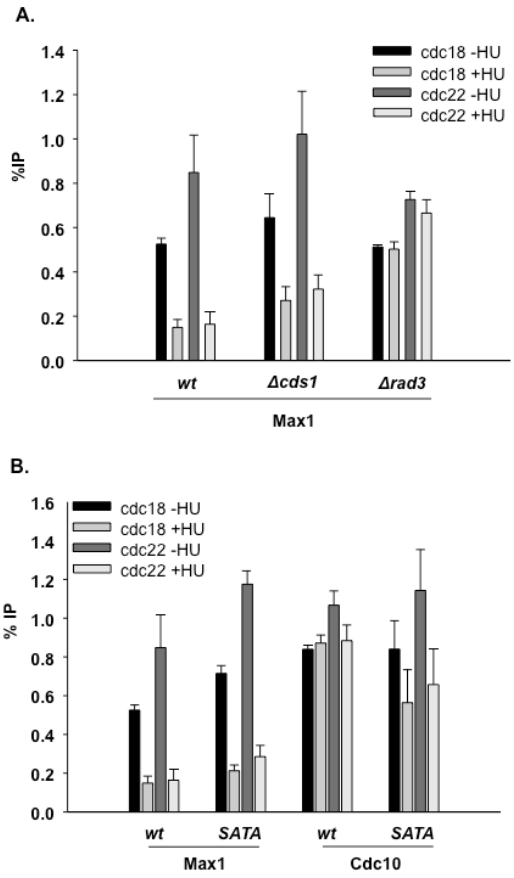


Figure 32. Representative ChIP data for Max1 occupancy at MBF genes promoters. **A**. Max1 occupancy at MBF promoters was measured in WT, Δ*rad3 and Δcds1* strains. **B.** Max1 and Cdc10-HA occupancy at MBF promoters was measured using α-Max1 and α-HA antibodies in two different strains: WT and Max1-SATA. Data was obtained from three independent experiments and are expressed as mean ± SD.

Role of the MBF transcriptional activation in response to replicative damage

If induction of MBF transcription is a part of the surveillance mechanisms of the checkpoint response, then transcription of those particular genes might be necessary for survival in front of replicative damage. To test which was the role of the MBF-induced transcription in the checkpoint response, we performed survival assays. We expected that cells with an impaired transcriptional response (SATA mutant) would have a compromised survival upon HU treatment. We analyzed how was survival of the SATA mutant on serial dilution spots (Fig. 33).

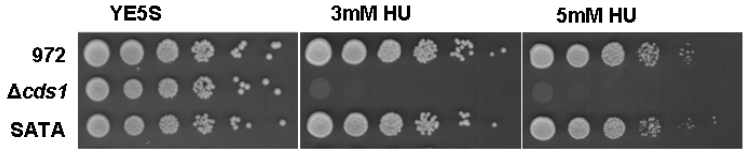


Figure 33. Survival of SATA mutant upon replicative damage. Liquid cell cultures from strains 972 (WT), Δ*cds1* and Max1-SATA were grown in YE5S and 10 to 10^5 cells were spotted into YE5S plates with different drugs at the indicated concentrations and incubated at 30ºC for 3 to 4 days.

Surprisingly, the strain carrying the SATA mutation did not have any grow defects in comparison to the wild type strain. Our control was the Δ*cds1* strain, which has the checkpoint response impaired and therefore is unable to survive in the presence of HU. The fact that SATA mutation was not lethal upon HU treatment would indicate that induction of transcription is not essential for the survival response.

There was still the possibility that the Max1 mutant was sensitive to HU, but not as much as to be detectable in the spots assays. So we tested the viability of the different strains in liquid assays by measuring the OD600 of the cultures (treated or not with HU), every 10 min.

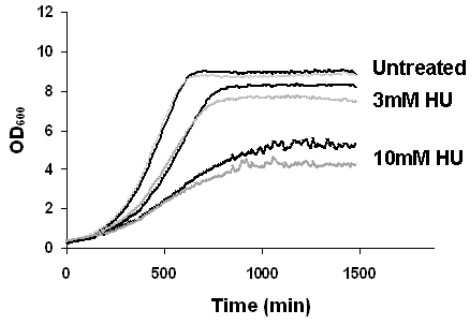


Figure 34. Growth curves of Wild Type and Max1-SATA in the presence of different concentrations of HU. Logphase cultures at an OD600 of 0.1 of WT (black) and Max1-SATA (grey) strains were treated or not with the indicated concentrations of HU, and grown into microculture wells. Growth was monitored by measuring OD600 every 10 min at 30º for 24 h.

Minor differences were noticeable in the growth curves, and the SATA mutant was able to overcome the stress situation despite a little delay in growth.

To make sure that the induction of transcription was not necessary for survival we tested other stress conditions besides HU. Some of the drugs we used are DNA damaging agents (γ irradiation, U.V. radiation, MMS and Phleomycin), but they have been reported to activate as well the replication checkpoint: as a consequence of the single and double strand breaks in DNA, stalling of replication forks occurs and the replication checkpoint is activated. Actually, there is a crosstalk between both pathways (DNA damage response and replication damage response) for most of the

damaging agents, although Δ*chk1* cells are more sensitive to the strictly DNA damaging agents, and Δ*cds1* cells is more sensitive to the drugs that directly affect DNA replication. However, the SATA mutant was not sensitive to any of the tested drugs.

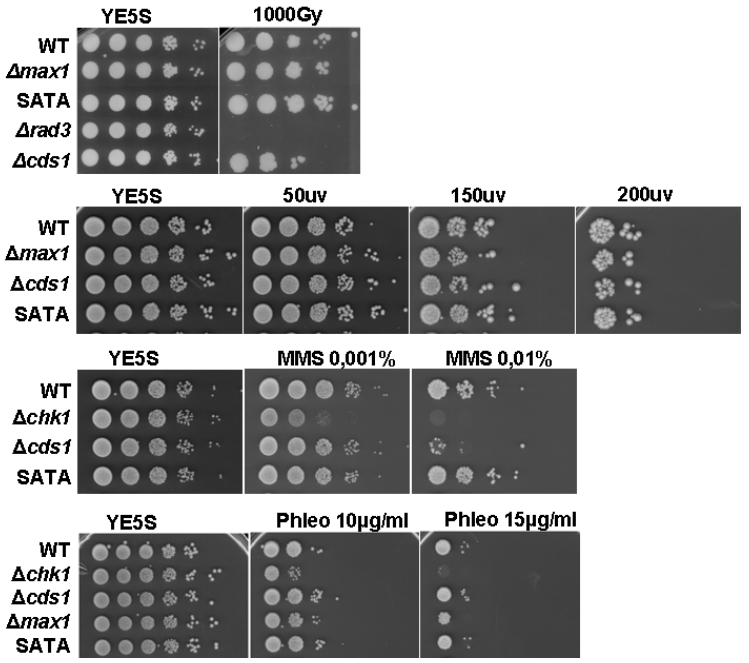


Figure 35. Survival of SATA mutant upon replicative damage. Cell cultures from strains 972 (WT), Δ*chk1*, Δ*cds1*, Δ*max1* and Max1-SATA were grown in YE5S, and 10 to 10^5 cells were spotted into YE5S plates with different drugs at the indicated concentrations and incubated at 30℃ for 3 to 4 days.

We hypothesized that since *S. pombe* cells spend most of the cell cycle in G2, cells could compensate during this period the lack of induction of MBF-dependent transcription, preventing aberrant mitosis that would lead to death. We decided to test our mutant in a background *wee1-50*, which has a short G2 phase when growing at 37ºC. We hypothesized that cells with a short G2 could have a compromised survival if they had problems in the transcriptional induction of MBF-dependent genes. As shown in Fig. 36 *wee1-50*

Max1-SATA cells are hypersensitive to HU, while *wee1-50* Δ*max1* have improved viability compared to the parental strain.

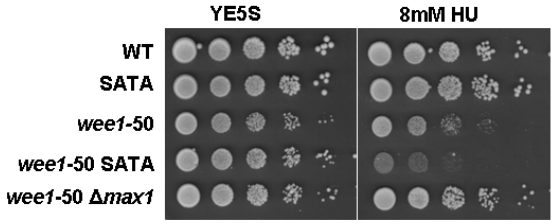


Figure 36. *wee1-50* Max1SATA phenotype upon HU treatment. Cell cultures from the indicated strains were grown in liquid culture at 25ºC and 10 to 10^5 cells were spotted into YE5S plates with 8mM HU and were grown at 37ºC for 3-4 days.

All the results we obtained regarding Max1 regulation by the replication checkpoint are attached as a manuscript in the appendix of this thesis.

Regulation of Max1 by CDKs

Since we observed a cell cycle-regulated binding of Max1 to MBF-dependent promoters, we wondered if this was regulated by the CDK activity, which could modulate MBF by activating or inactivating the binding capacity of Max1 to these promoters. Such phosphorylations could be dependent on two different CDK complexes depending on the phase in which they would take place: Cdc2/Cdc13, if Max1 was phosphorylated in G2/M, or Cdc2/Cig2 if it was phosphorylated in G1/S.

We noticed that Max1 has three putative consensus sites of phosphorylation by CDKs (Fig. 23, Fig. 37). Only the first site presents the extended consensus phosphorylation site (S/T-P-X-K/R), while the two latter are only TP sites. We did not know how the possible phosphorylations would modulate Max1, since they could be activating or inactivating phosphorylations, affecting binding of Max1 to promoters in one way or the other.

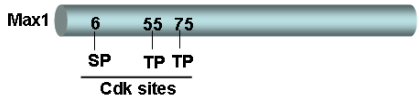


Figure 37. Schematic representation of Max1 phosphorylation sites for CDKs.

In vitro phosphorylation of Max1 by CDK

We decided to perform *in vitro* kinase assays using purified fusion proteins from *E.coli* (GST pull down) and immunoprecipitated the kinases from *S. pombe* native extracts. We immunoprecipitated

Cdc2-HA (Fig. 38A), and also the cyclins Cig2-HA (Fig. 38B), and Cdc13-GFP (Fig. 38C). Immunoprecipitating the cyclins we were immunoprecipitating their associated kinase activity (co-immunoprecipitating Cdc2 in a specific CDK complex). Cig2 was immunoprecipitated from asynchronous cultures (where the Cdc2/Cig2 activity is low), and from HU treated cultures (where the kinase activity of the complex is high). As substrates of the assay, we used Histone 1 as a control (known to be phosphorylated by both CDK complexes), and GST as a negative control.

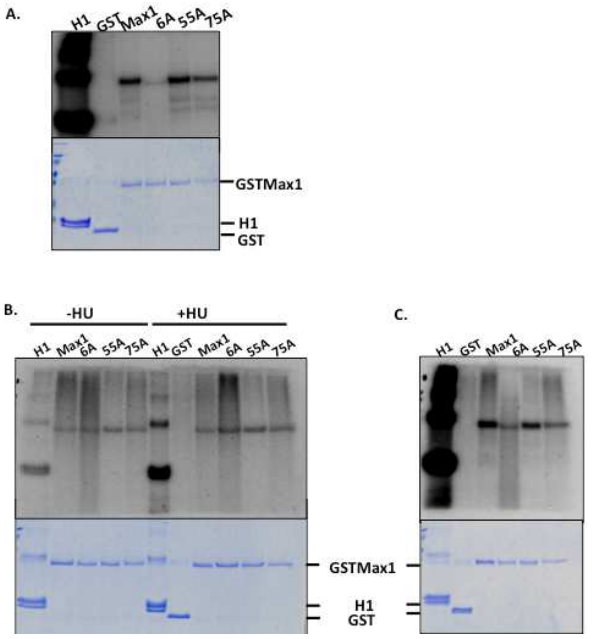


Figure 38. Kinase Assay of Max1 CDK-mutants. A. Kinase Assay of immunopurified Cdc2-HA over Histone 1 (H1), GST, and recombinant fusion proteins GST-Max1, GST-Max1-6A, GST-Max1-55A and GST-Max1-75A. **B.** Kinase assay of immunopurified Cig2-HA, from cultures treated (+HU) or not (-HU) with 10mM HU over the same substrates. **C.** Kinase assay of immunopurified Cdc13-GFP over over Histone 1 (H1), GST, and recombinant fusion proteins GST-Max1, GST-Max1-6A, GST-Max1-55A and GST-Max1-75A.

The different GST-Max1 fusion proteins purified from *E. coli* were incubated with the kinases. In Fig. 38A can be observed how Cdc2 phosphorylates Max1, and also the different mutants. However, phosphorylation of mutant Max1-6A is extremely reduced when compared to WT. From Fig. 38B we could discard that the complex Cd2/Cig2 was specifically phosphorylating Max1, since there was not an increase in the signal in the assay with the kinase purified from HU treated cultures. The other CDK complex assayed, Cdc2/Cdc13 (Fig. 38C), phosphorylated Max1, but the signal corresponding to the phosphorylation of Max1-6A was again reduced compared to wild type.

We concluded that, at least *in vitro*, the CDK complex Cdc2/Cdc13 could phosphorylate Max1 in serine 6. The implications of such a phosphorylation were very interesting since it still remains completely unknown how MBF is activated at the end of M phase. If Max1 was phosphorylated by Cdc2/Cdc13 *in vivo*, then this phosphorylation could be the switch ON of MBF activity: MBF would switch from a highly repressed state in G2, to an active complex in M phase by the phosphorylation (by the CDK) of the repressor Max1. Thus, MBF would be activated by a derepression.

In vivo phosphorylation of Max1 by CDK

To verify whether this phoshorylation does occur *in vivo*, we obtained CDK-mutants of Max1 performing site-directed mutagenesis. We obtained mutations to alanine (S/T to A) to obtain unphosphorilable forms of Max1, and mutations to aspartic (S/T to D) to mimic constitutively phosphorylated forms of the protein.

We started analyzing if we could detect a mobility shift when analyzing Max1 in SDS/PAGE. Since we were interested in verify if the complex Cdc2/Cig13 phosphorylates Max1 in serine 6 in mitosis, we synchronized cells in anaphase. To achieve this block in cell cycle, we used a *cdc13*Δ90 background. When thiamine is washed from the medium, cells get arrested in anaphase, with a constitutively active Cdc2/Cdc13 complex (see page 72). We constructed the strains *cdc13*Δ90, Max1-6A *cdc13*Δ90 and Max1-6D *cdc13*Δ90.

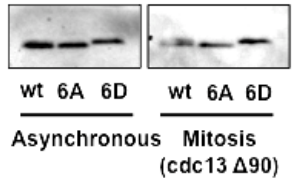


Figure 39. Electrophoretic mobility of Max1 CDK-mutants. Max1-Myc, Max1-6A-Myc and Max1-6D-Myc. Native extracts from asynchronous cultures and from anaphase blocked cultures (*cdc13*Δ90 strains, collected after 17 hours of growth without thiamine) were analyzed by western blot to check Max1 mobility.

Figure 39 shows that in asynchronous cultures (left panel) there is not any difference between Max1 and Max1-6A. However, Max1 migrates in the gel as a doublet in anaphase arrested cells (right panel), whereas this doublet is not present in the mutant 6A. Moreover, mutant 6D migrates as a slower migrating band (maybe due to a conformational change). This doublet might correspond to a phosphorylation that occurs in mitosis, and that disappears in the unphosphorilable version of Max1, Max1-6A.

To confirm the hypothesis that Cdc2/Cdc13 phosphorylates Max1 in serine 6, we used HU to block cells in S phase. Since we already knew that in HU-treated cells Max1 is highly

phosphorylated by the checkpoint response, we used the Max1-SATA mutant to get rid of the phosphorylation shift. We wanted to check if, once abrogated the checkpoint dependent shift, there was still some cell cycle regulated phosphorylation in serine 6.

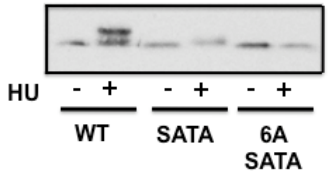


Figure 40. Electrophoretic mobility of Max1 CDK-mutants. Max1-Myc, Max1-SATA-Myc and Max1-6ASATA-Myc were analyzed by western blot. Native extracts from asynchronous cultures and from HU (10 mM) treated cultures were analyzed by western blot to check Max1 mobility.

We compared the Cds1-mutant Max1SATA with the CDK-mutant Max1-6ASATA. As can be observed in Fig. 40, the Max1-SATA mutant treated with HU still runs on the SDS PAGE as a slower migrating form of the protein. We thought this shift could correspond to a phosphorylated form of the protein. In the Max1SATA-6A, the phosphorylation shift is abrogated.

One interesting observation about this experiment is that Cdc2/Cdc13 complex is not active in S phase. However, an arrest with HU is not a physiological condition for the cells, and it does not correspond to a real S phase arrest. However, to make sure that the shift we observed in the HU treated cells was due to a phosphorylation, and more concretely, to a Cdc2/Cdc13 dependent phosphorylation, we constructed strains with the different CDK activities impaired. First, we analyzed the phosphorylation shift in a Δ*cig2* background. Also, since Cdc13 is essential, we used a

temperature sensitive allele *cdc13-117*. When this ts strain grows at 25ºC, Cdc13 is active, but when cells are blocked at 36ºC it becomes inactive.

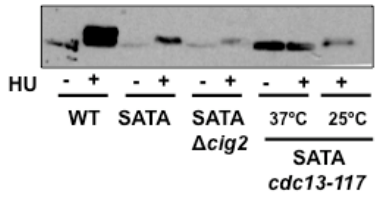


Figure 41. Electrophoretic mobility of Max1 CDK-mutants. Max1-Myc, Max1-SATA-Myc and Max1-SATA-Myc in Δc*ig2* and in *cdc13.117* backgrounds were analyzed by western blot. Native extracts from asynchronous cultures and from HU (10mM) treated cultures were analyzed to check Max1 mobility. *cdc13.117* strain was grown at 25ºC and then blocked at 36ºC for 4 hours, coinciding with the treatment with HU.

Deletion of Cig2 seemed not to have an effect on the phosphorylation of Max1. On the contrary, the shift of phosphorylation disappeared if Cdc13 was inactivated (37ºC), but not if it was active (25ºC).

We had previously reported that interaction between Max1 and MBF is lost in anaphase (Fig. 20) and we wandered if phosphorylation in serine 6 from the CDK complex Cdc2/Cdc13 was indeed the responsible of the loose of interaction. To test this hypothesis, we analyzed the co-immunoprecipitation of Max1-6A with Cdc10 in anaphase (Fig. 42).

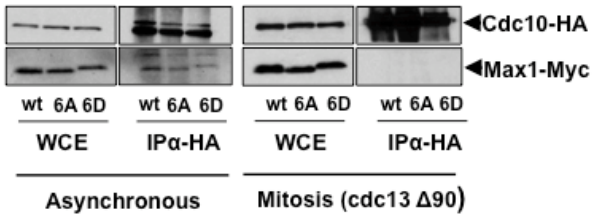


Figure 42. Max1-6A and Max1-6D binding to MBF. Extracts from strains expressing Max1-13Myc and Cdc10-HA were obtained from asynchronous cultures and from anaphase arrested cultures (*cdc13*Δ90 strains) in WT, Max1-6A and Max1-6D strains. Native extracts were immunoprecipitated (1mg) with α-HA antibody and proteins were detected by western blotting.

As can be inferred from Fig. 42, there were no differences between Max1 and the CDK-phosphorylation mutants (Max1-6A and Max1-6D). Both mutants interacted with Cdc10 in asynchronous cultures, but the interaction with MBF was lost, as in the WT strain, in anaphase. This meant that Max1 binding to Cdc10 was independent to the phosphorylation of Max1.

Effect on transcription of Max1 CDK-mutants

To characterize the role of the phosphorylation of Max1 in serine 6 by the CDK complex, we analyzed how was MBF regulation in cells which expressed the unphosphorylable Max1 mutant (6A). We extracted RNA from anaphase arrested cultures, and checked by northern blot *cdc18* mRNA levels. The mutant Max1-6A did not show any remarkable difference compared to wild type cells.

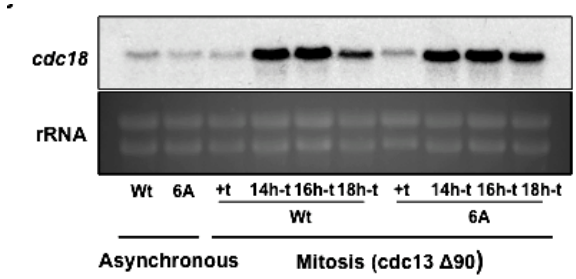


Figure 43. Transcriptional behaviour of Max1-6A CDK-mutant. Total RNA from anaphase arrested cultures (*cdc13⊿90* strains) from WT and Max1-6A strains was analyzed by Northern blot and hybridized with *cdc18* probe. Time after thiamine wash is indicated.

Since we did not observe any differences between WT Max1 and Max1-6A in regulation of transcription in mitosis, we decided to analyze MBF dependent transcription of different CDK mutants throughout the cell cycle. We constructed strains with the mutations S6A and S6D in a *cdc25-22* background.

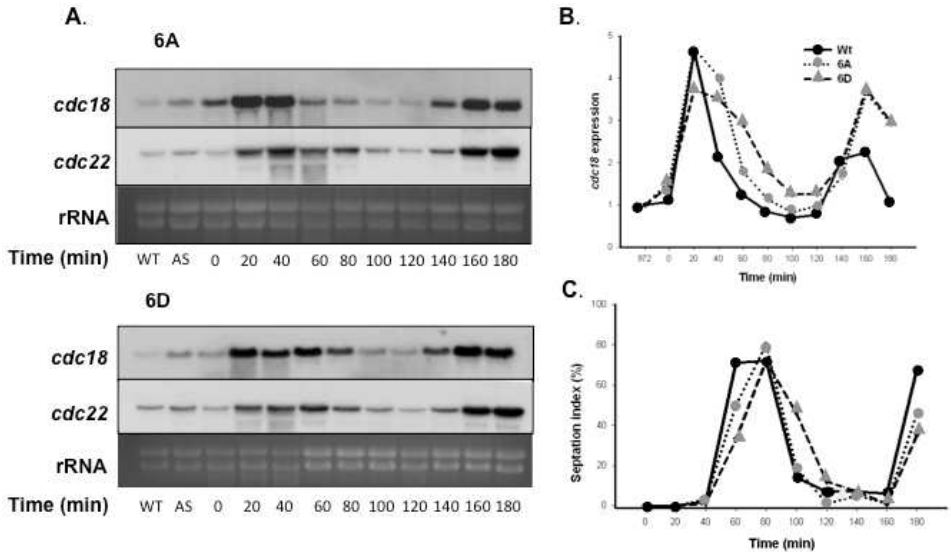


Figure 44. Transcriptional behaviour of Max1 CDK-mutants. A. Total RNA from *cdc25-22* synchronized cultures from WT, Max1-6A and Max1-6D strains was analyzed by Northern blot and hybridized with *cdc18* and *cdc22* probes. **B.** Quantification of the relative levels of *cdc18* expression was plotted relative to WT asynchronous expression. **C.** Septation index of the three strains was plotted to measure synchronicity.

As can be observed in Fig. 44, none of the mutations had any effect in transcription, cells showed minor differences compared to wild type in the periodicity of *cdc18* expression

To discard that the additional CDK consensus phosphorylation sites of Max1 (T55 and T75) could compensate the mutation in serine 6, we constructed a strain with the three mutations (S6A, T55A, T75A) and we analyzed its MBF transcriptional regulation. The triple mutant was as well wild type regarding *cdc18* expression (Fig. 45), with no defects on its induction neither on its repression.

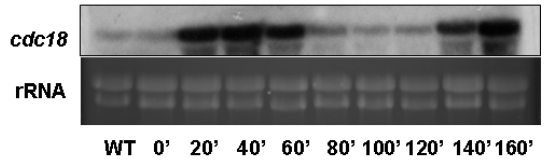


Figure 45. Transcriptional behaviour of Max1-6-55-75A. Total RNA from strain Max1-6-55-75A *cdc25-22* obtained from a synchronized culture was analyzed by Northern blot and hybridized with *cdc18* probe.

Although none of the mutants had an effect on MBF transcription, we checked how was Max1-6A binding to MBF dependent promoters (Fig. 45). There were some differences between Max1 and Max1-6A. Max1-6A was partly released from promoters at M phase, exactly as wild type Max1 did. Surprisingly, the differences were at the entry of Max1 to promoters during G2, which was partially impaired in the 6A mutant compared to wild type. The expected pattern of *cdc18* expression in the mutant 6A, raised from the ChIP experiments, would be an increased expression that we did not observe by northern blot.

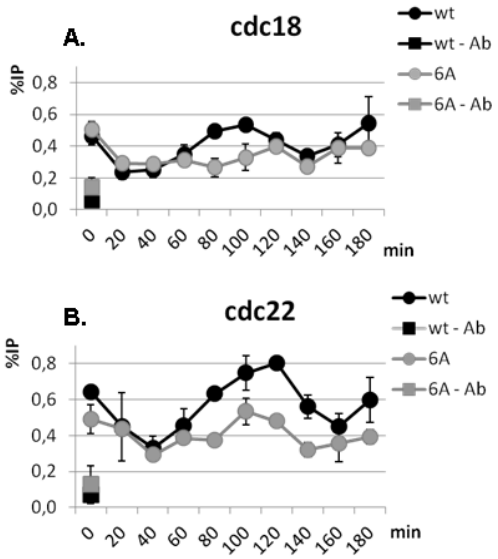


Figure 46. Max1 and Max1-6A promoter occupancy. Strains *cdc25-22* and Max1-6A *cdc25-22* were synchronized by block at 36ºC for 4 hours and release at 25ºC. ChIP data for occupancy on *cdc18* promoter (A) and *cdc22* promoter (B). Mean was obtained from three independent experiments and are expressed as mean ± SD.

All these data taken together indicate that Max1 might be phosphorylated in serine 6 by the CDK complex Cdc2/Cdc13. This phosphorylation might activate MBF transcription by inhibition of the repressor Max1. But we did not find any effect of such a phosphorylation on Max1. Since MBF is regulated at multiple levels, and phosphorylation of Max1 is not the only regulatory mechanism, we decided to investigate MBF regulation more deeply, and we started working with another repressor of MBF, Nrm1, to better understand the overlapping roles of both repressors.

Roles of Max1 and Nrm1

Max1 showed similarities to the other described repressor of MBF, Nrm1 regarding its role in the regulation of the transcription factor (de Bruin et al., 2006). To further investigate the overlapping roles of both repressors, we analyzed MBF dependent transcription of strains in which we deleted Max1, Nrm1 or both (Fig. 47). We realized that derepression was similar in both strains. In Δ*max1*Δ*nrm1* cells there was not a significant increase in the derepression. .

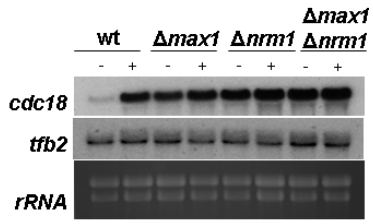


Figure 47. Regulation of MBF-dependent transcription by the repressors Max1 and Nrm1. Total RNA was prepared from untreated (-) or hydroxyurea-treated (+) cultures (3 hours at 30ºC) of wild type (wt), Δ*max1*, Δ*nrm1* and Δ*max1*Δ*nrm1* cells, and analyzed by hybridization to the probes indicated on the left. *tfb2* probe was used as a loading control.

Since we knew that both repressors were binding promoters with a similar periodicity (our data and Paper nrm1), we wanted to elucidate if they could be acting as heterodimers of a repressing complex. We tested by chromatin immunoprecipitation their binding to promoters. Surprisingly, in Δ*nrm1* cells, Max1 binding to promoters was abrogated (Fig. 48A). In Δ*max1* cells, on the contrary, Nrm1 was able to bind promoters normally (Fig. 48B). This result gave us an important clue regarding MBF regulation. Nrm1 seems to be necessary to load Max1 into the promoters. However, Nrm1 itself does not act as a repressor, since in Δ*max1*,

there is a derepression of transcription although Nrm1 is binding MBF.

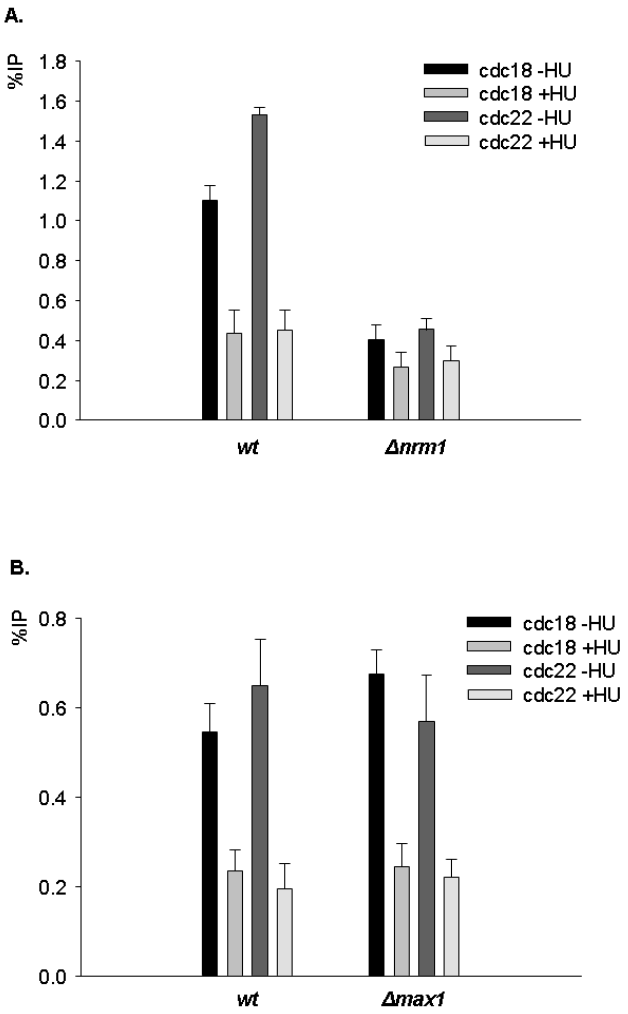


Figure 48. ChIP data for Max1 and Nrm1 occupancy at MBF genes promoters. A. Max1 occupancy at MBF promoters *cdc18* and *cdc22* was measured in WT and Δ*nrm1* strains, in the presence or absence of 10mM HU. **B.** Nrm1-HA occupancy at MBF promoters *cdc18* and *cdc22* was measured in WT and Δ*max1* strains, in the presence or absence of 10mM HU. Occupancy at MBF promoters was measured using α-Max1 and α-HA antibodies. Data was obtained from three independent experiments and are expressed as mean ± SD.

DISCUSSION

Identification of MBF interactors

Our main objective was to understand the regulation of the *S. pombe* transcription factor MBF. The mechanisms regulating the transcriptional program in G1 and S phases of mitotic cell cycle are highly conserved in yeasts and metazoans. The fact that E2F/DP, the transcription factor in metazoans, shows little homology to its functional homologues in yeasts emphasizes the importance of the regulation of the G1/S transcription.

We were able to succesfully purifiy MBF through affinity purification, and to indentify its main components using the iTRAQ labelling technology (Fig. 7 and 8). Among the putative interactors identified, we focused this work in Max1. However, there is a deeper analysis to be done. Other possible MBF regulators might be among the proteins that we purified with highest iTRAQ ratios, such as several chromatin remodelers (Set5 and FKBP), uncharacterized DNA-binding proteins (Nhp6 and SPBC28F2.11), or other proteins with no described function in *S. pombe* (Table II).

Also, we keep in mind the possibility to further use this technique to purify Cdc10 from extracts prepared from cells blocked at different phases of the cell cycle, to isolate specific activators and repressors and to better understand how MBF regulation is achieved through changes in the composition of the transcription factor. We are also interested in the functional characterization of MBF during meiosis, since the composition of the nuclear core of MBF also changes when *S. pombe* cells enter into meiosis (Ayte et al., 1997).

Characterization of Max1

In this thesis we describe a new MBF regulator, encoded by the *SPBC21B10.13C* gene, that we have named Max1 (MBF associated homeobox protein). The protein was described independently to our work (Aligianni et al., 2009), although they named it Yox1. The homology of Max1 to Yox1 in *S. cerevisiae* is little. Both proteins share a homeobox DNA binding domain, and both are transcribed by the MBF transcription factor, but there is not functional homology between both proteins. Yox1 in *S. cerevisiae* is a transcription factor that specifically binds to the ECB (early cell cycle box) elements in DNA, and regulates the transcription of the so called early cell cycle genes, which takes place in early G1. Transcription of the early cell cycle genes is MBF/SBF independent, and therefore Yox1 acts as independent transcription factor, and not as a regulatory subunit of MBF/SBF. The differences in the G1/S transcriptional regulation in both organisms are notable, and in *S. pombe* it depends exclusively on MBF. Because of the differences between Max1 and Yox1 in *S. cerevisiae* we decided to keep the name Max1.

We have shown that Max1 interacts with Cdc10 and requires the subunits Res1 and Res2 for the interaction (Figs. 9 and 10). Also, we have demonstrated that when it binds MBF it acts as a repressor, since the deletion of *max1* leads to a constitutive derepression of the MBF dependent transcription (Fig. 11 and 12). MBF is under several layers of control, with several repressing mechanisms described so far, like repression by the interaction of Nrm1 (de Bruin et al., 2006) and phosphorylation of Res1 by the CDK complex Cdc2/Cig2 (Ayte et al., 2001) . For Δ*nrm1* cells the

same constitutive depression was described (Fig. 47) (de Bruin et al., 2006). This gave us a hint that there is a connection between Max1 and Nrm1 roles, because deletion of one of them is enough for the complete loose of the periodic expression of MBF dependent genes, despite the presence of the other repressor. Regarding MBF repression by Cdc2/Cig2, this mechanism seems to have a role in a modulation of the gene expression, rather than a complete inhibition of transcription. It is interesting to notice that the three negative regulators, Max1, Nrm1 and Cig2, are themselves transcribed in a MBF dependent manner, so the cells ensure to shut down the MBF transcription by negative feedback loops.

It is clear that the maintenance of a periodic gene expression program is important, but deletion of *max1*, however, does not have severe consequences for cell viability. In Drosophila, studies of regulation of the two E2F complexes showed that deletion of both complexes does not affect cell viabilty neither normal cell division, although it leads to highly basal expresion of G1/S genes throughout the cell cycle. Therefore, periodicity in E2F dependent gene expression is not essential (Frolov et al., 2001). However, in mammals, RB mutations are frequently associated to cancer (Burkhart and Sage, 2008).

It is possible that Δ*max1* cells control the excess of G1/S transcripts by additional mechanisms. We actually did not check that increased levels of transcription corresponded to increased levels of the proteins coded by the mRNAs. The fact that deletion of *max1* leads to resistance to HU (Fig. 15), however, indicates that, at least in the case of *cdc22*, high levels of ribonucleotide

reductase (Cdc22), confere the resistance to HU. The same phenotype of partial resistance to HU was described for Δ*nrm1* cells (de Bruin et al., 2006).

We have reported that deletion of *max1* causes chromosome instability (Fig. 13), showing an increased rate (6-fold) of chromosome loss (0.35% of chromosome loss in Δ*max1* cells compared to 0.06% in wild type cells). Many different situations can lead cells to chromosome instability, like defects in chromosome segregation, DNA replication, spindle assembly and dynamics, cell-cycle regulation and mitotic checkpoint control, and mutations in more than 100 genes involved in all these processes have been reported to cause chromosomal instability in yeasts (Jallepalli and Lengauer, 2001). In the case of Δ*max1* cells, we hypothesize that there might be abnormalities during DNA replication because of the derepressed transcription of part of the replication machinery. However, we have not been able to demonstrate that Δ*max1* cells suffer defects in DNA replication.

Deletion of *max1* is not able to rescue the lethal phenotype of *cdc10-129* cells (Fig. 14), which have reduced transcription of MBF genes when growing at the restrictive temperature (36ºC). Overexpression of the DNA binding subunit Res1, however, was reported to rescue *cdc10.129* cells (Ayte et al., 1995). This would mean that Max1 repressing activity acts through Cdc10, and deletion of *max1* has not an effect in the absence of a functional Cdc10. Surprisingly, deletion of *max1* does recue the lethal phenotype of a different *cdc10* ts mutant, *cdc10-C4.* This mutant version of Cdc10 only lacks the amino part of the protein and when

growing at 36ºC, cells are arrested at START. Deletion of *max1* compensates partially this cell cycle arrest through an induction of transcription. This result indicates that the interaction between Cdc10 and Max1 is preserved in the C4 mutant.

We have shown, by western blot and by microscopy (Figs. 17, 18 and 19) that Max1 remains nuclear throughout the cell cycle, and that protein levels remain constant. However, its binding to MBF promoters is periodic (Fig. 21). Binding of Max1 to promoters is higher during G2, and coincides with the maximum repression of MBF. On the contrary, in M, G1 and S phases, when MBF genes are highly transcribed, Max1 is partially released from promoters, although not completely. Moreover, when we performed co-immunoprecipitations of Cdc10 and Max1 in mitosis (using anaphase arrested cells, Fig. 20), we confirmed that interaction between both proteins is lost in this phase of the cell cycle. Since protein levels remain constant throughout the cell cycle, we hypothesized that this periodicity might be achieved by a post-translational modification, such a phosphorylation.

Regulation of Max1 by the DNA synthesis checkpoint

We have demonstrated that the DNA synthesis checkpoint directly activates MBF dependent transcription through the phosphorylation of the repressor Max1. It has been previously reported that in mammalian cells there is a link between the DNA damage checkpoint and E2F/Retinoblastoma (Inoue et al., 2007; Stevens et al., 2003). In *S. pombe*, other two components of MBF have been recently described to be regulated by the DNA replication

checkpoint: Cdc10 (Dutta et al., 2008) and Nrm1 (de Bruin et al., 2008). The fact that the checkpoint machinery regulates MBF through three independent mechanisms enhances the robustness of the system, and indicates that induction of transcription is an important part of the checkpoint response.

The fact that in response to HU treatment there was an induction of MBF transcription (Fig. 11) was known for a long time, and it was attributed to a cell cycle arrest in S phase (HU induced), where MBF genes are thought to be actively transcribed. However, the recent findings of a direct regulation MBF through the checkpoint response indicate that induction of transcription upon HU treatment is not a consequence of the cell cycle arrest, but a checkpoint-mediated activation of transcription.

Part of the DNA replication checkpoint response consists in transcriptional induction of genes required for DNA synthesis and DNA repair. This is a conserved mechanism from prokaryotes (SOS response) to eukaryotes. In *S. cerevisiae* there is a well characterized transcriptional response, that involves the kinase Dun1 (Zhou and Elledge, 1993). However, it remained unclear how this induction of transcription is achieved in *S. pombe* and in metazoans. The transcriptional response in the different organisms includes transcription of genes required for DNA repair, and also genes required for DNA replication, since arrested replication must restart once cells overcome the damage, and part of the replication machinery must be synthesized *de novo*.

The mechanism by which phosphorylation of Max1 by the checkpoint induces transcription seems to be because of an

release of Max1 from the MBF complex, thus causing a derepression, what we have corroborated by co-immunopreciptation and by ChIP experiments (Fig. 25, 26 and 27). The proposed model is represented in Fig. 49.

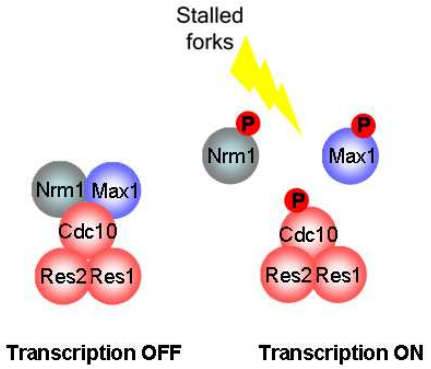


Figure 49. Model for the regulation of transcription in response to replicative stress.

We mutated the phosphorylation sites of Max1, and obtained an unphosphorilable form of the protein, Max1- SATA. As expected, the SATA mutant is not able to induce transcription upon HU treatment (Fig. 29). This was a remarkable finding, since the mutations of the other two substrates of the checkpoint, Nrm1 and Cdc10, to unphosphorilable forms of the proteins (Nrm1-8A and Cdc10-8A), were able to induce MBF transcription upon HU treatment (de Bruin et al., 2008; Dutta et al., 2008).

It is not clear whether Cdc10 is a direct substrate of the checkpoint. In the work of the Rhind Lab (Dutta et al., 2008) they show that mutations that mimic a checkpoint constitutive phosphorylated Cdc10, mutant *cdc10-2E*, shows checkpoint-induced levels of transcription in untreated conditions. However, no phenotype was observed for the unphosphorilable mutant, cdc10-8A, which is able

to induce transcription upon treated conditions. One explanation could be that cdc10-2E shows upregulation of transcription not because mimicking phosphorylation, but because of a conformational change that impairs proper binding of the repressors, either Nrm1 or Max1.

The fact that the SATA cells, although not being able to induce transcription upon HU damage, are not sensitive to the drug (Fig. 33, 34) has several explanations. One is that *S. pombe* cells spend most of the time in G2 phase of the cell cycle, which could compensate the lack of induction of transcription and prevent the aberrant mitosis. We show that in *wee1-50* cells, which have a short G2 phase, indeed *wee1-50* SATA cells are more sensitive to HU (Fig. 36) than *wee1-50* cells.

Also, SATA cells have an impaired transcriptional induction, but they have the rest of the checkpoint response completely preserved. They are able to stabilize replication forks, to prevent aberrant mitosis by arresting the cell cycle, and to prevent firing of replication origins. There are evidences in *S. cerevisiae* that the transcriptional response is not essential for survival (Tercero et al., 2003). This explains why only cells with a completely disrupted checkpoint pathway (Δ*rad3* or Δ*cds1* cells) are highly sensitive to HU. We provide here a new evidence that transcriptional response might not be an essential component of the checkpoint response.

Our proposed model of regulation, in which Max1 is phosphorylated by Cds1 in serine 114 and threonine 115 upon replicative stress, and as a consequence of the phosphorylation, is evicted from MBF promoters is however more complex. Our ChIP

data (Fig. 32B) shows unexpectedly that Max1-SATA is partially evicted from promoters upon HU treatment despite not being phosphorylated. On the contrary, Cdc10 is also partially evicted from promoters in the Max1-SATA cells, what could account for the lack of induction of MBF-dependent genes observed in these cells. This eviction of Cdc10 is a surprising result, because there is not any reported situation in which Cdc10 is released from MBF promoters. This release of Cdc10 in the SATA mutant is not checkpoint-dependent, since Cdc10 is not evicted in wild type cells. We hypothesize that phosphorylation of Max1 in serine 114 and threonine 115 might be reducing the affinity of Max1 for Cdc10. On the contrary, Max1-SATA maintains a high-affinity state of interaction with Cdc10, that provokes an eviction of Cdc10, together with Max1-SATA, from promoters, impairing the transcriptional response (Fig. 29.). This also would explain why both proteins interact in the immunoprecipitation experiments (Fig. 31).

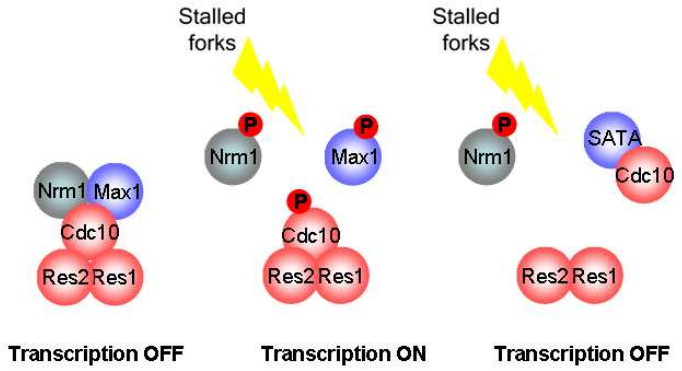


Figure 50. Model for the regulation of transcription in response to replicative stress in the SATA mutant.

Regulation of Max1 by CDK

The G1/S transcriptional program in mammals and in *S. cerevisiae* shows a common pattern of activation (Schaefer and Breeden, 2004), in which transcription factors E2F/DP and SBF are activated by the phosphorylation of a repressor. In *S. pombe*, however, it has not been described the mechanism that activates MBF, and we hypothesized whether Max1 could be the repressor that switches ON transcription.

We have shown that Max1 is released from MBF complex in anaphase: we show how the interaction with the complex is lost by co-immunoprecipitation (Fig. 20), and also we show how Max1 is periodically released from promoters (Fig. 21).

To prove our hypothesis that this release could be cell cycle regulated by the mitotic CDK complex Cdc2/Cdc13, we generated CDK-phosphoryation mutants. We have evidences that phosphorylation in serine 6 by CDK complex might occur, *in vitro* and *in vivo* (Fig. 38, 39, 40, 41). However, we have not been able to see an effect of such a phosphorylation on MBF regulation. Release of Max1 from promoters seems not to depend on a specific CDK phosphorylation, because our phosphorylation mutant Max1-6A is released from promoters as the wild type protein (Fig. 42 and 46). Moreover, Max1-6A cells do not have any defects on the regulation of transcription (Fig.43 and 44). We can not discard that Max1 might be phosphorylated in alternative phosphorylation sites, despite the mutation to alanine of the three consensus sites (6, 55, 75), does not have an effect on transcription (Fig. 45). Since MBF is a highly regulated complex, it is possible that

punctual mutations in one of the regulators, although might be affecting the function of this regulator in particular (ChIP experiment of the 6A mutant), might not have any effect on the global function of MBF (northern blot of the 6A mutant). Other regulators would keep MBF properly functional, or compensating mechanisms such as feedback loops would be activated. This is another example of the robustness of the MBF regulation: a system governed by multiple mechanisms that ensure the function even if some components fail.

In this thesis we also provide evidences that Nrm1 is not strictly a repressor of MBF, but rather a co-repressor together with Max1. Nrm1 is necessary to load Max1 into promoters. If *nrm1* is deleted, Max1 is not bound to promoters and MBF transcription is derepressed. However, in the absence of Max1, transcription is also derepressed despite Nrm1 is still binding MBF.

A better understanding of how MBF is activated in mitosis might raise from this new model of regulation that we propose, in which Nrm1 is necessary for the binding of the repressor Max1. CDK complex Cdc2/Cdc13 is possibly phosphorylating Max1 in serine 6, but a direct regulation directed to Nrm1 might also occur. If Nrm1 is released from promoters in mitosis because of an independent mechanism, then Max1 would be released from promoters as well, in a phosphorylation independent manner, and transcription would be activated (Fig. 51).

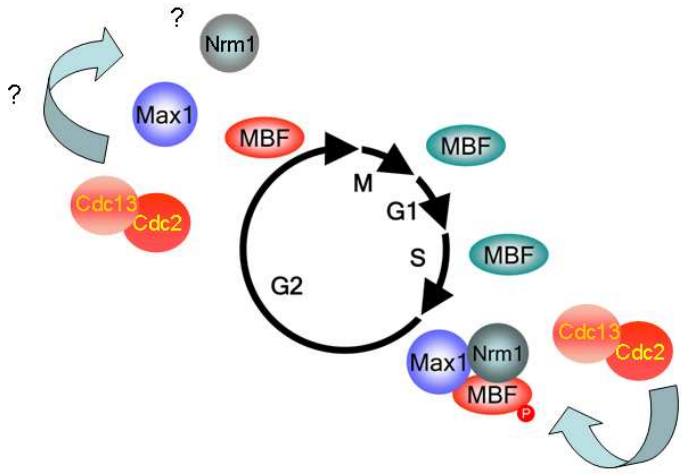


Figure 51. Model of regulation of MBF by its repressors.

Future experiments will be necessary to better understand the regulation of MBF by the different mechanisms, and particularly, to understand how activation of MBF in anaphase is achieved.

CONCLUSIONS

1. **Max1 interacts with the MBF transcription factor, and this interaction requires an intact MBF complex.**

2. **Max1 is a negative regulator of MBF. Deletion of *max1* leads to constitutive expression of MBF-dependent genes. Oscillations in the binding of Max1 to MBF promoters correlate with modulation in the expression of MBF genes.**

3. **Δ*max1* cells are partially resistant to HU.**

4. **Δ*max1* cells show genomic instability.**

5. **Max1 is phosphorylated in response to replicative stress. Upon HU treatment, the replication checkpoint kinase Cds1 phosphorylates Max1 in residues serine 114 and threonine 115.**

6. **Phosphorylation of Max1 abrogates its binding to MBF. Max1 is evicted from MBF promoters upon its phosphorylation.**

7. **The DNA replication checkpoint directly regulates MBF dependent transcription through Max1. Induction of transcription of MBF genes upon replicative stress depends on Max1 phosphorylation by the checkpoint.**

MATERIALS AND METHODS

Strains. All *S. pombe* straits are isogenic to wild-type 972h-. Media were prepared as previously described (Moreno et al., 1991). Hydroxyurea (HU) treatment (10mM) was carried out with midlog grown cells (3-4 x 10^6 cells ml $^{-1}$ cells), treated for three hours at 30ºC.

Cell Synchronization. Temperature-sensitive strains *cdc25-22* were cultured at the permissive temperature (25ºC) in a water shaker (INFORS HT) until mid log phase (3-4 x 10^6 cells ml $^{-1}$) before shifting to non-permisive temperature (36ºC) for 4 h as described. Synchronicity was messured by septation index using 4',6'-diamidino-2-phenylindole (DAPI) staining. For Δ*max1cdc25-22* experiments, a background Δ*mik1* was necessary since Δ*max1cdc25-22* were not viable at 25ºC. As a wild type control in this experiment, a Δ*mik1cdc25.22* strain was used.

Protein extraction and immunoprecipitation. Extracts were prepared in NET-N buffer (20mM Tris HCl pH 8.0, 100mM NaCl, 1mM EDTA, 0,5% NP40, 1mM dithiothreitol (DTT), 1mM phenylmethyl sulphonyl fluoride (PMSF), 5 μgml^{-1} aprotinin, protease inhibitor cocktail (Sigma, used as described by manufacturer), 2mM sodium fluoride (NaF), 0,2mM sodium orthovanadate (Na_3VO_4), 2mM β-glycerophosphate). Cells were broken with glass beads in a BioSpec Minibeadbeater. Immunoprecipitations (1 to 3 mg of whole-cell lysate) were performed with 10 μl of prot. G separose and 100 μl of tissue culture supernatant from the monoclonal hybridoma (HA or Myc). For HA immunoprecipitations, antibody was previously crosslinked to protein G separose. Immunoprecipitates were washed after 1

hour of incubation three times with the same buffer and resolved in 8%SDS-PAGE, transferred to nitrocellulose membranes and blotted with the indicated antibody.

Affinity purification and iTRAQ analysis. Max1 was isolated by affinity purification followed by mass spectrometry (AP/MS). Total protein extracts of two different strains (972-no tag- and JA242 -Cdc10HA-) were prepared from 30litres of asynchronous midlog grown cultures. Cells were frozen and then broken in a Retsch RM100 mortar grinder. Cell lysates were resuspeneded in 100 ml of NET-N buffer (described above) and centrifuged 5' at 3500rpm. Supernatant was collected and centrifuged in a Beckman centrifuge 40' at 14000rpm. Protein concentration was quantified by Bradford. 1,5 g of total protein of each strain were precleared to allow unspecific binding by incubation 1 hour at 4ºC with protein G-sepharose crosslinked to Myc antibody. Precleared supernatants were incubated 4 hours at 4ºC with protein G-sepharose crosslinked to HA antibody. Immunoprecipitates were washed 4 times in Bio-Rad Poly-prep Chromatography Columns with 5 ml of NET-N buffer, and eluted from columns with 5 washes of 1 ml of glycine pH 2. pH of the eluted fractions was neutralized with 1M TrisHCl pH 8.8. The presence of Cdc10 in eluates was checked by Western Blot and 1/5 of the selected eluate was loaded on a 12%SDS-PAGE followed by silver staining to compare the specificity of purification in both strains. The rest of the sample was dialyzed O/N with 20mM NH_4HCO_3 buffer using Spectra/Por dialysis membranes (Spectrum laboratories), and then lyophilized. Samples were analyzed by M/S and an ITRAQ labeling was

performed (as described by manufacturer) at the Proteomics Facility of the Universidad Complutense de Madrid.

Gene expression analysis. RNA extraction was performed as described (Moldon et al., 2008) and 10 µg of extracted RNA were loaded. *cdc18*, *cig2*, *tfb2*, and *his3* probes contained the complete ORFs of the genes.

Fluorescence microscopy. Samples of 1ml from 5 ml of exponentially growing yeast cultures were concentrated in 25µl, and 2ul were loaded on poly L-lysine-coated multiwell slides (the remaining suspension was immediately withdrawn by aspiration). Fluorescence microscopy was performed on a Nikon Eclipse 90i microscope at 100X magnification. Images were captured with an Orca II Dual Scan Cooled CCD camera (Hamamatsu), using Metamorph 7.1.2 software. Time lapse experiments were performed at the Microscopy Facility of the Universidad de Salamanca, with the technical advice of Dr. Pilar Pérez, using a Nikon Eclipse micorsocpe and Metamorph software. Images were processed with Image J software.

Flow Citometry. 1ml of Sodium Citrate (50 mM, pH 7) was added to 100µl of 70%EtOH fixed cells. 0.5 ml of Sodium Citrate (50mM, pH7) with 50 mg/ml of RNAse were added. Cells were incubated O/N with Rnase at 37ºC. 0.5 ml of Sodium Citrate with propodium iodide were added. Cells were vortexed and sonicated.

Chromatin Immunoprecipitation. ChIP experiments were performed as described (Moldon et al., 2008). All the experiments were plotted as the average of at least three different biological replicates ± SD

Liquid cultures. For survival on solid plates, *S. pombe* strains were grown in liquid YE5S medium to an optical density at 600 nm (OD600) of 0.5. Cells were then diluted in water, and 10 to 105 cells per dot in a final volume of 3 µl (metal replica plater) were spotted onto rich medium plates containing (or not) the indicated drugs. The spots were allowed to dry, and the plates were incubated at 30℃ for 2 to 4 days. To determine survival in liquid cultures, cells were grown in YE5S to an OD600 of 0.5. HU was added at time 0.

***In vitro* kinase assay.** Substrates were prepared as GST fusion proteins in *E. coli* as described (Ayte et al., 1997). Protein extracts (300 µg) from asynchronous cultures of strains with HA-tagged Cdc2 or GFP-tagged Cdc13 were immunoprecipitated as described above. Protein extracts (300 µg) from HU synchronous cultures of Cig2-HA strain were immunoprecipitated as described above. Immunoprecipitation was followed by three washes with NET-N buffer and one wash with kinase buffer (10mM Hepes pH7.5, 20mM $MgCl_2$, 4mM EGTA, 2mM DTT). Immunoprecipitates were incubated in kinase buffer containing 2µg of substrate and 10µCi of

[γ-^{32}P]ATP for 30 min at 30℃. Labeled proteins were resolved in 11% SDS-PAGE and detected by autoradiography.

Table of strains used in this work:

Strain	Genotype
972	h-
JA 37	cdc25-22 leu1-32, h-
PN663	wee1-50, leu1-32 h-
JA 242	cdc10-HA Kan+ leu1-32 h-
KGY629	cdc13-GFP in 972 h- background h-
JA 777	max1-13Myc-kan h+
JA 781	max1-GFP-kan h+
JA 783	cdc25-22 max1-GFP-kan leu1-32 h?
JA 784	max1-13Myc-kan cdc10-3HA-Nat leu1-32 h-
JA 793	cig2::ura4 max1-13My leu1-32
JA794	cdc13-117 max1-13Myc-kan
JA 802	max1-13Myc::ura4 ura4-D18 h+
JA 803	cdc10-C4 h+
JA 805	cds1::kan h-
JA 810	max1-13Myc-Nat cds1::kan ura4-D18 leu1-32 h+
JA 811	max1-13Myc-Nat rad3::kan ura4-D18 leu1-32 adeM-210 h?
JA 934	max1-13Myc-kan cdc10-3HA-Nat res2::ura4 ura4-D18 h?
JA 940	cdc25-22 mik1::kan max1::ura leu1-32 ura4-D18 h?
JA 941	cdc25-22 mik1::kan leu1-32 h?
JA 944	max1-13Myc-kan cdc10-3HA-Nat res1::ura4 ura4-D18 leu1-32 h?
JA 947	max1S6A-13Myc-kan h+
JA 948	max1S6D-13Myc-kan h+
JA 949	max1S114A-13Myc-kan h+
JA 956	max1-13Myc-Nat::ura4 cds1::kan ura4-D18 h+
JA 957	max1::kan cdc10-C4 ade6-M216 leu1-32 h?
HM6118	cdc2-L7□□cdc2-HA (ura4+) ura4-D18 leu1-32 h-
PN10633	tos4-GFP-kan+ ade6M210 h-
JA 973	max1S115A-13Myc-kan h?
JA 974	max1S114-115A-13Myc-kan h?
JA 975	maxS6A-S114.115A-13Myc-kan ura4-D18 h+
JA 977	max1-13Myc-kan nrm1-HA-Nat h+
JA 978	max1S114A-13Myc-kan nrm1-HA-Nat h+
JA 982	max1S114-115A-13Myc-kan cdc10-3HA-Nat leu1-32? h?
JA 983	max1-6A (no tag) ura4-D18 h+
JA 988	cdc25.22 max16A (no tag) h-
JA 994	wee1-50 max1::kan ura- leu1-32 h-
JA 995	wee1-50 max16A-114A-115A-13Myc-kan ura- leu- h-
JA190	cdc10-129 leu1-32 h-
JA 945	cdc10-129 max1::kan leu1-32 h-
HM1109	ade6-210 Ch16 h-
JA 1003	max1-13Myc::ura4 ura4-D18 ade6-210 Ch16 h-
JA 1005	max1-13Myc-kan cdc10-3HA-kan^{+} nmt44-cdc13Δ90 sup 3-5 ade6-704 h?
JA 1006	max1-6A-13Myc-kan cdc10-3HA-kan^{+} nmt44-cdc13Δ90 sup 3-5 ade6-704 ura4-D18 h-
JA 1007	max1-6D-13Myc-kan cdc10-3HA-kan^{+} nmt44-cdc13Δ90 sup 3-5 ade6-704 ura4-D18 h-

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APPENDIX

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