The DNA-damage and the DNA-synthesis checkpoints converge at the MBF transcription factor

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SUMMARY

Summary

DNA damage is an ongoing threat to both the ability of the cell to faithfully transmit genetic information to its offspring as well as to its own survival; thus, cells require a mechanism that allows them to stop cell cycle progression before fixing any problem. 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 (DDR). In fission yeast the two DDR pathways converge at the regulation of single transcriptional factor complex (MBF) resulting in opposite directions. When DNA replication is challenged, the effector kinase Cds1 is activated and inhibits M phase entry through inactivation of the phosphatase Cdc25, stabilizes stalled replication forks to prevent deleterious DNA structures and triggers transcriptional activation of S-phase genes. MBF is the complex controlling the transcription of genes required for the S phase and Max1/Yox1, a homeodomain-containing protein, binds and represses MBF-dependent transcription at the end of S phase in a cell cycle-regulated manner. We have shown that when the DNA-synthesis checkpoint is activated, Yox1 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 the replication challenge. In contrast, upon DNA damage, Chk1 the effector kinase of the DNA damage

checkpoint is activated and blocks the cell cycle progression, inducing DNA repair and repressing the MBF dependent transcription. We have revealed that Cdc10 is the target of the DNA-damage checkpoint and when cells are treated with the alkylating agent MMS or are exposed to IR, Chk1 phosphorylates Cdc10 inducing the exit of MBF from chromatin. The consequence is that under these conditions, MBF-dependent transcription is repressed. Thus, Yox1 and Cdc10 couple normal cell cycle regulation and the DNA-synthesis and DNA-damage checkpoints into MBF.

Resumen

El daño al ADN es una amenaza permanente en la célula que puede afectar a la fidelidad en la transmisión de su información genética a sus descendientes así como a su propia supervivencia; es por esto que las células requieren mecanismos que les permitan parar su ciclo celular para dedicarse a solucionar este tipo de daños. Para mantener su integridad genómica, los organismos eucariotas han desarrollado un mecanismo, muy conservado, que les permite detectar, señalizar y reparar el daño en el DNA, conocido como respuesta de daño a ADN (DDR). En la levadura de fisión hay dos DDRs que convergen para regular la actividad de MBF, un complejo transcripcional esencial para la progresión en el ciclo celular. Cuando la replicación del ADN está afectada, la kinasa efectora Cds1 se activa e inhibe la entrada en fase M a través de la inactivación de la fosfatasa Cdc25, estabiliza las bloqueadas horquillas de replicación para prevenir estructuras de ADN perjudiciales y activa la transcripción de los genes de fase S. MBF es el complejo que controla la transcripción de genes requeridos para el comienzo de la fase S y Max1/Yox1, una homeoproteina, se une al complejo y reprime la transcripción dependiente de MBF, al final de la fase S, de una manera regulada por el ciclo celular. Nosotros hemos demostrado que cuando el punto de control de síntesis de ADN se activa, Yox1 se fosforila por Cds1 y se libera de MBF. Como consecuencia, la transcripción de MBF se mantiene activa

hasta que las células son capaces de superar los problemas de replicación. Por el contrario, después de un daño en el ADN, se activa el punto de control de daño a ADN y se bloquea la progresión en el ciclo celular, se induce su reparación y se reprime la transcripción dependiente de MBF. Nosotros hemos demostrado que Cdc10 es el objetivo del punto de control de daño a ADN y cuando las células son tratadas con el agente alquilante MMS o son expuestas a IR, Chk1 fosforila a Cdc10 induciendo la salida de MBF de la cromatina. La consecuencia es que, bajo estas condiciones de estrés, la transcripción dependiente de MBF se reprime. Así, Yox1 y Cdc10 aúnan la regulación normal del ciclo celular y los puntos de regulación de síntesis de ADN y de daño a ADN en un único complejo transcripcional.

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INTRODUCTION

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 can proliferate in a haploid state. Therefore it has one single copy of the genome, which facilitates simple gene function analysis working with mutations and deletions.

A major reason for using *S. pombe* to study fundamental biological problems is to exploit the acquired knowledge to understand more complex organisms, especially humans. In many cases, molecular processes are so complicated in higher eukaryotes that it is impossible to unravel and understand them, without prior knowledge from more simple systems. In contrast, in yeast, the relatively simplicity of processes allows their characterization, with this information permitting subsequent analysis and understanding in higher eukaryotes.

S. pombe 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 easily

identifying 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 precise regulatory machinery that ensures that the events of the cell cycle occur in the correct order. How a cell duplicates and divides is a major area of interest, not only because the process is so incredibly accurate and complex, but also because defects in the process are the basis for many human diseases. The main events to be regulated are the duplication of genetic content and the distribution of those components into two identical daughter cells. Traditionally, the eukaryotic mitotic cell cycle is divided into four separate, consecutive, and distinct phases: S phase (where DNA replication occurs), M phase (where the chromosomes separate), and two gap phases G1 and G2. Gap phases are important for cell cycle regulation and contain key events to control the progression to the next phase.

From simple yeast to higher eukaryotes including humans, it has been revealed that the cell division cycle is controlled in many ways and at different levels. As a generalization, this complexity of control seems to be to ensure that cell division occurs in a highly reproducible and accurate way, with multiple levels of controls introducing `double check ` and `fail - safe` processes. The various types of control mechanisms include changes in protein activity through posttranslational modification (such as phosphorylation), changes in protein stability (in some cases through specific, targeted degradation), and changes in

protein distribution. Regulation of gene transcription is another important layer of control.

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.

Cell growth must also be regulated, to maintain the proper cell size. All the steps of regulation take place at particular moments of the cell cycle named checkpoints. When any trouble in the accomplishment of one of the phases of the cycle is detected in a checkpoint control, 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 short G1 phase, S phase where DNA is replicated, a long G2 phase where cells grow by length extension, followed by a rapid M phase where chromosomes are segregated. Mitosis is followed by formation of the septum at a central position in the cell, but this is a slow process that does not occur immediately 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.

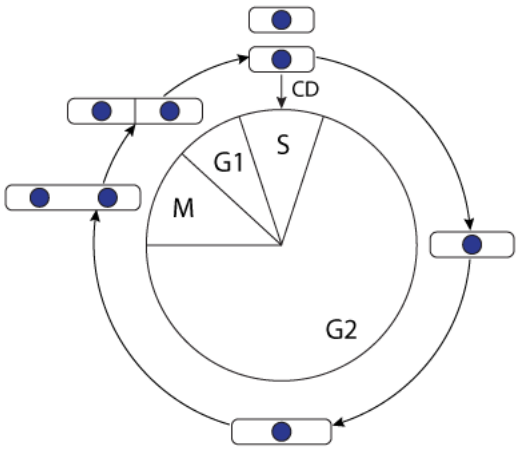

Fig 1. | The fission yeast cell cycle (Image from The CellLMProject)

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.

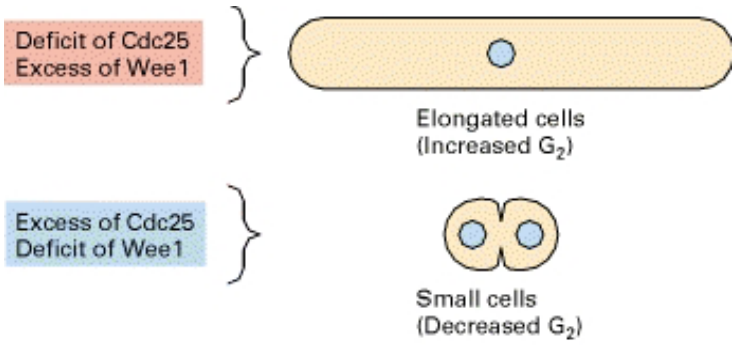

Fig 2. | Schematic representation of the *cdc* and the *wee* phenotypes (From Molecular Cell Biology, Lodish, Darnell et al.).

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 1994; 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 1994; Mondesert et al. 1996). 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 which are inhibited when phosphorylated by the complex, like Cdc18, which is part of the replication machinery (Lopez-Girona et al. 1998) and Res1, a component of the MBF complex (Ayte et al. 1995).

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 1994).

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; Coudreuse and Nurse 2010). 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 the Cdc2/Cdc13 complex (Broek et al. 1991; Fisher and Nurse 1996).

2.3. 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 nutrient levels are limited. In that case, they exit the vegetative cycle and enter into sexual cycle. In addition, in 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 in higher eukaryotes can lead to a misregulation in cell proliferation and is frequently associated to cancer (Pardee 1989)**.**

2.4. DNA replication and S phase

Chromosome duplication occurs in 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 (pre-RC) 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 committed to enter a new cell cycle at START, and CDK activity is required for this step.

The first step in forming pre-RC 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 (Stillman 1993; Antequera 2004). Then, other proteins of the pre-RC 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, and is preloaded in the pre-RC 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 when 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.

2.5. 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 identifying the main regulators of this transition. It is a mechanism based on regulatory phosphorylations that are conserved in higher eukaryotes.

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 for the inactivating phosphorylations of Cdc2 are Wee1 and Mik1, with redundant activities. The active state of Cdc2/Cdc13 is reached by means of dephosphorylation of Tyr-15 by the phosphatase Cdc25 (Russell and Nurse 1986; Millar et al. 1991).

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 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.

3. Transcriptional program in fission yeast

In fission yeast, four main waves of gene expression have been described. These fall across various phases of the cell cycle at G1/S, S phase, G2/M, and M/G1.

1. G1/S wave

The G1/S wave was the first to be identified in this organism and has been the most well characterized. It contains up to 20 genes whose encoded products have roles in DNA synthesis and cell cycle controls. All the genes contain MCB (MluI Cell Cycle Box) sequence motifs in their promoters, which is bound by a transcription factor complex called MBF (originally DSC1- from DNA Synthesis Control 1), with several components identified so far (Lowndes et al. 1992).

2. S-phase wave

A number of genes encoding histones are periodically expressed in fission yeast during DNA synthesis (Matsumoto and Yanagida 1985), with a promoter motif that is bound by the transcription factor Ams2, that positively regulate their expression (Takayama and Takahashi 2007)). Furthermore, a repressor named Hip1 has been characterized (Blackwell et al. 2004).

3. G2/M wave

Despite G2 contributes to a significant proportion of the mitotic cell cycle in fission yeast, and the G2/M transition being the major control point, only a smaller group of weakly induced genes at this cell cycle phase have been identified. These include *spd1*, *psu1*, and *rds1*, with a putative common promoter UAS (Rustici et al. 2004). This UAS has neither been confirmed experimentally, nor has the transcription factor that binds to it been identified.

4. M/G1 wave

An important wave of transcription occurs in fission yeast at the M/G1 interval. This group of genes numbers at least 20 genes, with the first identified being *cdc15* (Fankhauser et al. 1995; Anderson et al. 2002; Rustici et al. 2004). Most genes encode products required for processes at the end of the cell cycle, such as chromosome separation, cytokinesis, and septation. The promoter sequences and transcription factors required for their expression have been identified, named pombe cell cycle boxes (PCBs) and PCB-binding factor (PBF), respectively (Anderson et al. 2002). At least three components of PBF have been identified, and these include two forkhead-like transcription factors, Fkh2 and Sep1, and a MADS box-like protein, Mbx1 (Zilahi et al. 2000; Buck et al. 2004; Bulmer et al. 2004; Szilagyi et al. 2005). The two forkhead transcription factors have complementary and opposing roles in regulating gene expression: Fkh2, which is only bound to PCB promoters

when M/G1 genes are not being expressed, appears to have a repressive role; in contrast, Sep1 is only bound when genes are being expressed, and thus has a positive role (Papadopoulou et al. 2008).

3.1. Transcriptional program during G1/S

3.1.1. *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 multisubunit transcription factor comprised by Cdc10, Res1, Res2, and few other regulatory components. MBF mediates G1/S specific transcription of genes required directly or indirectly for DNA synthesis and S phase. A group of about 20 genes is known to be under MBF control. Among them are: *cdc22* (ribonucleotide reductase) (Lowndes et al. 1992), *cig2* (S phase cyclin) (Ayte et al. 2001), *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 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 three major MBF components: Cdc10, Res1 and Res2 have constant protein levels over the cell cycle (Simanis and Nurse 1989; Whitehall et al. 1999). Also, the MBF and more particularly Cdc10, has been found bound to its target promoters throughout the cell cycle (Wuarin et al. 2002), implicating that regulation of MBF dependent transcription is not achieved simply by modulating the DNA binding activity of the complex. It is still not completely clear how the complex is activated at M phase and inactivated at the end of S phase, and how it remains inactive during G2, but so far there are evidences that MBF is regulated by posttranslational modifications and by other regulatory subunits.

Cdc10

Cdc10 is considered as the active 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 complex.

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). Cdc10 is similar to Swi6 of budding yeast, in peptide sequence, predicted structure, and also contains ankyrin motifs, suggesting a common ancestry for this class of transcription factor (Aves et al. 1985). The ankyrin repeats are motifs present in a large number of functionally diverse proteins and are considered sites for protein-protein interaction; this is a sequence of about 30 amino acids repeated four or more times, and 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 with other proteins) more than in direct interactions Cdc10/Res1/Res2 (Ayte et al. 1995; Ewaskow et al. 1998; Whitehall et al. 1999).

The C terminus of Cdc10 is important for the regulation of MBF function (McInerny et al. 1995). A truncated form of the protein (Cdc10-C4), lacking the 61 amino acids in its C terminus, leads to a highly induced transcription of MBF genes throughout cell cycle.

Overexpression of Cdc10 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. 1994). But Res2 also forms part of the mitotic MBF complex (Miyamoto et al. 1994; Ayte et al. 1997; 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. 1994).

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 the phenotype of the *cdc10*-C4 mutant was due to loss of interaction with Res2, but it is shown that was not the case (Dutta et al. 2008).

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*(*Yox1*), *cig2*, and *mik1*,

which have wild type levels of expression. Δ*res1* cells have defects to induce transcription for a larger 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 components of MBF

Other components/interactors of the MBF complex include Rep1, Rep2, Cig2, Max1(Yox1) and Nrm1. Rep1 was first described as a component of the meiotic MBF, with no function in the control of mitotic transcription (Tanaka et al. 1992). 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 has an important regulatory role in controlling MBF activity in mitosis (Nakashima et al. 1995). 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 via Res2 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.*

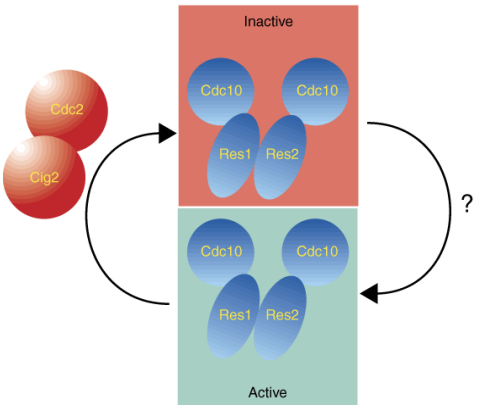

Fig 3. | Negative regulation of MBF by Cdc2/Cig2 phosphorylation (Ayte et al. 2001)

It has been recently shown that another two MBF regulated genes, Yox1 and Nrm1 are implicated in negative feedback loops (de Bruin et al. 2006; Aligianni et al. 2009; Gomez-Escoda et al. 2011). Nrm1 is the co-repressor (negative regulator of MBF targets) required to load the repressor Yox1 onto the MBF complex and thus inhibiting MBF dependent transcription. It was described that it requires the intact complex (Cdc10, Res1 and Res2) to bind DNA (de Bruin et al. 2008). Yox1 is not able to bind the MBF complex in the absence of Nrm1, which lead to up-regulated MBF-dependent transcription. However, in the absence of Yox1, transcription is also constitutively induced despite Nrm1

is still being able to bind the MBF complex. This second mechanism of MBF-dependent transcription inactivation at the end of each S phase, independent to the one carried out by Cig2, indicate the robustness of the regulation of the complex by different mechanisms, to ensure proper timing of transcription.

3.1.2. 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 a 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 S phase initiation, 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. 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 transactivation 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 (Simon et al. 2001; Horak et al. 2002). 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.

3.1.3. Metazoans: E2F/DP

E2F/DP is the functional homolog of yeasts MBF and SBF, and E2F transcription factors have critical roles in the control of transcription, cell cycle and apoptosis (DeGregori et

al. 1997). In mammals, E2F activity is generated by a large number of interconnected complexes — eight E2F genes (E2F1–8), two DP (DP1 and DP2) genes and three genes encoding RB related proteins (pRB, p107 and p130). All E2Fs share a conserved DNA-binding domain and can be divided into two classes based on whether they function as hetero- or homodimers (Fig. 4). E2Fs 1–6 require dimerization with a DP family member (DP1 or DP2), which are essential for the DNA binding of E2F (Trimarchi and Lees 2002), whereas E2F7 and E2F8 bind as homodimers, thus they have two DNA-binding domains and do not require a DP partner to bind to DNA. The E2F family members have also been divided into several subclasses based on the patterns of their expression and their transcriptional regulatory properties. E2F1, E2F2, and E2F3a are often considered to be activators and display maximal expression during S phase of the cell cycle. Members of a second class of E2F proteins, E2F3b, E2F4, and E2F5, are expressed throughout the cell cycle and are often referred to as repressors. Basically, E2F4 and E2F5 lack a transactivation domain and function mainly in combination with members of RB family of corepressors (Attwooll et al. 2004). E2F6, E2F7, and E2F8 are also classified as transcriptional repressors, but they function independently of the RB family (DeGregori and Johnson 2006).

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. E2F7-

E2F8 are an important arm of the E2F transcriptional network, which is responsible for regulating E2F1 activity upon DNA damage and, consequently, involved in regulating cell viability. An individual E2F can function to activate or repress transcription, promote or block cell cycle progression and enhance or inhibit cell death.

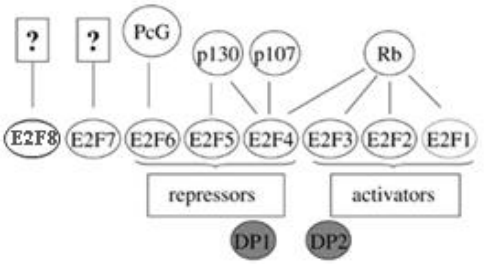

Fig 4. | Schematic representation of the E2F transcription factor subgroups, their function and specific binding partners (Attwooll et al. 2004)

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 cycle 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.

3.2. 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.

The mechanism of activation is better understood in *S. cerevisiae*, especially for SBF. Activation of SBF and MBF transcription in budding yeast depends on G1 CDK activity, being the complex Cln3/Cdc28 the primary activator and in

cells with reduced levels of Cln3, G1/S transcription is delayed (Dirick et al. 1995; Costanzo et al. 2004). Whi5 is the transcriptional repressor of SBF. It maintains the complex 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.

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, and thus allowing transcription activation (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 (with an extension of G1 phase), a phenotype for the Whi5 mutant was shown.

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. In 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, in 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 family of the retinoblastoma protein (RB). There are three RB 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.

RB is a transcriptional co-factor able to bind the different E2F transcription factors. pRB inhibits the activator E2F complexes, whereas p107 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 dissociates 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 SBF 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). So, one possibility is that the activation of MBF in fission yeast might also occur through phosphorylation of the repressor system.

3.3. Implication of misregulation of the G1/S gene expression

Loss of E2Fs regulation leads to defects in cell proliferation and in differentiation (Lukas et al. 1996; Frolov et al. 2001). 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 (Knudsen and Wang 1996; Hernando et al. 2004; van Deursen 2007).

4. DNA damage and DNA replication checkpoints

DNA in the living cell is subject to many chemical alterations. If the genetic information encoded in the DNA is to remain uncorrupted, any chemical changes must be corrected. 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.

4.1. Sources of DNA damage

4.1.1. Endogenous sources of DNA damage

DNA damage can be generated spontaneously during DNA metabolism. To maintain genomic integrity, DNA must be protected. Such kind of DNA alterations can be due to dNTP misincorporation during DNA replication, interconversion between DNA bases caused by deamination, loss of DNA bases following DNA depurinaton, and

modification of DNA bases by alkylation (Lindahl and Barnes 2000). Additionally, oxidized DNA bases and DNA breaks can be generated by reactive oxygen species (ROS) driven from normal cellular metabolism. Also 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 anticancer 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 are 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).

4.1.2. Exogenous sources of DNA damage

Besides the DNA damage produced by normal cellular processes, cells can receive insults from exogenous sources. Environmental DNA damage can be produced by physical or chemical agents. Physical genotoxic agents as ionizing radiation (IR) can induce oxidation of DNA bases and generate single and double strand breaks (SSBs and DSBs, respectively). Ultraviolet (UV) light 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. Chemical agents used in cancer chemotherapy can cause a variety of DNA lesions. The mutagen MMS (methyl methanesulfonate) generates mutations by methylation of bases in the DNA, which causes mispair in DNA synthesis and therefore point mutations. Other chemical agents, such as the topoisomerase inhibitor camptothecin (CPT) inhibit

topoisomerase I and induce DNA damage by trapping topoisomerase- DNA covalent complexes. Bleomycin produce double strand breaks, and hydroxyurea inhibits the ribonucleotide reductase enzyme, causing a depletion of nucleotides that provokes replication fork stalling.

4.2. DNA damage response

Damage to the genetic material of each living cell is an ongoing threat to both the ability to faithfully transmit genetic information to the offspring as well as its own survival. 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 (DDR). This regulatory mechanism allows cells to sense many types of damage and activate a proper response. It consists usually in the recruitment of repair proteins with a plethora of enzymatic activities that chemically modify DNA to repair DNA damage, including nucleases, helicases, polymerases, topoisomerases, recombinases, ligases, glycosylases, demethylases, kinases, and phosphatases. These repair tools must be precisely regulated, because each in its own right can rise to havoc on the integrity of DNA if misused or allowed to access DNA at the inappropriate time or place. **Thus, eukaryotic cells have developed strategies to recruit and activate the right factors, in the right place, at the right time**. The DDR is a

signal transduction pathway, mainly divided in three parts: first detected by sensors, then passed down through transducers and eventually the effectors receive the signal and execute various cellular functions- a choreographed response in order to protect the cell and ameliorate the threat to the organism (Harper and Elledge 2007; Jackson and Bartek 2009). 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).

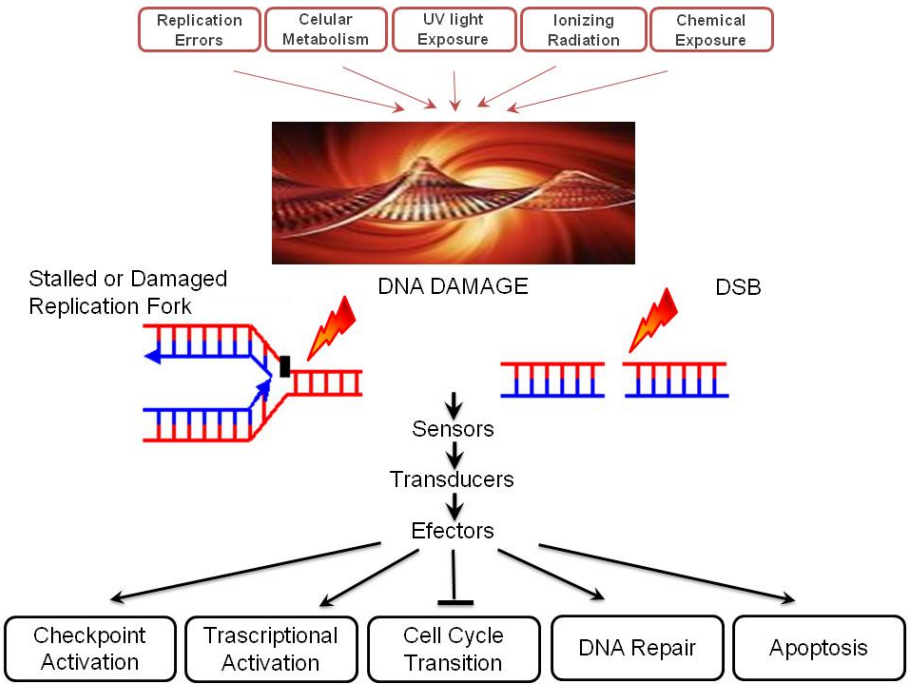

Fig 5. | Schematic representation of DNA damage: sources, types and cell effects

4.2.1. Sensors

The DDR is primary mediated by proteins of the phosphatidylinositol 3-kinase-like protein kinase (PIKK) family. In fission yeast the DDR is constrained mainly to a single sensor kinase Rad3 in contrast to higher eukaryotes, where more proteins are involved in sensing the DDR including ATM, ATR, and DNA-PK from the PIKK family, and members of the poly (ADP-ribose) polymerase (PARP) family. Much of the current understanding of the DDR is based on the study of ATM (Tel1 in *S.pombe*) and ATR (Rad3 in *S. pombe*), which detect the damage and bind DNA in the specific site, where the damage is produced.

The assembly of the DDR cascade is dependent on a broad spectrum of posttranslational modifications– acetylation, methylation, phosphorylation, ubiquitination, sumoylation - induced by the activation of the DDR (Harper and Elledge 2007; Bergink and Jentsch 2009; Kleine and Luscher 2009; Misteli and Soutoglou 2009; Ciccia and Elledge 2010). Although the sensor proteins share a PI-3-like kinase domain, they could not function as lipid kinases, but rather have strong preference to phosphorylate serine or threonine residues that are followed by glutamine (Gately et al. 1998; Rotman and Shiloh 1999; Abraham 2001). Once the DDR is activated, it drives a cascade of phosphorylations: the signal activates and recruits DNA repair proteins at the damaged sites, and also activates the effector kinases Chk1 (CHK1 in mammals) and Cds1 (CHK2 in mammals)- the

kinases responsible for the cell cycle arrest and the transcriptional response (Rhind and Russell 2000).

Despite clear conservation of biochemical activities, the precise division of labor between the sensor kinases in initiating the checkpoint response, as well as the regulatory connection between sensor and effector kinase orthologs is not strictly conserved between species (Fig. 6). In metazoans, the two kinases, ATM and ATR, have specialized functions: ATM is activated predominately in response to double strand breaks (DSBs) and specifically activates CHK2, while ATR is activated in response to stalled replication forks, seems to detect damage in single strand DNA (ssDNA) and activates CHK1 (Shiloh 2003). In fission yeast, in spite $Tel1^{ATM}$ is activated by DSBs, it is primarily involved in telomere maintenance (Rhind and Russell 2000; Harrison and Haber 2006; Sabourin and Zakian 2008). On the other hand the vast majority of the checkpoint responses to all genotoxic insults, including DSBs, is dependent on $Rad3^{ATR/Mec1}$, which activates the two effector kinases: Cds1 and Chk1 (similarly as it happens in *S. cerevisiae*, where Mec1 activates Rad53 and Chk1). And this may be is an adaptation to the rapid processing of DSB ends to ssDNA that occurs in yeast.

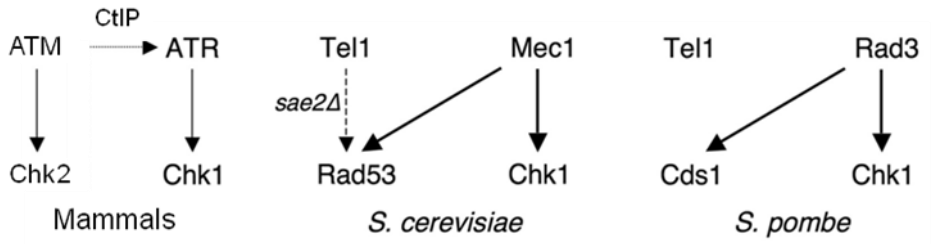

Fig 6. | Regulatory connections between ATM/ATR and CHK1/CHK2 orthologs in mammals, *S. cerevisiae* and *S. pombe*. (Limbo et al., 2011)

- **Mechanism of DDR activation**

When DNA damage is detected, chromatin that flanks this damage is marked by the DDR. This is a challenge, both in terms of accessibility to the lesion and maintenance of genome stability. Therefore DNA damage responses require that DNA repair and checkpoint proteins work in concert with factors that bind to or modify chromatin at DNA lesions (Stucki and Jackson 2006; Harper and Elledge 2007). Histones, the main protein component of chromatin, can be subjected to a variety of post-translational modifications (PTMs) that impact on genome function by either directly affecting nucleosome stability or providing a docking site for distinct regulatory proteins. Central to this integration of chromatin metabolism and DNA repair, the checkpoint kinases Tel1 and Rad3 phosphorylate the carboxyl terminus of histone H2A (H2AX in mammalians) of chromatin surrounding the damaged DNA. Phosphorylated H2A (γH2A) signaling is the initial step of the checkpoint response and acts as a scaffold for the recruitment of other proteins of the

checkpoint cascade in the surroundings of the damaged sites (Williams et al. 2010).

- **Post-translational modifications**

In metazoans there are additional regulatory mechanisms involved in activation of the DDR. Post-translational modifications may regulate the activity of PIKKs as well as localization. Indeed, activation of ATM involves autophosphorylation, an event that may help convert an inactive ATM dimer into active monomers (Bakkenist and Kastan 2003). However, there is no data indicating that the oligomerization status of ATR-ATRIP is regulated. It seems likely that ATR activation is dependent upon continued stimulation by TOPBP1 (Cut5 in *S. pombe*).

- **Signal amplification**

Auto-amplification might be also important in the ATM response and is mediated through ATM-dependent phosphorylation of H2AX. MDC1, a BRCT-domain containing protein needed for ATM activation, binds to γH2AX (the phosphorylated form of H2AX) through its tandem BRCT domains and brings more ATM to the DNA damage site. Brc1 in fission yeast was described to be the major H2A binding protein in replication stress responses (Williams et al. 2010). In the ATR pathway, the interaction between ATR and TOPBP1 may provide a point for signal auto-amplification.

- **Redundancy and crosstalk**

Interestingly, most substrates can be phosphorylated either by ATR or by ATM, and the major functions of ATR and ATM in cell cycle control are overlapping and redundant- an important point of crosstalk (Siliciano et al. 1997; Cortez et al. 1999; Tibbetts et al. 1999; Tibbetts et al. 2000). However there is an evidence of some unique specificities (in particular, CHK1 and CHK2 may be exclusively ATR and ATM substrates, respectively). Even though ATR primarily is a replication stress response kinase, it is also activated by DSBs; certainly ATM does signal at collapsed replication forks where DSBs are often formed (Brown and Baltimore 2003; Jazayeri et al. 2006). In particular, the ability of one DNA damage type to be converted into another, the crosstalk between the pathways suggests both unique and interdependent roles for these kinases. Recently it has been proposed an as called biphasic mechanism of checkpoint signaling at DSBs that can operate whenever Ctp1/CtIP recruitment is significantly delayed relative to Tel1/ATM (Limbo et al. 2011) (Fig. 7). In mammalian cells, both ATR and ATM are critical for CHK1 activation in response to DSBs (Jazayeri et al. 2006; Myers and Cortez 2006) and ATM-to-ATR switch, coincide with formation of SSOs. The hand-off between Tel1 and Rad3 can ensure that checkpoint responses are both fast and yet can be maintained, while DSBs are processed for HR repair. From the data currently available (Limbo et al. 2011), the only crucial difference between fission yeast and mammalian cells with respect to

resection and checkpoint signaling is that Tel1 is not required for efficient DNA end processing, hence is not required for Chk1 activation by Rad3 in fission yeast.

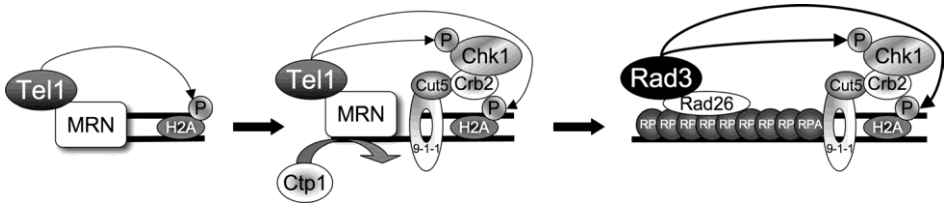

Fig.7 │ Biphasic DNA damage checkpoint (Limbo et al. 2011)

- **Implication in human health**

Understanding and regulating this path of the response is from extreme importance, as in humans responding to and repairing DNA damage is critical for cell viability and disease prevention. Mutations in ATM predispose carriers to cancer and are found in approximately 0.5–1% of the human population (Swift et al. 1987; Renwick et al. 2006). People with mutations in both alleles of ATM suffer from the neurodegenerative and cancer predisposition disorder ataxia-telangiectasia (Savitsky et al. 1995). Mutations in ATR are rare and probably only compatible with viability when heterozygous or hypomorphic. While the only clear link between ATR gene mutation and disease is in a few patients with the rare Seckel syndrome characterized by growth retardation and microcephaly (O'Driscoll et al. 2003); disruptions in the ATR pathway do cause genomic instability. ATR is activated by most cancer chemotherapies making

ATR signaling a promising target for cancer drug development (Collins and Garrett 2005; Kaelin 2005).

4.2.2. Transducers

Among the complexes recruited to the damage sites, there are some multi-protein complexes such as the Mre11-Rad50-Nbs1^{Xrs2} (MRN) protein complex which directly binds DSB ends and thus allows Tel1^{ATM} association. The Rad family members: Rad1, Rad9, Rad17, Rad26, and Hus1; where Rad26 (ATRIP in mammals) is a partner protein interacting with Rad3^{ATR}; Rad17-RFC is the clamp loader and the sensor complex 9-1-1 (Rad9, Rad1, Hus1) is a heterotrimeric ring surrounding the affected DNA, which acts as a tether, linking the upstream kinases (Tel1 and Rad3) to the downstream targets. And then a series of adaptator proteins like Cut5, Crb2, that form a platform for the recruitment and activation of the effector kinases Cds1 and Chk1 (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)

In fission yeast the functions of ATM and ATR orthologs are intimately tied to the detection and nucleolytic processing of DSBs, through nonhomologous end joining (NHEJ), in which DNA ends are directly ligated, and homologous recombination (HR) (Shrivastav et al. 2008).

After DSBs Tel1 localizes at the damaged place by interacting with the MRN complex and repair is initiated by 5`-3`resection of the broken ends to form 3`ssDNA overhang. The MRN complex has from one side DNase activities driven by the Mre11 subunit, and also recruits Ctp1 DNA end-processing factor to DSBs (CtIP in mammals) (Lloyd et al. 2009; Williams et al. 2009). Thus 3`-single-strand overhangs (SSOs) are generated. RPA binds to SSOs, before being displaced by $Rhp51^{Rad51}$, creating a nucleoprotein filament in

a process requiring Rad22^{Rad52} and is essential for HR repair of DSBs. It is also important for recruiting Rad3$^{ATR/Mec1}$, which interacts with RPA through its regulatory subunit Rad26$^{ATRIP/Ddc2}$ (Zou and Elledge 2003; Cimprich and Cortez 2008). Rad3^{ATR}-Rad26^{ATRIP} phosphorylate and activate the downstream kinases Chk1 and Cds1 (Martinho et al. 1998; Edwards et al. 1999). Rad3^{ATR} also phosphorylate Rad26^{ATRIP}, Rad9, Hus1 (9-1-1 subunits) and the checkpoint adaptor proteins Crb2 and Mrc1 (Caspari et al. 2000; Alcasabas et al. 2001; Tanaka and Russell 2001; Furuya et al. 2004).

Mrc1 is already pre-loaded with the replisome during normal S phase, which on its turn phosphorylates many chromatin bound factors to promote fork stability and restart of stalled or collapsed replication forks. Rad9 can be phosphorylated by Rad3^{ATR} and to a lesser extent by Tel1^{ATM} after S phase arrest and DNA damage in G2, and this promotes its association with Cut5. All this promotes formation of a checkpoint complex required for Chk1 activation and arrest of mitosis.

Interestingly, in mammals the scenario is different: ATM in collaboration with the MRN complex recognizes DSBs. Conversely, ATR and its partner protein ATRIP, recognize ssDNA coated with replication protein A (RPA)- an intermediate of many DNA transactions (Cortez et al. 2001; Zou and Elledge 2001; Zou et al. 2002).

4.2.3. Effector kinases

Once the DDR is in action, it finally leads to the activation of the two effector kinases Cds1 and Chk1, which depending on the responding pathway will elicit their effector function.

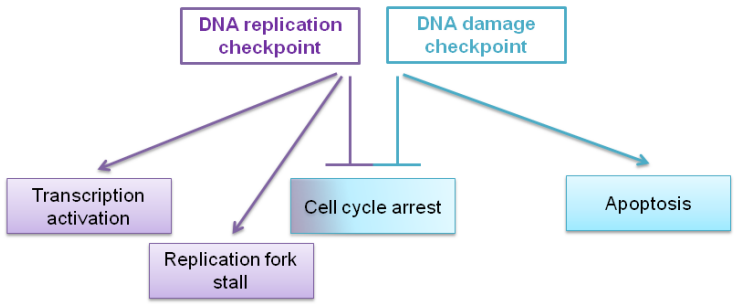

Fig.8 | DNA damage effector responses

4.3. DNA replication checkpoint

The DNA replication checkpoint response is that branch of the DNA damage which is activated upon replication fork stalling. In fission yeast this checkpoint converges in a single effector kinase Cds1. Failure to properly overcome it, leads to an inability to complete chromosome duplication and can lead to mitotic catastrophe, complex chromosomal rearrangements, and cell death.

The replication checkpoint response consists in:

- Cell cycle arrest
- Stabilization of stalled replication forks
- Activation of a transcriptional response

4.3.1. Cell cycle arrest upon replication stress

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. As *S. pombe* spends most of his time in G2 phase, the arrest occurs at G2/M transition. The G2 checkpoint response is conserved in all eukaryotes, including yeasts (Kuntz and O'Connell 2009) and when a damage is detected in S and in G2 phases, the entry into mitosis is blocked, to avoid segregation of damaged chromosomes. 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 fission yeast 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. And this phosphorylation state is regulated either by the two kinases Wee1 and Mik1, or negatively by the Cdc25 tyrosine phosphatase (Coleman and Dunphy 1994). This is an inhibitory phosphorylation of Cdc2, which renders a Cdc2/Cdc13 CDK complex with an intermediate kinase activity, not enough to trigger mitosis. So, the checkpoint role is to maintain that Tyr-15 phosphorylated and this is achieved through several mechanisms (Rhind et al. 1997; Rhind and Russell 1998).

Upon replication stress Cds1 inhibits Cdc2 (Rhind and Russell 1998) either through inhibition of Cdc25 phosphatase (Zeng et al. 1998) or targeting the two kinases Wee1 and Mik1 (Murakami and Okayama 1995; Boddy and Russell 1999). Cds1 associates with and phosphorylates Wee1 and also is required for the large accumulation of Mik1 (Boddy et al. 1998), suggesting that Cds1 might enforce replication by increasing the activity of Wee1 and Mik1 kinases.

4.3.2. Stabilization of stalled replication forks

Cds1 is also involved in the stabilization of stalled replication forks, to avoid lethal fork collapse. However once the damage is repaired, stabilized forks are able to resume replication. Checkpoint also prevents 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. Late origins are maintained in a pre-replicative state until they are necessary for the completion of replication once the damage is repaired. Replication forks have a role in both, sensing the damage and signalling it as effectors of the response. This role of Cds1 is extremely important as defective mutants cause irreversible collapse of replication forks and cell death (Tercero et al. 2003). 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).

4.3.3. Activation of a transcriptional response.

Upon replication stress the transcription of genes with role in DNA repair and replication is highly induced. 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. What is the significance of this regulation for the survival of the cell? The role of this transcriptional induction is to provide resistance to the replication stress and to prepare cells to resume replication, once the damage is repaired.

All MBF dependent genes are upregulated in response to checkpoint activation (Dutta et al. 2008), and the product of those genes are directly or indirectly required for DNA synthesis. Δ*cds1* and Δ*rad3* mutants are not able to upregulate MBF-dependent transcription upon HU treatment. Also, the checkpoint response is affected upon deletion of each component of MBF (Δres1, Δ*res2*, and *cdc10-C4* cells)

There are three MBF targets of Cds1 that have been lately described: Cdc10, Nrm1 and Yox1. Cdc10 has several Cds1 consensus phosphorylation sites in its C-terminus region, with a crucial role in Cdc10 regulation (Dutta et al. 2008). However the mutant *cdc10-8A*, which cannot be phosphorylated by the kinase, is perfectly able to induce

transcription upon HU treatment pointing that those phosphorylation sites are not important for the checkpoint response. On the contrary, mutations that mimic a checkpoint constitutive phosphorylation have indeed a remarkable phenotype: *cdc10-2E* allele actually shows constitutively upregulated transcription. Consistent with this, *cdc10-2E* mutation confers resistance to HU, and partly rescues the lethality of Δ*cds1* cells, pointing that transcriptional response could have a role in survival upon replicative stress in *S. pombe.*

Nrm1 was also described to play an important role in DNA replication checkpoint response (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, therefore more bound to promoters, and transcription is partially repressed. However the mutant Nrm1-8A behaves as wt upon HU treatment. Anyhow cells deleted in *nrm1* are partly resistant to HU, as one of the subunits of the ribonucleotide reductase (*cdc22*) is an MBF target.

It has been also described recently that Yox1 and Nrm1 bind and repress MBF dependent gene transcription at the end of S phase (Aligianni et al. 2009; Caetano et al. 2011; Gomez-Escoda et al. 2011). Yox1 is phosphorylated by Cds1

at two residues Ser-114 and Thr-115 which leads to its release from MBF, triggering transcriptional induction of MBF dependent genes (Purtill et al.; Caetano et al. 2011; Gomez-Escoda et al. 2011). The fact that Yox1 mutant (Yox1.SATA) is not remarkably sensitive to HU despite being unable to induce MBF dependent gene transcription could lead to think that the transcriptional response induced by the DNA damage checkpoint has a minor contributing role for survival in fission yeast, as also observed in budding yeast (Tercero et al. 2003).

In budding yeast, the checkpoint promotes the induced expression of G1-S genes. It is likely that the mechanism is conserved between the two yeasts. 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). However in mammals, so far there is no published data of E2F/DP regulation by CHK1 in response to replicative stress.

The current model for G1/S transcription up-regulation by the DNA replication checkpoint in the different organisms is based on recent findings in *S. pombe* (de Bruin and Wittenberg 2009) (Fig. 9).

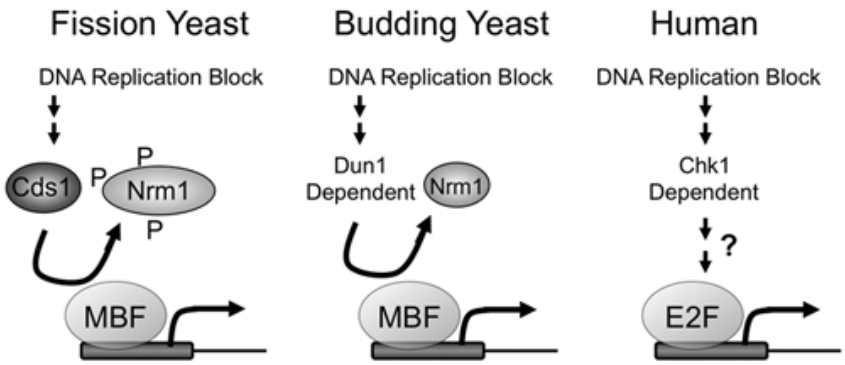

Fig. 9 | The DNA replication checkpoint promotes persistent expression of cell cycle regulated transcripts in eukaryotes (de Bruin and Wittenberg 2009).

4.4. DNA damage checkpoint

The DNA damage checkpoint Chk1$^{CHK2/Chk1}$ is one of the major players in response to DNA damage. Is activated in response to DSBs and acts to both facilitate DSB repair and delay the cell cycle, thus preventing cell cycle progression prior to completion of repair (Lazzaro et al. 2009). DSBs can occur at each stage of the cell cycle, including either G1/S, the intra-S or G2/M, anyhow all leading to Chk1 activation and respectively to cell cycle arrest, preventing sister chromatids separation. When DNA damage occurs, a signal transduction pathway cascade is activated in which sensor proteins recognize the damage and transmit signals that are amplified and propagated by adaptors/mediators to the downstream effectors, which connect the checkpoint with the cell cycle machinery and final cell fate.

The DNA damage checkpoint response consists in:

- Cell cycle arrest
- Activation of transcriptional response

4.4.1. Cell Cycle arrest upon DNA damage

As has been already mentioned above in fission yeast, when DNA damage occurs, its main aim is the negative regulation of Cdc2 by maintaining Cdc2 Tyr-15 and thus blocking cell cycle progression until the problem is solved. Chk1 also inhibits Cdc25 through phosphorylation, leading to its relocalization in the cytoplasm, and thus preventing Cdc2 activation. This is achieved through interaction with Rad24 and Rad25, proteins belonging to the 14-3-3- family. Not only nuclear exclusion, but also an inactivation of Cdc25 is required to arrest the cell cycle (Lopez-Girona et al. 1998). This mechanism is conserved in mammals, where a target of fission yeast Chk1 is also the human CDC25 (Peng et al. 1997; Sanchez et al. 1997). Similarly, CDC25 control is preserved in mammals, where phosphorylation of CDC25A by CHK2 prevents it from activating CDK2, which plays an essential role in G_1 to S transition (Falck et al. 2001). In a similar manner, CHK2 phosphorylation of CDC25C interferes with activation of CDK1 activity thus resulting in G_2/M cell cycle arrest (Ahn and Prives 2002). Apart of Cdc25, fission yeast Chk1 also targets the two kinases Wee1 and Mik1 (Rhind and Russell 2001; Kuntz and O'Connell 2009).

4.4.2. Activation of transcriptional response

So far, in *S. pombe* there is no evidence for Chk1 driven transcription regulation of MBF- dependent genes upon DNA damage. The mechanism is better understood in higher eukaryotes and to date it has been shown that human CHK2 plays a role in responding to DNA replication–associated DNA damage.

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). In normally growing cells, pRB exists in a predominantly phosphorylated state, which is dissociated from E2F, allowing E2F-dependent transcription. In the early

stages of DNA damage, pRB is dephosphorylated at CDK sites, and is also directly phosphorylated by CHK2. Thus pRB becomes active and repress E2F-1 transcriptional activity. Consequently, pRB-dependent cell cycle arrest and repression of apoptosis occur (Inoue et al. 2007). However, if DNA damage is severe, pRB is cleaved by caspase (Tan et al. 1997) and becomes inactive, leading to activation of E2F-1, and subsequent induction of apoptosis (Attwooll et al. 2004).

So, after DNA damage the two effector kinases in fission yeast Cds1 and Chk1 strictly regulate the cell cycle progression by arresting the cells in G2/M phase. However how the transcriptional control during G1/S transition is regulated by the checkpoints, was a challenge we tried to answer with this work.

OBJECTIVES

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

1. To characterize the MBF regulation under replication stress focusing on Yox1.

2. To determine how the MBF transcription factor is regulated upon DNA damage.

RESULTS

Chapter I

The DNA synthesis checkpoint activates the G1/S phase transcription program through MBF

MBF, the single transcriptional factor complex required for the regulated expression of all the S phase genes and thus for the normal G1/S cell cycle progression, is activated in response to DNA replication stress. Upon replication arrest, cells induce a checkpoint response based on the activation of Cds1, the effector kinase, which stops the cell cycle, stabilizes the replication fork and also activates the MBF dependent transcription. The S phase transcription programme is induced, because Yox1, the MBF repressor, is inactivated and released from MBF through direct checkpoint phosphorylation. This allows the cell to complete its replication programme once the damage is repaired.

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Chapter II

The DNA damage checkpoint reppreses the G1/S phase transcription program through MBF

The G1/S transcriptional factor complex MBF is negatively regulated by the DNA damage checkpoint response. Upon severe damage and DSB, Chk1, the DNA damage effector kinase, is activated and induce an arrests in cell cycle progression. This checkpoint seems to be responsible for a new role: the repression of MBF dependent gene transcription, which is achieved through targeting the core element Cdc10. Upon phosphorylation by Chk1 on its carboxy terminal domain, Cdc10 is released from its target promoters and as a consequence, the MBF complex is inactivated. This probably is a part of a safe mechanism of the cell to protect its genomic integrity untill the damage is repaired.

The DNA-damage and the DNA-synthesis checkpoints convey at the MBF transcription factor

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ABSTRACT

In fission yeast cells, Cds1 is the effector kinase of the DNA-synthesis checkpoint. We have previously shown that when the DNA-synthesis checkpoint is activated, Yox1 is phosphorylated by Cds1 resulting in the activation of the MBF-dependent transcription until cells are able to overcome the replication challenge. Here we show that the MBF core itself (i.e. Cdc10) is a target of the DNA-damage checkpoint. When fission yeast cells are treated with DNA damaging agents (MMS, ionizing radiation) Chk1 phosphorylates Cdc10 at its carboxi terminal domain resulting in a repression of MBF-dependent transcription. Thus, Yox1 and Cdc10 couple normal cell cycle regulation and the DNA-synthesis and DNA-damage checkpoints in a single transcriptional complex.

Keywords: Chk1 / DNA Damage Checkpoint / G1-to-S transcriptional program / MBF / MMS

INTRODUCTION

Genomic integrity is constantly threatened by many processes that take place in any living cell. Reactions like transcription and DNA replication or the exposure to external or internal damaging agents suppose for the cell an increased risk of rearrangements in DNA or single nucleotide substitutions, defects that are the hallmark of cancer cells (Ciccia & Elledge, 2010; Elledge, 1996). In order to maintain genomic integrity, all eukaryotes have developed a highly conserved mechanism to detect, signal and repair damage in DNA, known as the DNA damage response (DDR) (Elledge, 1996; Hartwell & Weinert, 1989; Rhind & Russell, 1998). When DNA replication is challenged, cells activate a DNA synthesis checkpoint blocking cell cycle progression until they are able to overcome the replication defects (Boddy & Russell, 1999; Murakami & Okayama, 1995). Similarly, in response to damage to DNA, cell cycle must be arrested through the DNA damage checkpoint (Rhind & Russell, 2000). In the fission yeast *Schizosaccharomyces pombe* there are two effector kinases, Cds1 and Chk1, which are activated by the DNA synthesis and the DNA damage checkpoint, respectively. The main aim of these kinases is to block cell cycle progression before cells enter into mitosis; they do so by phosphorylating and inhibiting the phosphatase Cdc25 which prevents the fully activation of Cdc2 (Furnari et al, 1997; Walworth et al, 1993).

In fission yeast, activation of Cds1 also invokes a transcriptional response that ultimately increases the concentration of the deoxynucleotides required to complete the DNA synthesis. This response is achieved by activating the transcription factor MBF (Dutta et al, 2008), which in a normal –unperturbed– cell cycle is responsible for the transcription of a set of genes that are required for the S phase of the cell cycle (Lowndes et al, 1992). MBF, which is the functional homologue of mammalian RB/E2F, is a high molecular weight complex whose core elements are the product of the Start gene *cdc10*, and Res1 and Res2 which form a heterodimeric DNA-binding domain (Ayte et al, 1995; Lowndes et al, 1992; Miyamoto et al, 1994; Obara-Ishihara & Okayama, 1994; Simanis & Nurse, 1989; Tanaka et al, 1992). Under replicative stress, the activation of the MBF-dependent transcription is the consequence of the phosphorylation of several components of the MBF complex, including Cdc10 (Dutta et al, 2008), the co-repressor Nrm1 (de Bruin et al, 2008) and the repressor Yox1 (Caetano et al, 2011; Gomez-Escoda et al, 2011; Purtill et al, 2011). Specifically, phosphorylation of Yox1 by Cds1 disrupts the binding of Yox1 to the MBF complex, activating MBF-dependent transcription. Mutants in which Yox1 cannot be phosphorylated lack the proper transcriptional response under replicative stress (Gomez-Escoda et al,

2011). In this work, we demonstrate that there is also a direct link between the DNA damage checkpoint and the MBF complex, which contrary to what happens after the activation of the DNA synthesis checkpoint, is in charge of inactivating the transcription of the S phase genes. This is achieved by direct phosphorylation of Cdc10 at serine-720 and serine-732 by the efector kinase, Chk1

RESULTS

Cdc10 is targeted by the DNA damage checkpoint.

While investigating the effect of the DNA synthesis checkpoint on the regulation of the transcription factor MBF, we noticed that when cells were treated with hydroxyurea (HU) on a Yox1 mutant (Yox1.SATA) background that cannot be phosphorylated by the DNA synthesis checkpoint effector kinase, Cds1, MBF-dependent induction of transcription was abrogated (Fig. 1A) (Gomez-Escoda et al, 2011). Under these conditions, the core MBF element, Cdc10, was released from chromatin (Fig. 1B), in parallel to the release of the repressor Yox1 (Fig. 1C). We could observe this release independently of the presence of Cds1, since in cells lacking Cds1, Cdc10 was also released when they were treated with HU, and to a similar extent than in the Yox1.SATA cells. Unexpectedly, this release of Cdc10 was abrogated in the absence of Chk1 (both *Δchk1* and *Δchk1Δcds1* strains) or when Chk1 could not be activated in cells that lack the sensor kinase (*Δrad3* strain). Interestingly, we could observe that Chk1 was phosphorylated (which is a hallmark of its activation) when either Yox1.SATA or *Δcds1* cells were treated with HU, pointing to the fact that in these specific genetic backgrounds both the DNA synthesis and DNA damage checkpoint were activated by HU (Fig. 1D).

It was previously shown that besides Cdc10, other components of the MBF complex (Nrm1 and Cdc10) could be phosphorylated by Cds1 when cells were under replicative stress (de Bruin et al, 2008; Dutta et al, 2008). However, our previous results pointed to the possibility that some MBF components could also be targeted by Chk1 when the DNA damage checkpoint was activated. To further investigate the signaling from this checkpoint to the MBF factor, we treated fission yeast cells with different DNA damaging agents, like MMS (Fig. 2A) or with different doses of irradiation (Fig. 2B). Indeed, any of the damaging agents that we used were able to induce the release of Cdc10 from two of the better characterized MBF-dependent promoters, *cdc18* and *cdc22*.

The effect of the DNA damage checkpoint on MBF is dose-dependent.

To further characterize the response to MMS, we treated cells with increasing concentrations of the drug (from 0.002% to 0.1%). At the lower doses, we could not observe any affect on Cdc10 since it remained bound to the canonical promoters that we tested. In fact, Cdc10 was not released from chromatin unless cells were treated with the higher concentrations of MMS (0.05% and up) (Fig. 2C). On the contrary, when we measured the effect on the repressor system (Nrm1 and Yox1), we could clearly observe that the

repressor system was released from chromatin (and consequently from the MBF complex) at the lower MMS concentrations (Fig. 2C). This effect on the Nrm1/Yox1 repressor system paralleled a noticeable induction of the transcription of the MBF genes at these low MMS concentrations (Fig. 2D). Interestingly, we could detect a further release of both Yox1 and Nrm1 when we treated cells with 0.1% MMS (and higher concentrations), which is probably due to some remaining Yox1/Nrm1 that was still bound to the MBF complex that under these high MMS concentrations was released from chromatin while still bound to Cdc10. This second wave of Nrm1 and Yox1 release correlates with a repression of MBF-dependent transcription at higher MMS doses (Fig. 2D). To determine whether release of the MBF complex from chromatin was due to cell death, we measured the viability of the cells during the timing of the treatment. As shown in Fig. 2E, the MMS concentrations used (and even higher concentrations) barely affect cell viability.

Next, we wanted to further characterize the signaling from the DNA damage checkpoint to MBF. To confirm that the release of Cdc10 was exclusively due to the activation of the DNA damage checkpoint (and that the DNA synthesis checkpoint was not involved in this release), we analyzed the binding of Cdc10 and Yox1 to *cdc18* and *cdc22* promoters in fission yeast cells with impaired signaling in any or both of these checkpoints. As shown in Fig. 3A, the release of Cdc10 that we observed in cells lacking Cds1 was similar to the observed in wild type cells. However, in cells lacking either Chk1 or the upstream activating kinase, Rad3, Cdc10 was not released after the treatment with MMS. Under these concentrations of MMS, Yox1 release from chromatin paralleled the release of Cdc10, pointing to the possibility that it was the MBF complex as a whole that was released from chromatin (Fig. 3B). If the DNA damage checkpoint was able to induce a release of the MBF complex from chromatin, we should be able to observe a transcriptional down-regulation of the MBF-dependent genes. As a prove of concept, fission yeast cells were exposed during different time with a fixed MMS concentration (Fig. 3C) or with increasing MMS concentrations (Fig. 3D). When we treated *Δyox1Δnrm1* cells, which have an already induced transcription of the MBF-dependent genes in their basal condition, we could clearly observe a repression of MBF-dependent genes when treated with MMS.

Cdc10 phosphorylation by Chk1 inactivates MBF-dependent transcription.

We then decided to focus on the possibility that Cdc10 itself could be a target of Chk1. In fact, Cdc10 has already been described as a target for Cds1, although no clear phenotype has been associated to

Cdc10 mutants in the residues that are *in vitro* phosphorylated by Cds1 (Dutta et al, 2008). Since Cds1 and Chk1 can phosphorylate similar target sequences (O'Neill et al, 2002; Seo et al, 2003; Xu & Kelly, 2009), we set out to determine whether Cdc10 was *in vitro* a bona-fide target for Chk1 phosphorylation. Cdc10 has 4 putative sites that can be phosphorylated (ser-563, ser-603, ser-720 and ser-732). While the first two are in close proximity to the ankyrin domain, which mediates protein-protein interactions, the last two residues are in the C-terminal region of Cdc10, which is essential for loading the Yox1/Nrm1 repressor system onto chromatin (Supplementary Fig. 1). In our *in vitro* Chk1 kinase assays, a Cdc10 construct lacking the last 61 amino acids was not phosphorylated. Conversely, a construct containing only the carboxy-terminal 61 amino acids (and thus containing the last two putative phosphorylation sites) was consistently phosphorylated (Fig. 4A). When serines 720 or 732 were mutated to alanine, the phosphorylation was diminished. Furthermore, in the double mutant, Chk1 phosphorylation was completely abolished (Fig. 4A and 4B). It is worth noting that these phosphorylation sites are different from the described Cds1 phosphorylation sites in Cdc10 (Dutta et al, 2008).

To test whether Cdc10 phosphorylation by Chk1 is essential for the *in vivo* regulation of Cdc10/MBF binding to its target promoters upon activation of the DNA damage checkpoint, we introduced the serine-to-alanine mutations in fission yeast, replacing the endogenous copy of *cdc10*. When treated with MMS, the strains that carry single mutations (including those next to the ankyrin domain) were responding in a similar manner to a wild type strain, that is, Cdc10 was released from its target promoters, with the exception of the mutant S732A that showed a partially impaired exclusion from *cdc18* promoter (Fig. 4C). However, in a strain that carries the double mutation S720AS732A (Cdc10.2A) and that cannot be phosphorylated by Chk1, the release of Cdc10 was impaired from *cdc18* promoter after the treatment with MMS (Fig. 4C). Interestingly, we could not observe any effect on its binding activity to *cdc22* promoter, pointing to the fact that Chk1 might differentially regulate the binding of Cdc10 to only a subset of MBF-dependent genes. To test the consequences of the Chk1-mediated regulation of Cdc10 binding to chromatin, we measured the effect on transcription. As expected, when a strain in which the two Chk1 phosphorylation sites were mutated to alanine (Cdc10.2A) was treated with increasing doses of MMS, *cdc18* transcription was unaffected, while *cdc22* decreased to a similar extent as in the wild type counterpart (Fig. 4D).

We hypothesized that a strain, in which the transcriptional response of the DNA damage checkpoint was abolished, should have survival problems when confronted with a damaging agent, like

MMS. As can be seen in Fig. 4E, a strain carrying the double mutation was sensitive to MMS, even not to the same extend as a strain in which chk1 was deleted.

DISCUSSION

The MBF complex is an essential transcription factor that fission yeast cells need for the normal and controlled expression of the S phase transcription program. When DNA replication is challenged (i.e. after treating cells with HU), fission yeast cells activate their effector kinase (Cds1) and, among many other effects, are able to maintain a high level of MBF-dependent transcription (Gomez-Escoda et al, 2011). Since ribonucleotide reductase (Cdc22) is the target of HU and its expression is directly regulated by MBF (Lowndes et al, 1992), hyperactivation of the complex might help to overcome the block to DNA replication inflicted by the drug. The main MBF target of Cds1 is Yox1, the repressor of the MBF complex (Aligianni et al, 2009; Gomez-Escoda et al, 2011). It has recently been described that Yox1 phosphorylation by Cds1 results in its inactivation (Caetano et al, 2011; Gomez-Escoda et al, 2011; Ivanova et al, 2011; Purtill et al, 2011). We now report here that the DNA damage checkpoint exerts a new a new layer of control on the MBF complex. However, instead of exerting a positive effect on MBF, Chk1, the effector kinase of the DNA damage checkpoint, is responsible of inactivating MBF-dependent transcription (Fig. 5). This is achieved by direct phosphorylation of one of the core components of the MBF complex, Cdc10, at two different sites on its carboxi-terminal domain. This phosphorylation induces the exit of Cdc10 from the chromatin and thus the repression of the transcription of the MBF-dependent genes. Interestingly, low doses of MMS are able to induce MBF dependent transcription (through phosphorylation of Yox1) whilst high doses repress the same set of genes by directly phosphorylating Cdc10. In fact, under such severe damage there is no active MBF complex associated with the corresponding promoters (Supplementary Fig.2). Our hypothesis is that cells that have to cope with severe DNA damage must stop any attempt to initiate DNA synthesis which will worsen its situation; this is achieved by switching off the S phase transcriptional program. However, fission yeast cells sense discrete or minor DNA damage (low MMS concentration, HU) at least partly as a block to DNA synthesis, activating the DNA synthesis checkpoint. Consequently, these cells need to maintain activated the transcriptional S phase program until they manage to fully complete the duplication of its genome. In conclusion, MBF would be double targeted by the DNA synthesis and the DNA-damage checkpoints with an outcome that goes in opposite directions: while the DNA damage checkpoint aims to Cdc10 and causes a repression, the DNA synthesis checkpoint aims to Yox1 and induces an activation of transcription. Interestingly, while all the MBF-dependent genes are induced upon a challenge to DNA replication (Dutta et al, 2008; Gomez-Escoda et al, 2011), only a subset of them seems to be under the

control of the DNA damage checkpoint (Fig. 4C-D). We do not know mechanistically how this is achieved, but it has been long known that not all MBF-dependent genes are regulated in the same manner; for example, in synchronized cultures, transcription of *cdc18* is induced in anaphase, while *cig2* takes place later in the G1-to-S transition (Baum et al, 1997). Thus, the differential regulation of the MBF-dependent genes by the DNA damage checkpoint may be due to intrinsically differences in the chromatin structure of the two groups of MBF dependent genes; alternatively, we have not excluded that other components or regulators of the MBF complex can be overlapping targets for Chk1. Further work will be required to characterize this differential regulation.

While up-to-now in higher eukaryotes it has not been demonstrated a clear link between the DNA synthesis checkpoint and the regulation of the expression of S phase genes, previous reports have shown a connection between the DNA damage checkpoint and E2F/Retinoblastoma, which is the functional homologue of fission yeast MBF and budding yeast MBF/SBF (Inoue et al, 2007; Stevens et al, 2003; Zalmas et al, 2008). Initially it was reported that E2F-1 was phosphorylated and activated in response to DNA damage resulting in apoptosis (Stevens et al, 2003). However, a recent report demonstrated that irradiation causes phosphorylation of Rb (by Chk1/2) and repression of E2F-dependent transcription (Inoue et al, 2007). We propose that the checkpoint regulation of transcription through Cdc10/Rb is conserved across eukaryotes, with the same final outcome (repression of transcription after DNA damage) but divergent mechanisms: while in higher eukaryotes the phosphorylation tethers the repressor (Rb) to the transcription factor (E2F-1), in fission yeast decreases the binding of the transcription factor to its cognate promoters.

METHODS

Strains and media. All *S. pombe* strains are isogenic to wild type 972h-. The strains used in this work are listed in the supplementary information. Media were prepared as previously described (Moreno et al, 1991). HU (10mM), MMS and γ-irradiation treatment were carried out on midlog grown cultures (3-4x10^6 cells/ml) in YE5S media. To analyze sensitivity to HU and MMS on plates, *S. pombe* strains were grown in liquid YE5S media to an OD_{600} of 0.5. Cells were then diluted in YE5S and spotted onto YE5S media agar plates. Plates were incubated at 30°C for 3–4 days.

Viability assays. To determine cell viability, *S.pombe* strains were grown in liquid MM or YE5S media to an OD_{600} of 0.5 and the cultures were treated with the corresponding MMS concentration for a curtain time. Cells were then washed, diluted in YE5S and spotted onto YE5S media agar plates. Plates were incubated at 30°C for 3–4 days.

FACS. For cell cycle progression, *S.pombe* strains were grown in liquid MM or YE5S media to an OD_{600} of 0.5 and the cultures were treated with the corresponding MMS concentration for a curtain time. ~5×10^6 cells for each sample were fixed in 70% ethanol; washed with 1ml 50 mM Sodium citrate (pH 7), resuspended in 0.5ml of the same buffer with 100μg/ml RNase and incubated overnight at 37°C. DNA was stained with 1μg/ml of Propidium iodide, mixed vigorously and sonicated. For viability tests cells were grown in liquid YE5S media to an OD_{600} of ~0.3 and the cultures were treated with the corresponding MMS concentration for a curtain time; aliquots of the same cultures as above were stained with either propidium iodide or phloxine B. For propidium iodide staining, cells were centrifuged, washed twice with PBS, and incubated with 3 μg/ml of the dye for 40 min on ice in darkness. Regarding phloxine B, cells were incubated with 5 μg/ml of the dye for 2 h with shaking at 30°C in darkness, centrifuged and washed twice with PBS. 10,000 cells from each sample were scanned using channel FL3 for propidium iodide and channel FL2 for phloxine B with FACSCalibur (Becton-Dickinson).

Protein extraction. Extracts were prepared in NET-N buffer [20 mM Tris HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP40, 1mM dithiothreitol (DTT), 1 mM phenylmethyl sulphonyl fluoride (PMSF), 5 μg/ml aprotinin, protease inhibitor cocktail (Sigma), 2 mM sodium fluoride (NaF), 0.2 mM sodium orthovanadate (Na_3VO_4), 2 mM β-glycerophosphate]. Cells were broken with glass beads in a BioSpec Minibeadbeater.

***In vitro* Chk1 kinase assay.** Substrates were prepared as GST fusion proteins in *E. coli* as described (Dutta et al, 2008). Protein extracts (1mg) from MMS-treated cultures of a strain with HA-tagged Chk1 were immunoprecipitated as described (Ayte et al, 2001), 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 5μg of substrate and 10μCi of [γ-^{32}P]ATP for 30 min at 30°C. Labeled proteins were resolved in 12% SDS-PAGE and detected by autoradiography.

Gene expression analysis. RNA extraction was performed as described (Moldon et al, 2008) and 10 μg of extracted RNA were loaded on agarose gels and analyzed by northern blot. *cdc18*, *cdc22*, and *tfb2* probes contained the complete ORFs of the genes.

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

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Author contributions: TI, BG-E and IA-R performed the experiments; JAD made monoclonal antibodies; NR made and provided strains; TI, IA-R, EH and JA analysed the data and commented on the manuscript; TI, EH and JA wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

FIGURE LEGENDS

Figure 1. Cdc10 is targeted by the DNA Damage Response. **(A)** Total RNA was prepared from untreated (-) or HU-treated (+) cultures of wild type (WT) and Yox1.SATA (SATA) cells and analyzed by hybridization to the probes indicated on the left. *rRNA* is shown as loading control. **(B)** Loading of Cdc10 on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or HU-treated (10 mM HU, 4h at 30°C) cultures of wild type (WT), Yox1.SATA (SATA), Δ*cds1*, Δ*chk1*, Δ*cds1*Δ*chk1* and Δ*rad3* cells. Cdc10 is HA tagged and the levels of binding were quantified on anti-HA immunoprecipitated DNA. The average of three individual experiments (±s.d.) is plotted. **(C)** The same chromatin extracts were analyzed for Yox1 binding with polyclonal antibodies anti-Yox1. The average of three individual experiments (±s.d.) is plotted. **(D)** Phosphorylation level of endogenous Chk1-HA in native extracts prepared from untreated (−) or HU-treated (+) cultures of wild type (WT), Yox1.SATA (SATA) and Δ*cds1* strains. Proteins were resolved in an 8% SDS–PAGE and anti-HA western blotted to detect Chk1.

Figure 2. Chk1 effect on Cdc10 is MMS-concentration dependent. **(A)** Loading of Cdc10 on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or treated with methyl methanesulfonate (MMS) (0.1% for 1h at 30ºC) cultures of a wild type (WT) strain. The average of three individual experiments (±s.d.) is plotted. **(B)** Same as in (A), but cells were irradiated (100Gy). The average of three individual experiments (±s.d.) is plotted. **(C)** Loading of Cdc10, Yox1 and Nrm1 on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or treated with the indicated MMS concentrations (1h at 30ºC) cultures. Cdc10 and Nrm1 are HA tagged and the levels of binding were quantified on anti-HA immunoprecipitated DNA, while Yox1 was determined with polyclonal antibodies anti-Yox1. The average of three individual experiments (±s.d.) is plotted. In the inset, phosphorylation level of endogenous Chk1-HA in native extracts prepared from untreated (−) or MMS-treated (1h at 30°C) cultures. Proteins were resolved in an 8% SDS–PAGE and anti-HA western blotted to detect Chk1. **(D)** Total RNA was prepared from untreated (-) or MMS-treated cultures of wild type cells and analyzed by hybridization to the probes indicated on the left. *rRNA* is shown as loading control. **(E)** Wild type cells were treated with different concentrations of MMS for the indicated time. Viability was measured by propidium iodine staining of the cells.

Figure 3. Cdc10 is released from chromatin upon DNA damage. (A) Loading of Cdc10 on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or treated (0.1% MMS, 1h at 30ºC) cultures of wild type (WT), Δ*cds1*, Δ*chk1*, Δ*cds1*Δ*chk1* and Δ*rad3* cells. Cdc10 is HA tagged and the levels of binding were quantified on anti-HA immunoprecipitated DNA. The average of three individual experiments (±s.d.) is plotted. **(B)** Loading of Yox1 on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or treated (0.1% MMS, 1h at 30ºC) cultures of wild type (WT), Δ*cds1*, Δ*chk1*, Δ*cds1*Δ*chk1* and Δ*rad3* cells. The average of three individual experiments (±s.d.) is plotted. **(C)** Total RNA was prepared from untreated (-) or 0.1% MMS-treated cultures of Δ*yox1*Δ*nrm1* cells for the indicated time and analyzed by hybridization to the probes indicated on the left. *rRNA* is shown as loading control. **(D)** Total RNA was prepared from untreated or MMS-treated (increasing doses, 1h at 30°C) cultures in *Δyox1Δnrm1* strain, and analyzed by hybridization with the probes indicated on the left. *rRNA* is shown as loading control.

Figure 4. Cdc10 Ser-720 and Ser-732 are phosphorylated by Chk1 inactivating MBF-dependent transcription. (A) Chk1 was immunoprecipitated from MMS-treated cultures and *in vitro* kinase activity (in arbitrary units) was measured in the same protein extracts, using wild type Cdc10 or the mutants indicated on top as substrates. Coomassie staining of the gel is shown at the bottom. **(B)** Amino acid sequence of the Cdc10 region that is phosphorylated by Chk1. The phosphorylation consensus is indicated at the bottom. **(C)** Loading of Cdc10 on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or treated (0.1% MMS, 1h at 30ºC) cultures of wild type Cdc10 (WT) or the mutants indicated at the bottom. The average of three individual experiments (±s.d.) is plotted. *P<0.05, t test. **(D)** Total RNA was prepared from untreated or MMS-treated (increasing doses) cultures in a Cdc10.2A or its parental (*Δyox1Δnrm1*) strains, and analyzed by hybridization with the probes indicated on the left. *rRNA* is shown as loading control. Above relative mRNA levels are shown **(E)** Cell cycle progression was measured by FACS analysis from untreated or MMS-treated (increasing doses and timing) cultures of wild type (WT), *Δchk1* and Cdc10.2A, using propidium iodide staining of the cells.

Figure 5. Cartoon depicting a model for the integration of the DNA damage and the DNA synthesis checkpoint on the MBF complex. Upon replicative stress, fission yeast cells activate the effector kinase Cds1. Among its targets, the repressor Yox1 is phosphorylated, which no longer can bind the MBF

complex alleviating the transcriptional repression of genes required for DNA synthesis. Upon DNA damage, the effector kinase Chk1 phosphorylates another component of the MBF complex, Cdc10. The outcome of this phosphorylation is, contrary to what happens under replicative stress, the repression of MBF-dependent transcription.

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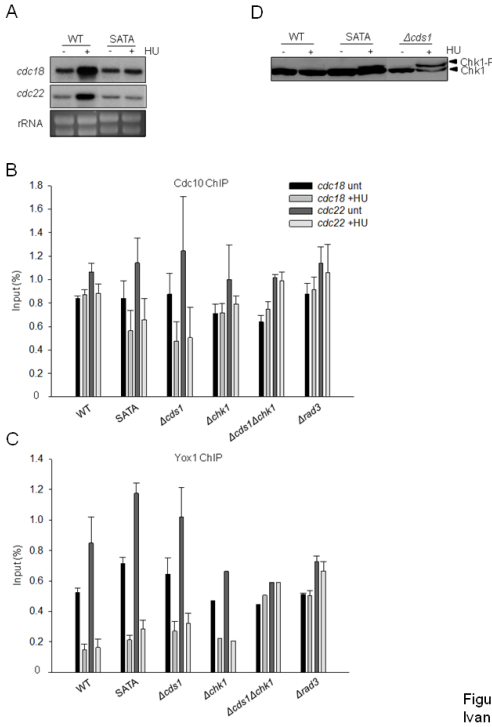

Figure 1
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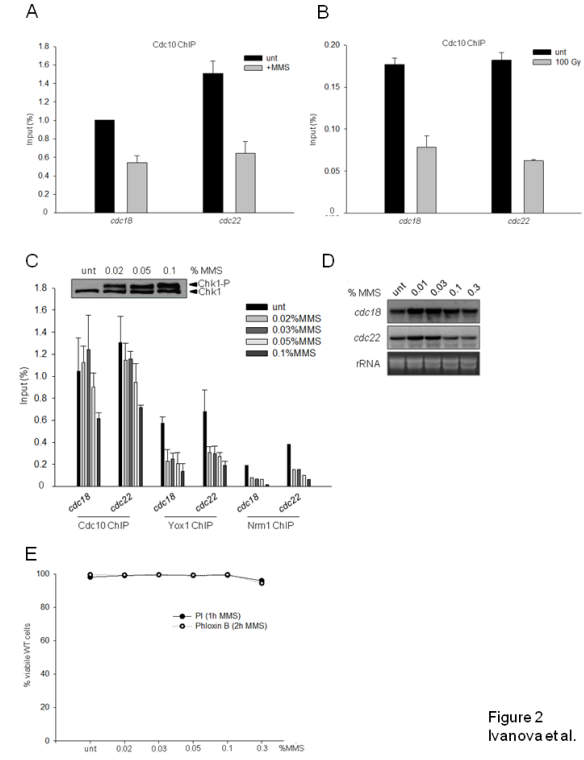

Figure 2
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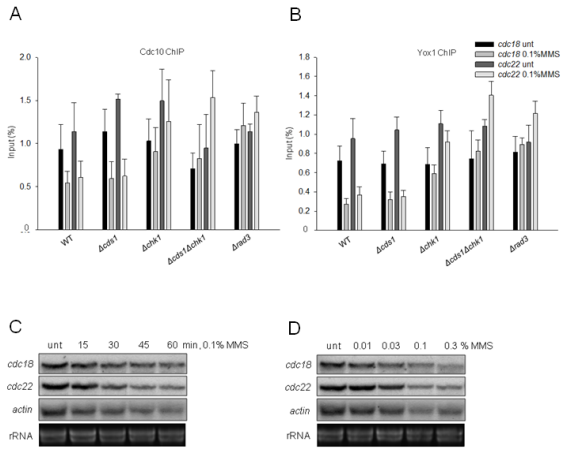

Figure 3
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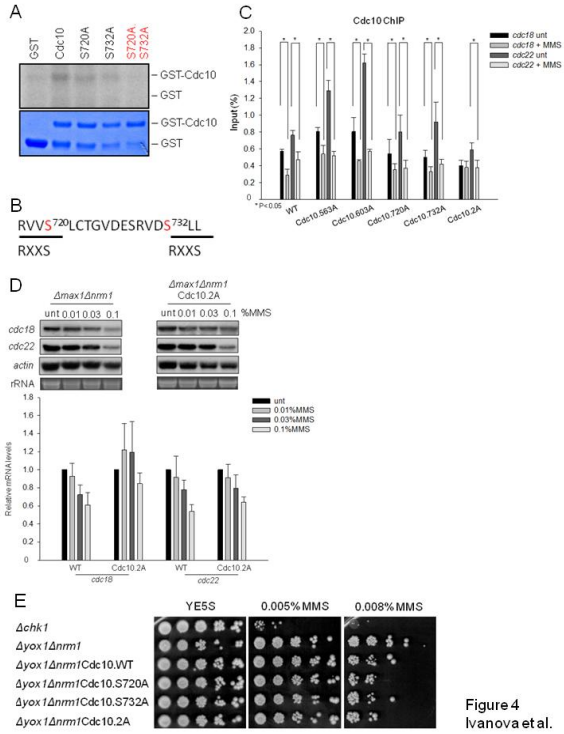

Figure 4
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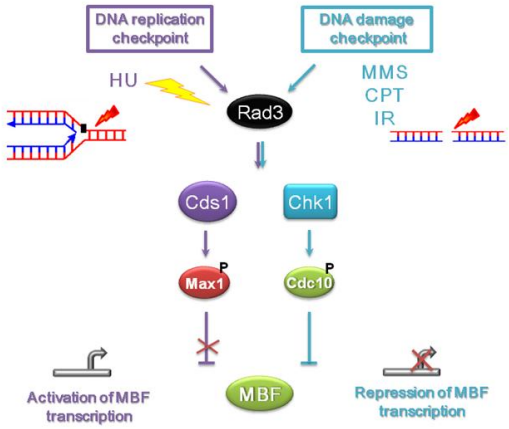


Figure 5
Ivanova et al.

SUPPLEMENTARY INFORMATION

Strains used in this work

Strain	Genotype	Figure
972	h-	1A,1D,2D,1E,4C,4E,s1,s2
JA974	max1S114-115A-13Myc-kan h-	1A
JA1077	9myc2HA6His:ura4+ ura4-D18 leu1-32 h-	1D,2C,4A
JA1405	chk1:9myc2HA6His:ura4+ cds1::kan ura4-D18	1D
JA1406	chk1:9myc2HA6His:ura4+ max1S114-115A-13Myc-kan ura4-D18	1D
JA770	cdc10-HA::Nat+ leu1-32 h	1B,1C,2A,2C,3A,3B,s2
JA1078	cdc10-HA-Nat+ yox1-114A-115A h+	1B,1C
JA1070	cds1::Kan+ cdc10-HA::Nat+ leu1-32 h-	1B,1C,3A,3B
JA1090	cdc10-HA-Nat+ chk1::ura4 ura4-D18 h?	1B,1C,3A,3B
JA1089	cdc10-HA-Nat+ cds1::Kan chk1::ura4 ura4-D18 h?	1B,1C,3A,3B
JA1069	rad3::Nat+ cdc10-HA::Kan+ h-	1B,1C,3A,3B
JA977	max1-13Myc-kan nrm1-HA-Nat h+	2C
JA672	chk1::ura4 ura4-D18 h+	1E,4E
JA1269	Cdc10-Kan h-	1E,2B,4C
JA1270	Cdc10-S3-Kan h-	1E,4C
JA1271	Cdc10-S5-Kan h-	1E,4C
JA1272	Cdc10-S3,S5-Kan h-	1E,4C,4E
JA1143	cdc10-8A:kanMX6 h?	1E
JA1016	nrm1::kan max1-13Myc-Nat::ura4 ura4-D18 h?	3C,3D
JA1407	chk1-HA-Kan+ h-	4A
JA1408	Cdc10-S563A-Kan h+	4C
JA1409	Cdc10-T603A-Kan h-	4C
JA1412	max1::Pheo nrm1::Nat Cdc10-Kan h+	4D,4E
JA1413	max1::Phleo nrm1::Nat Cdc10-S3A-Kan h-	4E
JA1414	max1::Phleo nrm1::Nat Cdc10-S5A-Kan h-	4E
JA1415	max1::Pheo nrm1::Nat Cdc10-S3,S5-Kan h-	4D,4E
JA1411	max1::Phleo nrm1::Nat h+	4E
JA803	cdc10-C4 h+	s1

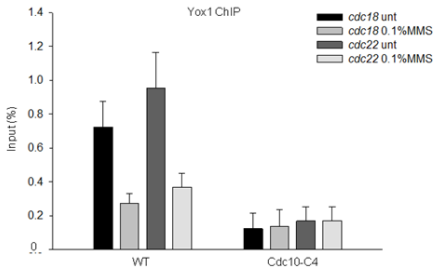
SUPPLEMENTARY METHODS

Protein extraction. Extracts were prepared in NET-N buffer [20 mM Tris HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP40, 1mM dithiothreitol (DTT), 1 mM phenylmethyl sulphonyl fluoride (PMSF), 5 μg/ml aprotinin, protease inhibitor cocktail (Sigma), 2 mM sodium fluoride (NaF), 0.2 mM sodium orthovanadate (Na_3VO_4), 2 mM 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, previously crosslinked with α-HA monoclonal antibody. Immunoprecipitates were washed after 1 hour of incubation three times with NET-N buffer and resolved in SDS-PAGE, transferred to nitrocellulose membranes and blotted with the indicated antibody.

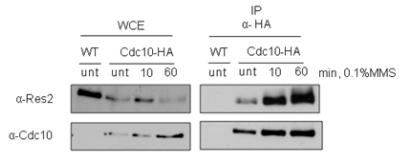
SUPPLEMENTARY FIGURES

Supplementary Figure 1. C-terminal region of Cdc10 loads Yox1/Nrm1 repressor system onto chromatin. Loading of Yox1 on *cdc22* and *cdc18* promoters was measured in untreated or MMS-treated (0.1% MMS, 1h at 30ºC) cultures of WT and *Cdc10-C4* strain by ChIP. The average of three individual experiments (±s.d.) is plotted.

Supplementary Figure 2. Cdc10 preserves interaction with Res2 after DNA damage. Extracts (2.5mg) from WT (972) and Cdc10-HA strains (with or without MMs treatment for the time indicated on the top) were immunoprecipitated with α-HA antibody, western blotted and analyzed for the presence of Res2 and Cdc10 with specific antibodies. WCE (whole cell extract).



Suppl Figure 1
Ivanova et al.



Suppl Figure 2
Ivanova et al.

Chapter III

1. Architectural hints of the MBF complex

Fission yeast cells can live with at least one of the two DNA-binding components of the MBF complex, Res1 or Res2. We decided to characterized Cdc10 binding to its target promoters in the absence of each one of this DNA-binding proteins. We have determined that binding of Cdc10 is dependent on an intact MBF complex. The absence of either Res1 or Res2 abolishes Cdc10 promoter association even in basal conditions. Furthermore, the level of binding keeps unchanged after DNA damage (Fig.1).

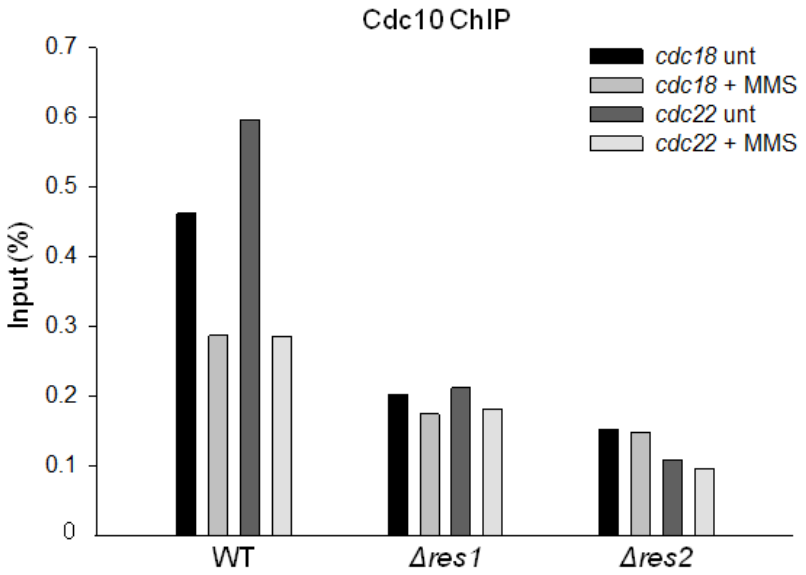


Fig.1 l Cdc10 binding is dependent on intact MBF complex. Loading of Cdc10 on *cdc18 and cdc22* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or treated (0.1% MMS, 1h at 30ºC) cultures of wild type (WT), Δ*res1* and Δ*res2* cells.

2. Crosstalk between the two effector kinases, Cds1 and Chk1

When DNA synthesis is challenged, the checkpoint kinase Cds1 responds both to block cell cycle progression and to regulate the MBF dependent gene transcription in order to overcome the block avoiding any DNA damage. Recently, it has been shown by our group (Gomez-Escoda et al., 2011) that Yox1 is phosphorylated by Cds1 at Ser-114 and Thr-115 in cells treated with HU. This phosphorylation releases Yox1 from MBF, triggering the transcriptional induction of all the MBF-dependent genes. Interestingly, we have demonstrated that a mutant Yox1 that cannot be phosphorylated by Cds1 at these residues (Yox1.SATA) remains bound to MBF (in particular to Cdc10) and cells cannot trigger the appropriate transcriptional response: the transcription is not de-repressed upon induction of the DNA-synthesis checkpoint (Fig.2 A). However, when measuring Yox1 promoter association by ChIP, and to our surprise, Yox1.SATA was released to almost the same extent as wild type Yox1. Interestingly, we could observe the same results in a *Δcds1* mutant. However Yox1 was retained in a *Δrad3* background (Fig.2 B).

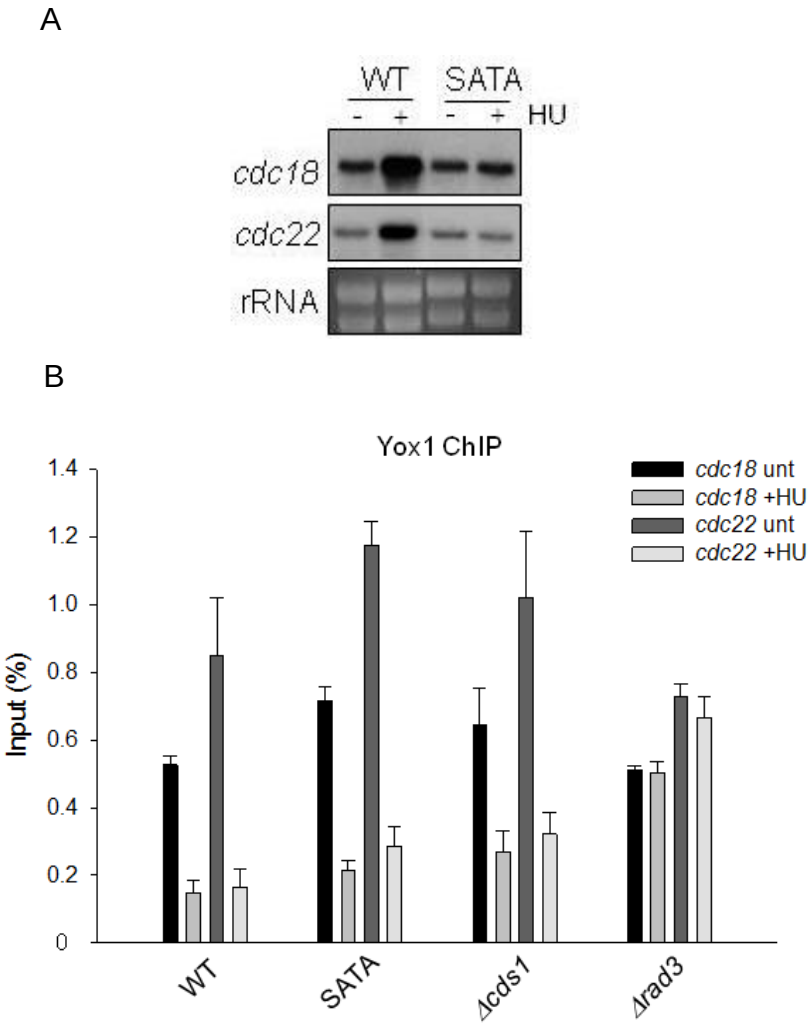


Fig.2. | Yox1 is released from MBF dependent gene promoters upon replication stress. A) mRNA levels of *cdc22* and *cdc18* were measured by northern blot analysis. Total RNA was prepared from untreated or HU-treated (+ 10mM HU, 4h at 30 ºC) cultures in wild tipe (WT) and SATA (Yox1.SATA) strains, and analyzed by hybridization with a *cdc18 and cdc22* probes. rRNA is shown as loading control. B) Loading of Yox1 on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or HU- treated (+ 10mM HU, 4h at 30 ºC) cultures of WT, SATA, *Δcds1* and *Δrad3* cells. The average of three individual experiments (±s.d.) is plotted.

This observation was very interesting and difficult to reconcile with the fact that transcription was not induced in a Yox1.SATA background. Therefore, we next explored the promoter binding of the MBF complex core element, Cdc10. Up till now, it has been always reported that MBF and, in particular, Cdc10 is constitutively bound to its target promoters (Wuarin et al., 2002; and WT in a Fig.3). Surprisingly, we observed that either in the SATA mutant background or in *Δcds1* cells, Cdc10 was released upon HU treatment. This release was dependent on the activity of the other effector kinase, Chk1: when Chk1 activity is abrogated (either upon deletion, like in a *Δchk1* strain or in a *Δcds1Δchk1* strain; or not activated, like in *Δrad3* strain), Cdc10 remains bound to its target promoters (Fig.3).

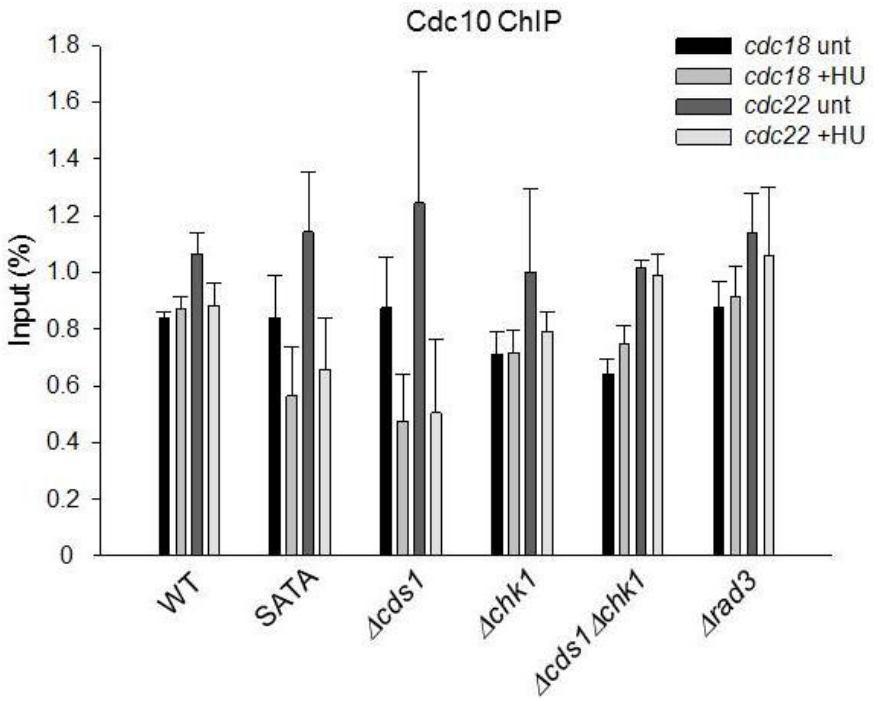
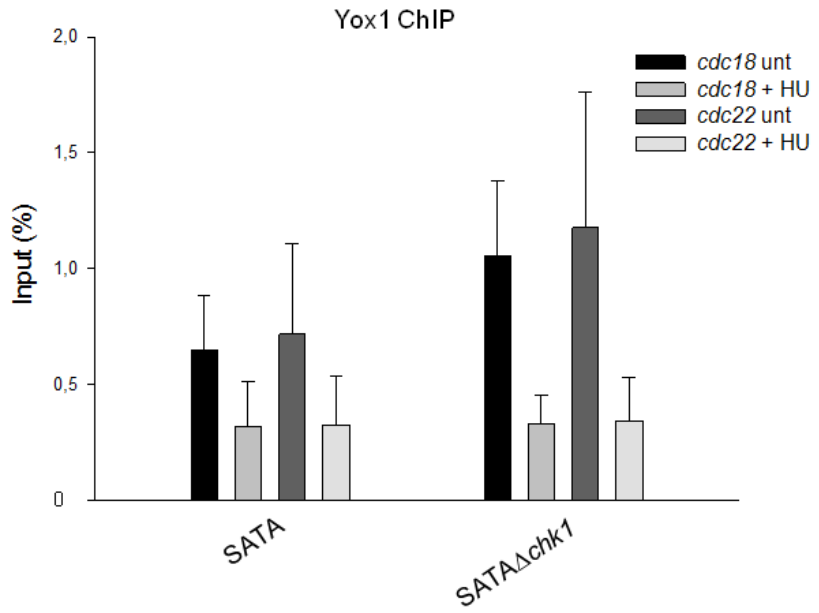


Fig.3. | Chk1 driven Cdc10 promoter eviction upon replication stress. Loading of Cdc10 on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or HU-treated (+ 10mM HU, 4h at 30 ºC) cultures of WT, SATA, *Δcds1, Δchk1, Δcds1Δchk1* and *Δrad3* cells. The average of three individual experiments (±s.d.) is plotted.

Since the single left available checkpoint kinase in conditions when the replication checkpoint response was abolished is Chk1, we hypothesized that there could be a crosstalk between both checkpoint responses that helps to prevent major damage to the cells. And this pointed immediately the question of could Cdc10-or other core component of MBF- be a target of Chk1? As we have shown, Yox1 is released from its target promoters in Yox1.SATA

background after HU treatment. However, Chk1 activation under these conditions, is able to phosphorylate Cdc10. Indeed as the two proteins (Yox1 and Cdc10) keep interacting (Gomez-Escoda et al., 2011), this phosphorylation leads to the dissociation of the whole complex Cdc10-Yox1.SATA. The final outcome is impaired MBF gene transcription. If this was the case what we were expecting is that this release will be abrogated in a *Δchk1* background. Surprisingly, the two proteins Yox1 and Cdc10 were also evicted from promoter binding, even to less extend, when the DNA damage checkpoint response is abrogated in Yox1.SATA *Δchk1* cells (Fig.4), when we have observed that normally in *Δchk1* cells the Cdc10 component is constitutively bound to its promoters (Chapter III, Fig.3). Additionally, there is no induced transcription after HU treatment, as there is no active MBF complex associated with the corresponding promoter (measuring the *cdc22* gene transcription). However, in this genetic background, Yox1.SATA*Δchk1*, Cdc10 release is probably due to compensating effect of Cds1, as in conditions when the complete checkpoint response is abrogated (in *Δcds1Δchk1* or *Δrad3* cells), neither Cdc10 nor Yox1.SATA mutant are evicted from promoter association. And this is again another confirmation of the existence of a crosstalk between the two checkpoint responses in order to ameliorate the danger to the cell, by ensuring the robustness of the system.

A


Yox1 ChIP
cdc18 unt
cdc18 + HU
cdc22 unt
cdc22 + HU
Input (%)
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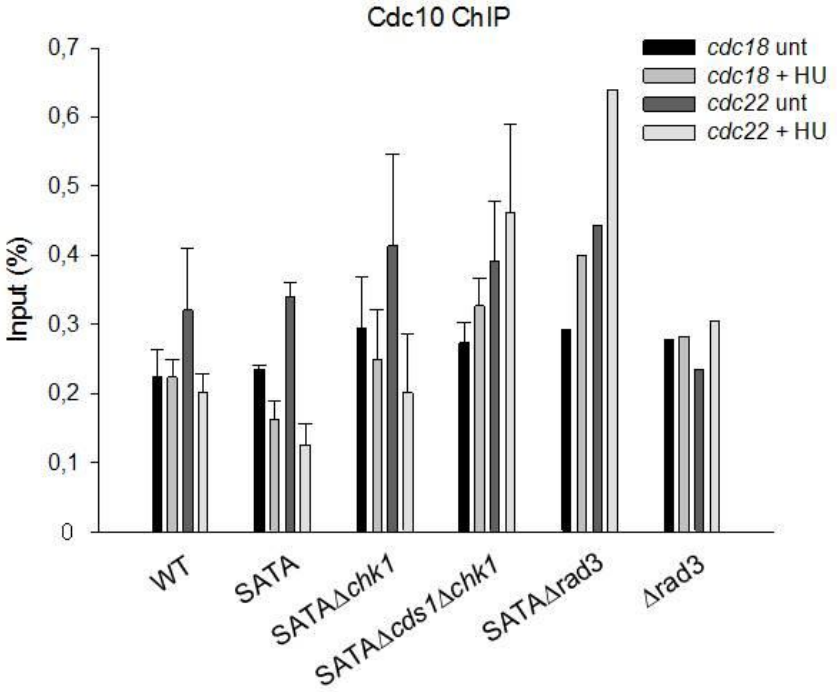
1,5
1,0
0,5
0
SATA
SATAΔchk1

B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cdc18 unt
cdc18 + HU
cdc22 unt
cdc22 + HU
Input (%)
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0,6
0,5
0,4
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SATAΔchk1
SATAΔcds1Δchk1
SATAΔrad3
Δrad3

C

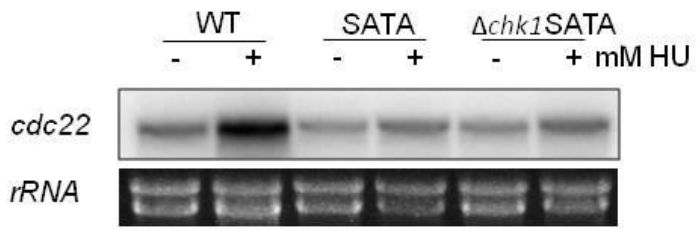


Fig.4 l Checkpoint crosstalk upon replication stress. A) Loading of Yox1 protein on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or HU-treated (+ 10mM HU, 4h at 30 ºC) cultures of SATA and SATA*Δchk1* cells. B) Loading of Cdc10 protein on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or HU- treated (+ 10mM HU, 4h at 30 ºC) cultures of WT, SATA, SATA*Δchk1,* SATA*Δcds1Δchk1*, SATA*Δrad3* and *Δrad3* cells. C) *cdc22* mRNA levels were measured by northern blot analysis. Total RNA was prepared from untreated or HU-treated (+ 10mM HU, 4h at 30 ºC) cultures in WT, SATA and *Δchk1*SATA strains, and analyzed by hybridization with a *cdc22* probe. rRNA is shown as loading control.

3. The effect of DNA damage is dose dependent

We measured the binding of three components of the MBF complex, Cdc10, Yox1 and Nrm1, to their target promoters after applying increasing doses of the DNA damaging agent MMS. As we can observe, the three of them were released from their target promoters, with Cdc10 requiring the highest MMS concentration to be released,

while Yox1 and Nrm1 are released even when minimal MMS doses were added to the cell cultures (Fig.5).

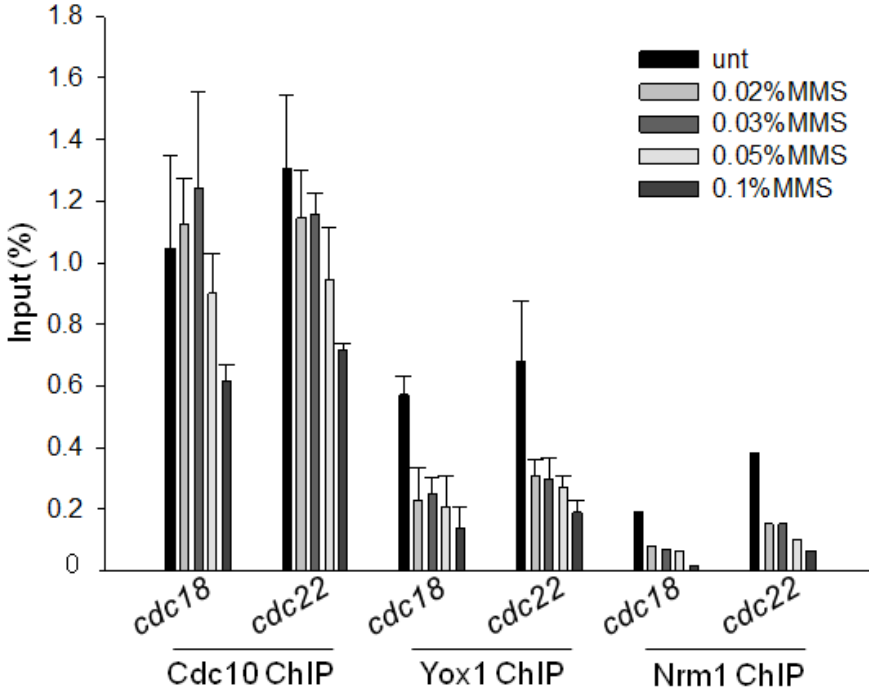


Fig.5 I Chk1 effect is MMS-concentration dependent. Loading of Cdc10, Yox1 and Nrm1 on *cdc22* and *cdc18* promoters was measured in untreated or MMS-increasing doses treated (1h at 30ºC) cultures by ChIP. Cdc10 and Nrm1 are HA tagged and the levels of binding were quantified on anti-HA immunoprecipitated DNA. The average of three individual experiments (±s.d.) is plotted.

This release of the MBF repressor system Nrm1-Yox1 explains the accumulation of *cdc18* mRNA that we observed at the low MMS doses, both in a wild type or in *Δchk1* cells. However, higher doses of the drug lead to transcriptional repression as neither Nrm1-Yox1, nor Cdc10 could bind any more (Fig.6. A). In parallel we have measured also γ- H2A

levels, used as a marker of DNA damage, and there is a considerable increase of phosphorylated γ-H2A after applying MMS treatment (Fig.6 B).

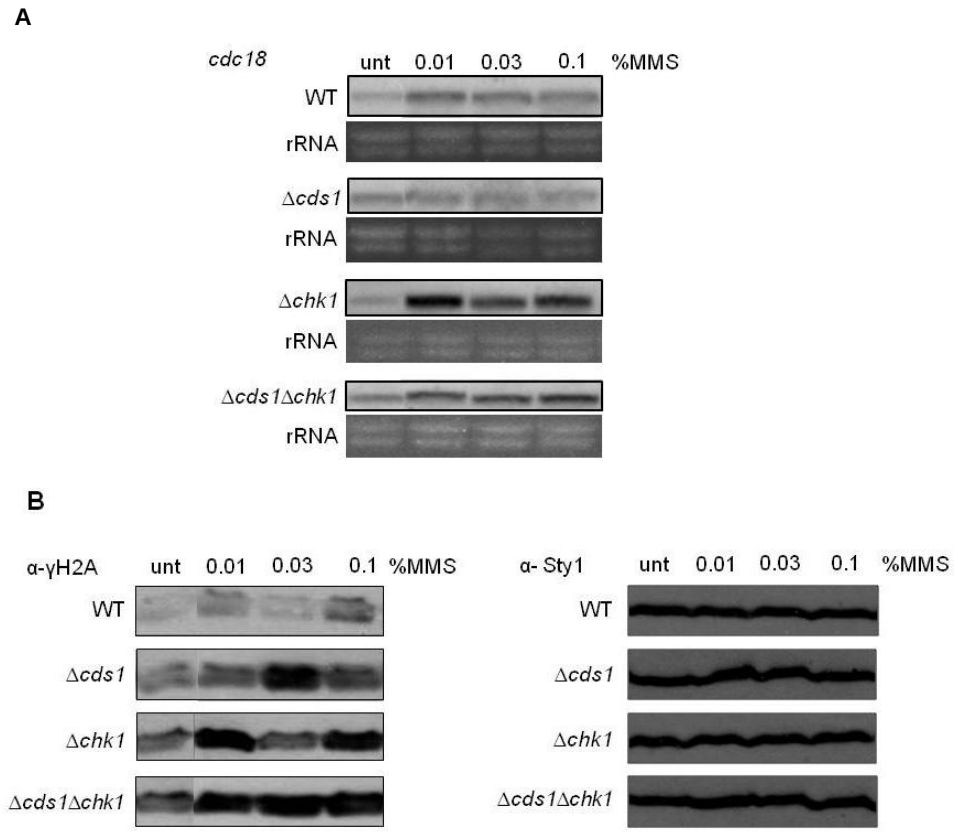


Fig.6 I Repression of MBF dependent transcription upon DNA damage. A) *cdc22* mRNA levels were measured by northern blot analysis. Total RNA was prepared from untreated or MMS-treated (increasing doses) cultures in wt, *Δcds1, Δchk1, Δcds1Δchk1* strains, and analyzed by hybridization with a *cdc18* probe. rRNA is shown as loading control. B) Phosphorylation level of H2A at Ser129 in native extracts prepared from untreated (−) or MMS treated cultures of wt, *Δcds1, Δchk1, Δcds1Δchk1* strains. Proteins were resolved in a 15% SDS–PAGE and anti-H2AP-S129 western blotted to detect phosphorilation. Anti- Sty1 was used as loading control (the panel on the left).

Moreover, as Yox1 and Nrm1 are released from MBF promoters at low MMS doses, and as both proteins are Cds1 targets upon replication stress, we next examined the kinetics of Yox1 promoter association in the absence of the replication checkpoint. We could observe that Yox1 dissociation was diminished, especially for the low MMS doses, in a *Δcds1* background (Fig.7). This more permanent association of Yox1 with its target promoters correlates with constitutive repression of MBF dependent gene transcription (Fig.6. A).

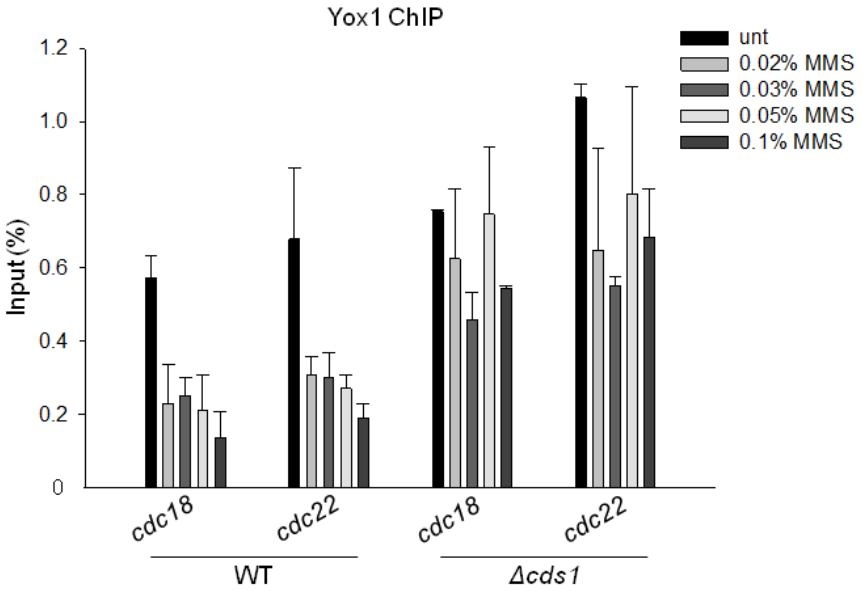


Fig.7 l Cds1 dependent release of Yox1 upon DNA damage
Loading of Yox1 on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or MMS-treated at different concentrations (1h at 30ºC) cultures of wild type (wt) and Δ*cds1* cells. The average of three individual experiments (±s.d.) is plotted.

4. Cdc10 is phosphorylated by Chk1

To determine the specific sites on Cdc10 that were phosphorylated, we took advantage of our previous observation that the *cdc10-C4* strain, which lacks the last 61 amino acids of Cdc10 and is insensitive to MMS. The carboxi-terminal region of Cdc10 contains two consensus phosphorylation sites capable of being phosphorylated by Chk1. Additionally there are two more consensus sites in the middle of the protein, next to an ankyrin repeat sequence (Fig.8).

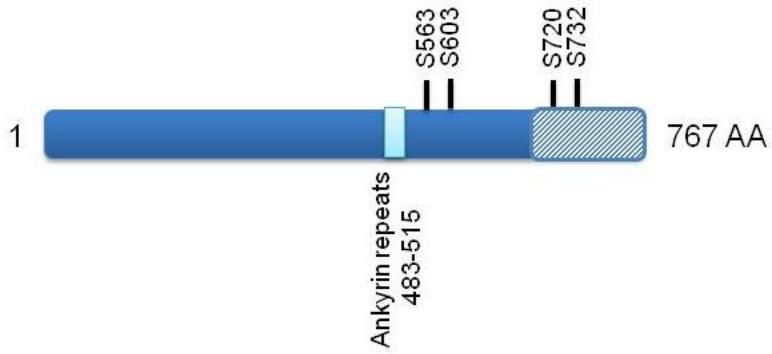
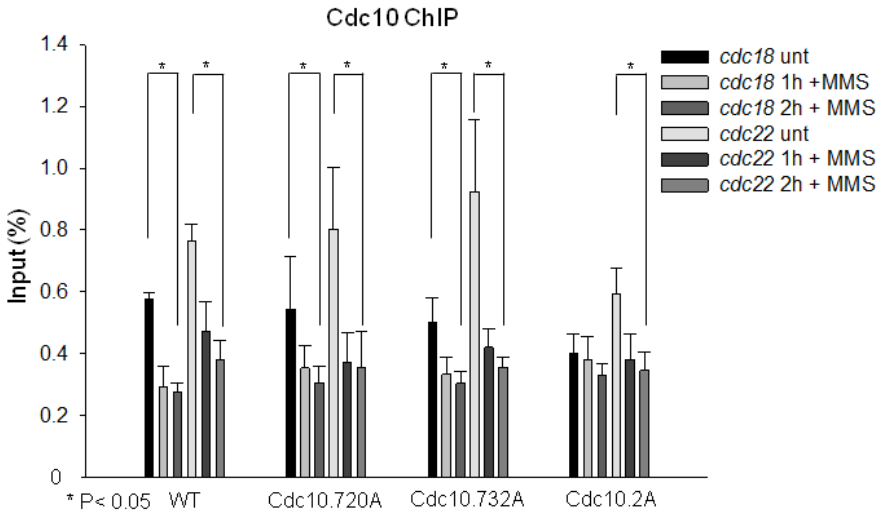


Fig.8 I Schematic representation of Cdc10 phosphorylation sites.

To test whether Cdc10 phosphorylation by Chk1 is essential for the regulation of the DNA damage checkpoint, we introduced serine-to-alanine mutations in fission yeast, replacing the endogenous copy of *cdc10*. When treated with MMS, the strains that carry the single mutations were responding in a similar manner to a wild type strain, in the sense that Cdc10 was released from its target promoters, and

this release was significantly different with p<0.05. However, a strain that carries a double mutation of the two carboxy-phosphorylation sites at S720 and S732 (Cdc10.2A) cannot release Cdc10 from the *cdc18* promoter after MMS treatment, as the difference is not significant (Fig.9 A). In contrast, we couldn`t observe the same binding capacity of Cdc10 on *cdc22* promoter, pointing that the two MBF genes could be differently regulated. Similar promoter binding profile of Cdc10 was obtained for Cdc10.2A mutant also after IR (Fig.9 B)

A



B

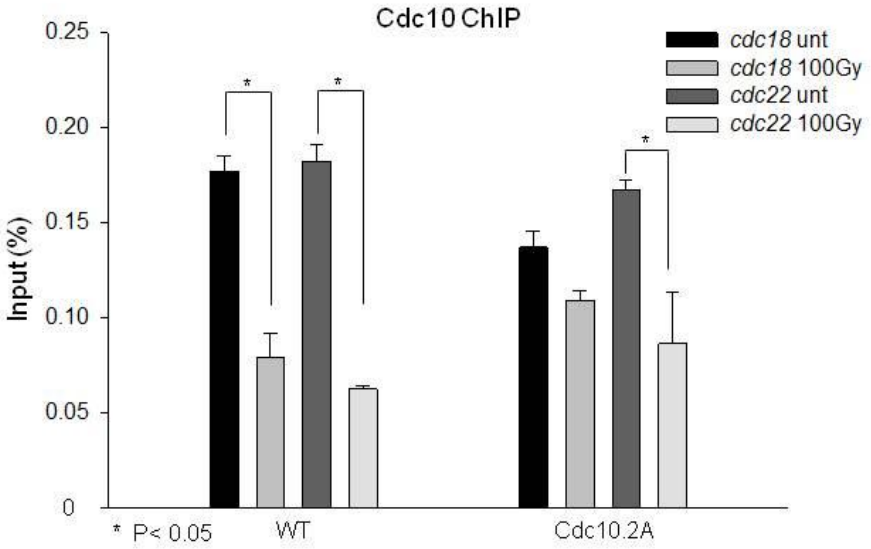


Fig.9 I Phosphorylation of S720 and S732 release Cdc10 from chromatin after DNA damage A) Loading of Cdc10 on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or MMS-treated (0.1%MMS, 1h at 30ºC) cultures of WT, Cdc10.S563A, Cdc10.T603A, Cdc10.S720A, Cdc10.S732A and Cdc10.2A cells. The average of three individual experiments (±s.d.) is plotted. B) Loading of Cdc10 on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or IR (100Gy) cultures of WT and Cdc10.2A cells. The average of three individual experiments (±s.d.) is plotted, * p<0.05, t test.

Furthermore, to determine which is the *in vivo* effect of the DNA damage checkpoint on Cdc10.2A mutant, which we have seen cannot sense the DDR, we performed spot analysis using different drugs. Indeed, the double mutant was slightly sensitive to CPT, but not to MMS or IR (all of which induce DSBs), neither to HU (Fig.10).

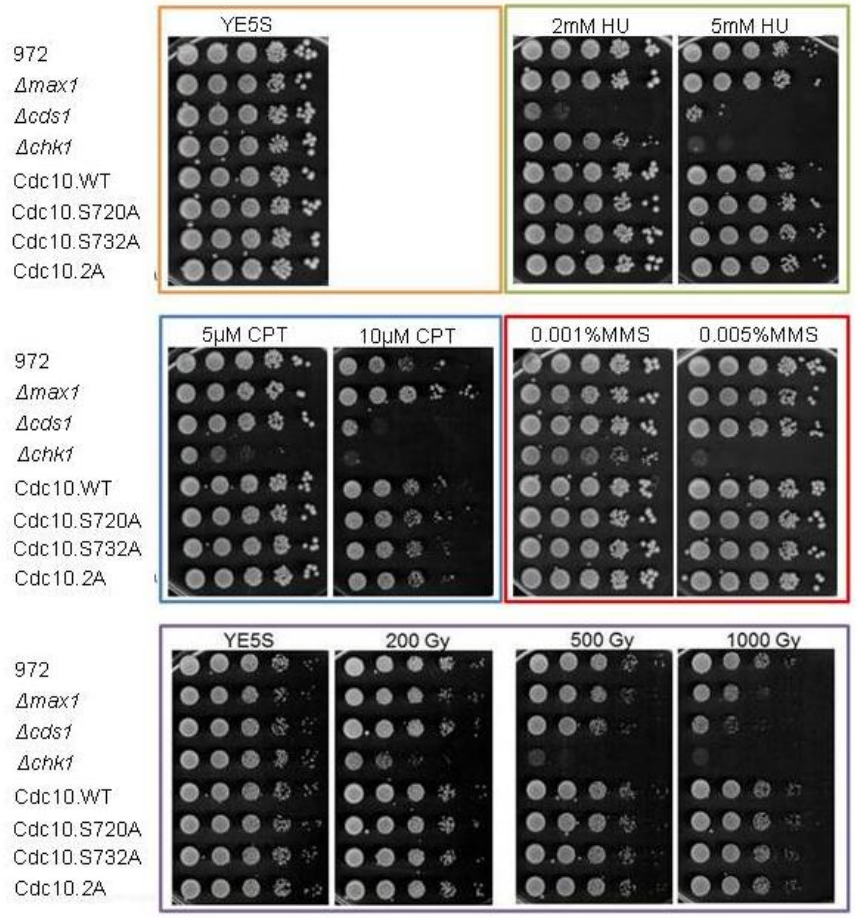


Fig.10 I Slight effect of S720 and S732 on survival after CPT induced DNA damage. Survival was performed by spotting 10 to 10^5 cell of WT, *Δyox1*, *Δcds1*, *Δchk1*, Cdc10.WT, Cdc10.S720A, Cdc10.S732A, Cdc10.2A strains onto YE5S plates with different drugs at the indicated concentrations and incubated at 30°C for 3 to 4 days.

In order to find out also an *in vivo* effect on survival, but of the DNA damaging agent MMS, Cdc10 mutants in *Δyox1Δnrm1* background were tested on spots, starting with an already upregulated MBF gene transcription. We could

detect again slight sensitivity of Cdc10 mutants against the highest MMS dose treatment (Fig.11).

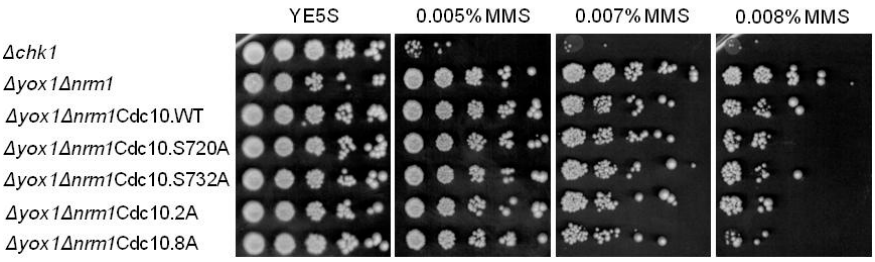


Fig.11 l *In vivo* effect of S720 and S732 on survival after MMS induced DNA damage. Survival was performed by spotting 10 to 10^5 cell of *Δchk1*,*Δyox1Δnrm1*,*Δyox1Δnrm1*Cdc10.WT,*Δyox1Δnrm1*Cdc10.S720A, *yox1Δnrm1*Cdc10.S732A,*Δyox1Δnrm1*Cdc10.2A, *Δyox1Δnrm1*Cdc10.8A strains onto YE5S plates with the indicated MMS concentrations and incubated at 30°C for 3 to 4 days.

5. *In vivo* determination of the Cdk- and Cds1-phosphorylation sites in Yox1 and Nrm1

In *S. pombe* the MBF repressor system Yox1/Nrm1 seems to be regulated through phosphorylation either by the main Cdk kinase Cdc2 during the cell cycle, but also by the replication checkpoint Cds1 under DNA-synthesis stress.

The protein levels of the three MBF components Cdc10, Res1 and Res2 are constant over the cell cycle. Lately we have shown that a Yox1 protein level also does not change through the cell cycle. However, when measuring Yox1 promoter association (for example at *cdc18* gene) Yox1 fluctuates, showing a maximum binding at G2/M and

dissociaton at G1/S phase, when exactly the transcription is induced (Fig.12).

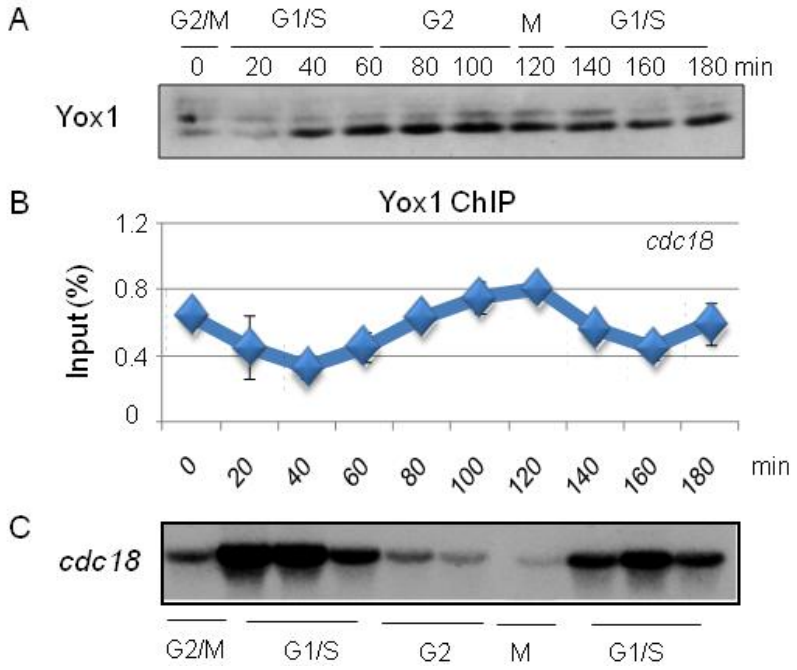


Fig.12 l Yox1 protein levels and chromatin oscillation during cell cycle. A) Cellular abundance of Yox1 protein was monitored in synchronous cultures after *cdc25-22* block (4h at 36°C) and time (minutes) after the release at 25°C is indicated. Native extracts were western blotted and detected with α-Myc antibody. B) Loading of Yox1 on *cdc18* promoter was measured by ChIP analysis of chromatin extracts isolated from synchronized cells after *cdc25-22* block. The average of three individual experiments (±s.d.) is plotted. C) *cdc18* mRNA levels were measured by northern blot analysis. Total RNA was prepared from synchronized cells after *cdc25-22* block.

As we observed a cell cycle-regulated binding of Yox1 to MBF-dependent promoters, we wondered if a Cdk driven phosphorylation could modulate the activity of Yox1. We

have noticed that Yox1 has three putative Cdk sites at positions S6, T55 and T75 and by *in vitro* kinase assay we have shown that only the S6 (which is also the only one full consensus Cdk site) is phosphorylated by the complex Cdc2/Cdc13 (Fig.13).

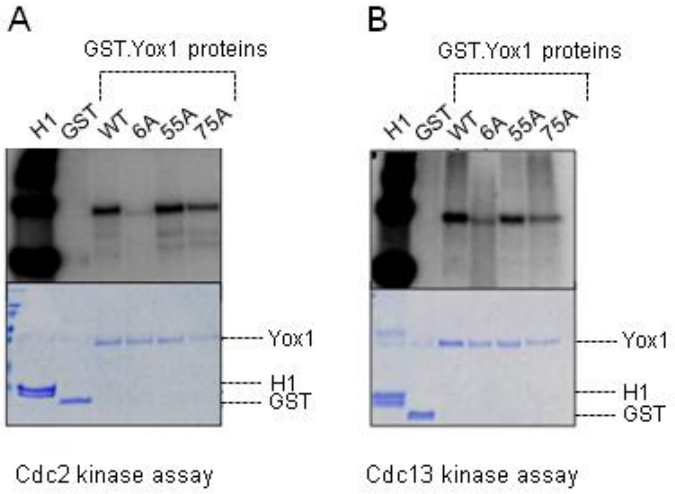


Fig.13 l Kinase assay of Yox1 Cdk-mutants. A) Kinase Assay of immunopurified Cdc2-HA over Histone 1 (H1), GST, and recombinant fusion proteins GST-Yox1, GST-Yox1-6A, GST-Yox1-55A and GST-Yox1-75A. B) Kinase assay of immunopurified Cdc13-GFP over Histone 1 (H1), GST, and recombinant fusion proteins GST-Yox1, GST-Yox1-6A, GST-Yox1-55A and GST-Yox1-75A.

However this phosphorylation did not show to have any significant role as in *in vivo* experiments the mutant Yox1.S6A, which cannot be phosphorylated by the Cdk, has no effect either on association of the protein with the MBF complex nor on regulation of MBF gene transcription.

Since we have previously shown that Nrm1 is required for Yox1 loading onto MBF (Gomez-Escoda et al., 2011) we

decided to determine whether Nrm1 could be targeted by Cdk activity and thus involved in MBF regulation. After *cdc25-22* block and release, Nrm1 protein levels did fluctuate (Fig.14.A), with an accumulation at G1/S/G2 and rapid depletion at late G2/M phase. This can be detected at least in two consecutive cell cycles. In addition, the timing of Nrm1 protein accumulation follow exactly its promoter binding, peaking at G2; and its dissociation at G1/S, which coincides with activation of MBF dependent gene transcription (Fig.14.B,C).

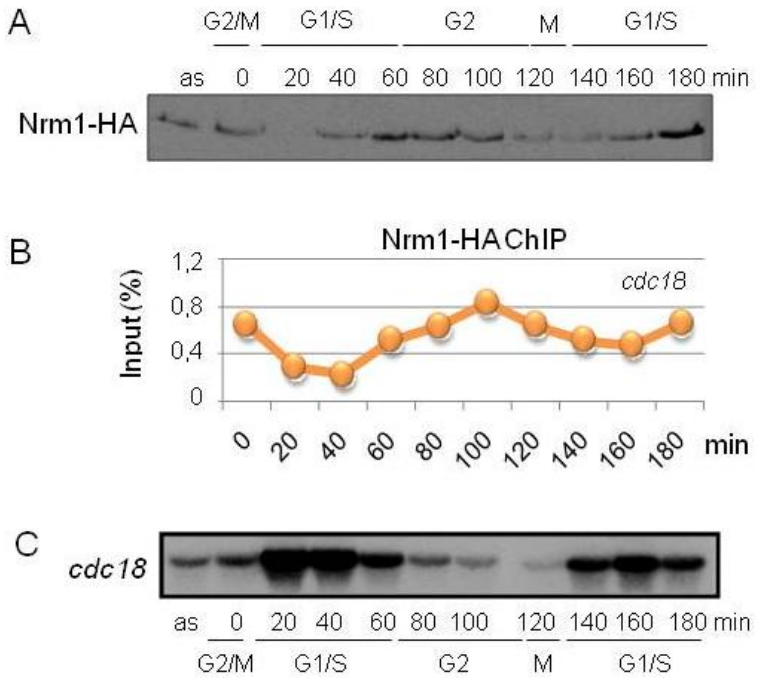


Fig.14 l Nrm1 protein levels and chromatin oscillation during cell cycle. A) Cellular abundance of Nrm1 protein HA tagged in its C-terminus was monitored in synchronous cultures after *cdc25-22* block (4h at 36°C) and time (minutes) after the release at 25°C is indicated. Native extracts were western blotted and detected with α-HA antibody. B) Loading of Nrm1 on *cdc18* promoter was measured by ChIP analysis of chromatin extracts isolated from synchronized cells after *cdc25-22* block.

The average of three individual experiments (±s.d.) is plotted. C) *cdc18* mRNA levels were measured by northern blot analysis. Total RNA was prepared from synchronized cells after *cdc25-22* block.

Since this rapid and considerable decrease in Nrm1 protein levels occurs concomitantly when there is a highly active complex of Cdc2/Cdc13 in the cell, we wondered if Nrm1 could be targeted by the proteasome upon Cdc2/Cdc13 phosphorylation. In order to answer this, we measured Nrm1 protein levels either after total inhibition of the proteasome by the drug MG-132 (Fig.15.A), or after depleting different components of the proteasome, in *mts2-1* or *mts3-1* (ts) strains. We could observe an accumulation of Nrm1 protein level, which was Mts3 dependent (Fig.15.B). Because, many regulating proteins are coordinately degradated by the ubiquitin-proteasome pathway, we next determined the E3 ligase through which Nrm1 is ubiquitinated . As we can see in Fig.15.C, Nrm1 ubiquitin mediated proteolisis is driven by the APC/C (anaphase-promoting complex/cyclosome) and not by SCF (Skp1-Cullin-F-box protein complex), as we could detect a clear increase of Nrm1 protein level in *slp1-1* (a component of the APC/C), and not in *skp1-A7* (part of the SCF) ts strains.

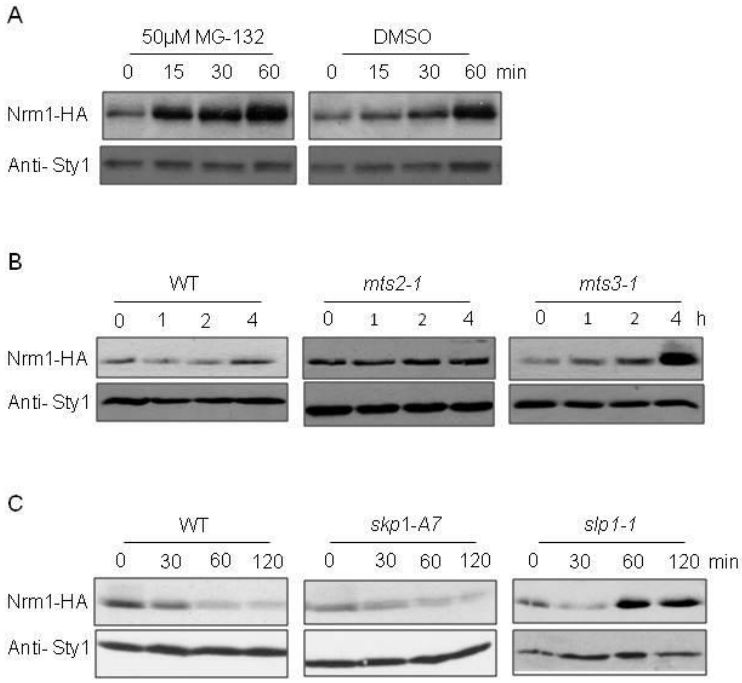


Fig.15 l Nrm1 protein degradation. A) Levels of Nrm1 protein HA-tagged were monitored in cells treated with 50μM MG-132 inh. or with DMSO as a control, and time (minutes) after the treatment is indicated. Native extracts were western blotted and detected with α-HA antibody. α-Sty1 was used as a loading control. B) Levels of Nrm1 protein HA-tagged were monitored in WT, *mts2-1* and *mts3-1* cells and time (hours) after the shift to 36˚C is indicated. Native extracts were western blotted and detected with α-HA antibody. α -Sty1 was used as a loading control. C) The same as in B), monitoring WT, *skp1-A7* and *slp1-1* strains.

Further, as APC substrates are targeted by the complex through their degradation motifs, principally a Destruction box (D-box; R/KxxL/I/M/VxxI/LxN) and KEN-box, we looked for the presence of such motifs in Nrm1 protein. Nrm1 has one KEN- box and three D boxes (two in tandem, and one in the middle of the protein) (Fig.16). We mutated those motifs, but this work is still in progress.

1 MDRSMEPLTPSRLNTLGERPTNEVYEYGKGKNVQHLFPITPMQRPLGKEN
51 AAPGTISPIAVRSRNVRAVEIADENACEEPVLKIKSVSSTESEEEKESST
101 EIGEKQEKETHLEPKTPVQTNTNNNHLDDIQCCAKNLRLRLELAMYKVQV
151 NQTFSPLQDLPIVAKTKLHNCPNSEPVTSIWNQRSLSSGKPPSLHLSGNR
201 RLSMGSPTKSIYDQNGLTTPRPIGSDDLTHMYDPYTSPLRTPSRTLSRSS
251 SHYLWVRHGKLTRSVSLLQHKTPRRIRPKSLSKSNSTPLKHLSAQKPNSN
301 YYTGPPTPVSISNTPENIHPSSSEVRRIASHSKQFSDYGLIR

Fig.16 l Nrm1 protein sequence. Red lined box KEN-box, blue lined boxes D-box sequences.

Next, to figure out if Nrm1 degradation is driven because of Cdk phosphorylation, we looked for the presence of Cdk sites. Nrm1 has 7 putative consensus sites, two in the amino part, and five in its carboxy part (Fig.17).

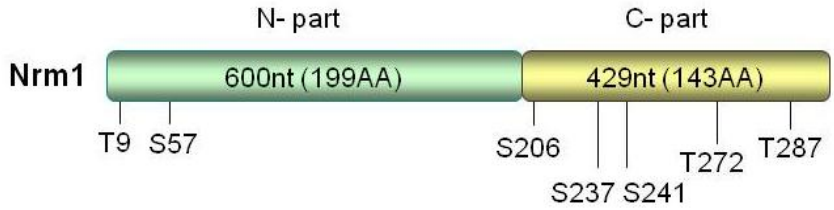


Fig.17 l Schematic representation of Nrm1 Cdk phosphorylation sites. In green and yellow filled bars are represented the N- and C- part of Nrm1.

On *in vitro* kinase assay we encountered the problem that either the full length or the C-terminal part, when expressed in bacteria, is extremely unstable. When analysing the N-terminal region, we found that both T9 and S57 are good substrates of Cdc2/Cdc13 kinase (Fig.18).

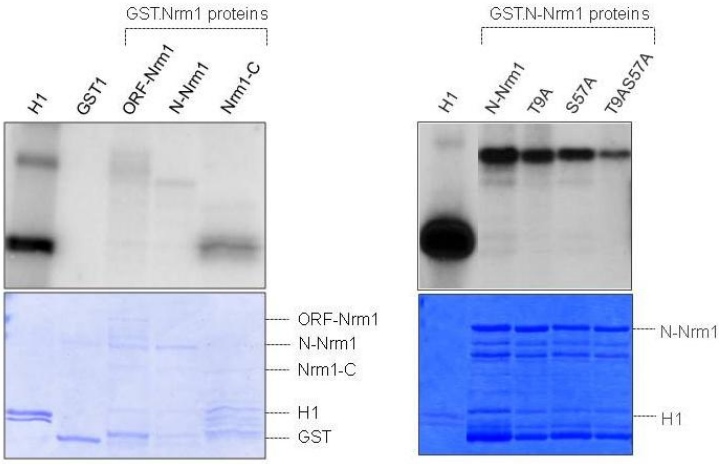
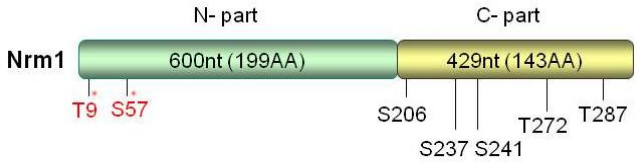


Fig.18 I Kinase assay of Nrm1 Cdk-mutants. On the left kinase assay of immunopurified Cdc2-HA over Histone 1 (H1), GST, and recombinant fusion proteins GST.Nrm1: ORF-Nrm1 (full length Nrm1), N-Nrm1 (amino part of Nrm1, 199AA), T9A (amino part of Nrm1 Thr9 mutated to Ala), Nrm1-C (carboxy part of Nrm1 143AA); On the right kinase assay of immunopurified Cdc2-HA over Histone 1 (H1), GST, and recombinant fusion proteins GST.N-Nrm1, GST.N-Nrm1.T9A, GST.N-Nrm1.S57A and GST.N-Nrm1.T9AS57A.

In order, to solve our problem of obtaining good substrates to map Nrm1phosphorylation sites, we searched but in *in vivo* conditions. To do so, we purify Nrm1 from HU-treated cells by IP, run the imunoprecipitated Nrm1 on SDS-PAGE, excise the band corresponding to Nrm1 and send it out to be analysed by MS/MS. We obtained 55% of sequence coverage, including phosphopeptides of residues T9 and S57 (Fig.19).



1 MDRSMEPLTPSRLNTLGERPTNEVYEYGKGKNVQHLFPITPMQRPLGKEN
51 AAPGTISPIAVRSRNVRAVEIADENACEEPVLKIKSVSSTESEEEKESST
101 EIGEKQEKETHLEPKTPVQTNTNNNHLDDIQCCAKNLRLR LELAMYKVQV
151 NQTFSPLQDLPIVAKTKLHNCPNSEPVTSIWNQRSLSSGKPPSLHLSGNR
201 RLSMGSPTKSIYDQNGLTTPRPIGSDDLTHMYDPYTSPLRTPSRTLSRSS
251 SHYLWVRHGKLTRSVSLLQHKTPRRIRPKSLSKSNSTPLKHLSAQKPNSN
301 YYTGPPTPVSISNTPENIHPSSSEVRRIASHSKQFSDYGLIR

Fig.19 I Schematic representation of Nrm1 Cdk phosphorylation sites by MS. MS analysis of Nrm1 protein after immunopurification; in green and yellow background are represented the N- and C- part of Nrm1; red letters indicate sequence coverage; red marked (*) point- detected phosphorylation sites.

Unfortunately, we have not been able to improve the sequence coverage, although this work is still in progress. Also we are currently working on the *in vivo* role of these phosphorylations, and how they may regulate the MBF-dependent transcription.

DISCUSSION

Different types of DNA damage, depending on its origin, drive different responses. And once damage occurs, cells activate a proper DNA damage response in order to preserve their genetic information. In *S. pombe* the two DNA damage pathways initiate with the activation of the single sensor kinase Rad3, which once is activated, drives a phosphorylation cascade leading to the activation of the two effector kinases (Cds1 and Chk1), being Cds1 activated in response to DNA replication challenges and Chk1 after either single or double stranded breaks. The main aim of these two checkpoints is to block the cell cycle progression before cells enter into mitosis. Furthermore, while Chk1 is involved also in activating DNA repair, Cds1 is involved in stabilization of stalled replication forks.

The aim of this work was studying how fission yeast responds to different types of DNA damage in order to ameliorate this threat. More specifically, we examined the regulation of a single transcriptional factor complex MBF in response to DNA damage. With this study we have shown how Yox1/Max1, the MBF repressor, and how Cdc10, the core active element of MBF, are targeted by the DDR resulting in two distinct fates of MBF dependent gene transcription.

1. MBF regulation and cell cycle progression

MBF is a multimeric complex involved in the regulation of the G1/S phase transition, activating the transcription of a set of genes required for DNA replication. The transcript levels of

MBF-dependent genes fluctuate periodically throughout the cell cycle, peaking in G1 and S phases; thus, these genes have to be precisely induced and repressed at the right time and this is achieved through regulation of MBF, as the complex is active only during the G1-to-S phase transition. In fact MBF is inactivated once cells exit S phase through a negative feedback loop that includes Res1 phosphorylation by the cyclin Cig2 (Ayte et al., 2001). In this study we have shown that two proteins, Yox1 and Nrm1, are forming another MBF repressor system (Gomez-Escoda et al., 2011). We have also demonstrated that Yox1 cannot bind to MBF gene promoters in the absence of Nrm1, leading to up-regulated gene transcription. However Nrm1 does not need Yox1 to bind to the same promoters, associating equally in the absence of Yox1, despite the transcription is up-regulated. The outcome of these results is that the real repressor in this system is Yox1, while Nrm1 is only required to load Yox1 onto the MBF complex.

The G1/S transcriptional program in higher eukaryotes and in *S. cerevisiae* has a common pattern of activation, in which the transcription factors E2F/DP and SBF are activated by the phosphorylation of a repressor. However, it is still not known the mechanism that activates MBF at the end of mitosis in fission yeast. At the beginning of our work, we hypothesized whether Yox1 could be the repressor that switches ON the MBF-dependent transcription. However, we have observed that despite Yox1 is phosphorylated by Cdc2 at S6 residue, this modification does not have any effect on

the endogenous MBF regulation. However, since Nrm1 is required to load Yox1 onto the MBF complex, we wondered if Nrm1 itself could be involved in the activation of MBF. In fact, Nrm1 is the only component of the MBF complex, whose protein levels fluctuate throughout the cell cycle. Intriguingly, Nrm1 is almost undetectable in late mitosis, which corresponds to the timing of Yox1 promoter dissociation and activation of the MBF dependent transcription. In fact, we have been able to show that this decrease in Nrm1 protein level is the consequence of protein degradation driven by the APC/C. We still have not been able to determine the mechanistic of this degradation, but it is work still in progress; however it has been recently published that in budding yeast, NRM1 is targeted by the APC through its D-box (Ostapenco 2011). What we have been able to show is that Nrm1 is also phosphorylated by the single CDK, Cdc2, at least at two different residues in its amino part (other phosphorylation sites in the carboxy- region are to be confirmed). One possibility that we are considering is that this phosphorylation may signal Nrm1 to be degraded in late mitosis by the APC/C-proteasome complex. The consequence is that the repressor Yox1 is released from the MBF complex and the corresponding gene transcription is up-regulated during G1/S phase. At the end of S phase as Yox1 and Nrm1 are de novo synthesized, Nrm1 binds again to MBF, allowing Yox1 association and thus repression of the MBF gene-transcription. Thus based on the findings in this work, we speculate that Nrm1 could be the cornerstone involved in

linking CDK activity to MBF-dependent transcription. Further work is still required to fully characterize this regulation, but we do not exclude that other regulators could be also involved in this activation.

2. Checkpoints and MBF regulation

So far, above is described our hypothesis of how MBF is regulated in an unperturbed cell cycle. However, when the DNA synthesis is challenged, the single effector kinase involved in that response, Cds1, up-regulates the MBF dependent transcription. Recent studies implicated Cdc10 and Nrm1 as putative Cds1 targets under replication stress (Dutta et al., 2008; de Bruin et al., 2009). Here we have shown that this activated transcription is Yox1 dependent, as upon its deletion there is a constitutive up-regulated gene transcription (Gomez-Escoda et al., 2011). Moreover we have shown that Yox1 is phosphorylated by Cds1 upon HU treatment at two residues Ser-114 and Thr-115. This phosphorylation is responsible for the release of Yox1 from MBF and to induce MBF-dependent transcription. As the *cdc22* gene, encoding for ribonucleotide reductase, is also an MBF dependent gene, its induced expression can restore the dNTP pool in the cell (counteracting the effect of DNA synthesis inhibitors, like HU) and thus allows re-starting the DNA synthesis once the stalled replication fork are fixed. Thus, Yox1 is the main target of the replication checkpoint, if not the single one.

Under replicative stress, the signal is specific and exclusively activates the replication checkpoint response. However, in conditions when this pathway is abolished (either in the mutant Yox1.SATA, which cannot be phosphorylated by Cds1, or in a *Δcds1* background), or when the DNA damage is more severe, there is an activation of the other pathway, the DNA damage checkpoint. As a consequence, instead of activating the MBF-dependent gene transcription, we have shown that there is a repression of the same set of genes. This was caused because there was a release of the MBF complex from the corresponding promoters. In parallel to the release of the core complex, we could also observe the release of the repressor system, Yox1 and Nrm1, while still associated to Cdc10. This release is dependent on the activity of Chk1. We propose here that Cdc10 is phosphorylated by Chk1 leading to this promoter eviction.

While a previous report has shown that Cdc10 is a putative target of Cds1, we have shown here that upon DNA damage, Cdc10 is targeted by the DNA damage checkpoint. We have shown that after induction of the damage, Chk1 is activated and can phosphorylate Cdc10 at two residues in the C-terminal region (specifically at positions S720 and S732). As a consequence of this phosphorylation, Cdc10 (and the whole MBF complex) is released from its target promoters and MBF-dependent transcription is shut down. Surprisingly, in a Cdc10 mutant background that cannot be phosphorylated, MBF is not released from some promoters (i.e. *cdc18*), but is still released from others (i.e. *cdc22*). This

observation could mean that not all the MBF dependent genes are controlled by Chk1 and only specific set of genes are regulated after DNA damage. Unfortunately, until the moment we have not been able to find other *cdc18* like genes. Why *cdc18*? Cdc18 is part of the pre-replicative complex during the G1/S transition, thus regulation of *cdc18* could allow the cell to preserve its genetic integrity, until the damage is fixed.

3. Crosstalk between the checkpoints

In fission yeast the single sensor kinase, Rad3, is common for the two checkpoint pathways and in general each response is very specific. However, activation of the DNA damage checkpoint upon HU exposure in conditions when the replication checkpoint pathway is totally or partially abolished is a clear example for the existence a crosstalk between the two checkpoint responses. The existence of such checkpoint crosstalk is even clearer in a *yox1.SATA Δchk1* background, in which we could detect a Cdc10 release from chromatin after exposing cells to HU; in this case is probably due to a compensating effect of Cds1. In fact the two checkpoints recognize the same consensus sequence LXRXXS/T. The two checkpoints can substitute/replace each other in some specific conditions.

Interestingly, weak DNA damage like low doses of MMS treatment, in which the Cdc10 promoter occupancy is still not affected, is able to induce Cds1 activation. This leads to the dissociation of Yox1/Nrm1 and respectively to the induction of

the MBF dependent transcription. The relevance of this response could be that when the DNA damage is not so harsh, cells are getting prepared to re-enter the cell cycle by increasing the dNTP pool in case the damage is properly fixed. However, if the DNA damage is severe what is better for the cell is to stop the cell cycle progression, blocking any attempt for DNA synthesis, in order to prevent damage accumulation. We do not exclude also the possibility that on top of Cdc10, other components or interactors of the MBF complex could be also targeted by the Chk1 kinase.

Some aspects of the mechanism of DNA damage checkpoint response are better understood in higher eukaryotes. With the findings of this work, we report a conserved regulation between organisms. In mammals, even E2F-1 can be activated by CHK2 phosphorylation driving to apoptosis, the repressor of the complex Rb can be also targeted by CHK2, conversely leading to E2F transcriptional repression. This is final outcome that we report for *S. pombe*, a repression of MBF transcription. In conclusion, in the model that we propose, Yox1 and Cdc10 couple normal cell cycle regulation and the DNA-synthesis and DNA-damage checkpoints in a single transcriptional complex. Upon DNA replication stress, Yox1 is phosphorylated by Cds1 leading to its dissociation from MBF and activation of MBF-dependent transcription. In contrast, upon DNA-damage, Cdc10 is phosphorylated by Chk1 resulting in the release of MBF from chromatin and repression of MBF-dependent transcription.

Future experiments will be necessary to better understand the regulation of MBF by other cell cycle components.

Ivanova,T., B. Gomez-Escoda, et al. (2011). G1/S transcription and the DNA syntheis checkpoint, common regulatory mechanisms. CellCycle 10(6):912-5

CONCLUSIONS

1. In fission yeast, there is a crosstalk between DNA synthesis and DNA damage checkpoint responses.

2. Upon a challenge to DNA synthesis, Yox1/Max1 is targeted and phosphorylated by the checkpoint. As a result, it is released from MBF and MBF-dependent transcription is activated.

3. Cdc10 is targeted by the DNA damage checkpoint. Upon DNA damage, Chk1 is activated and phosphorylates Cdc10 at ser-720 and ser-732 residues.

4. Cdc10 phosphorylation by Chk1 induces its release from a subset of MBF-dependent promoters.

5. The effect of the DNA damage checkpoint on MBF is dose dependent. Cdc10 requires higher doses of MMS (higher damage) to be released from its target promoters, while Yox1 and Nrm1 are evicted even after lower doses MMS.

6. MBF dependent gene transcription is repressed after severe DNA damage, as no active MBF complex is associated with its corresponding promoters. However, low MMS doses lead to up-regulated gene transcription, because only the Yox1/Nrm1 repressor system is released.

7. Cells in which the signalling from DNA damage to Cdc10 has been abrogated are sensitive to DNA damage, showing survival problems after insults to DNA.

MATERIALS AND METHODS

Strains and media. All *S. pombe* strains are isogenic to wild type 972h- and are listed in the Table of strains. Media were prepared as previously described (Moreno et al, 1991). HU (10mM), CPT, MMS and γ-irradiation treatment were carried out on midlog grown cultures (3-4x10^6 cells/ml) in MM or YE5S media. To analyze sensitivity to DNA damage sources on plates, *S. pombe* strains were grown in liquid YE5S media to an OD_{600} of 0.5. Cells were then diluted in YE5S and 10 to 10^5 cells per dot in a final volume of 3 µl (metal replica plater) were spotted onto YE5S media agar plates containing (or not) the indicated drugs. Plates were incubated at 30°C for 3–4 days.

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.

Protein extraction. Extracts were prepared in NET-N buffer [20 mM Tris HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP40, 1mM dithiothreitol (DTT), 1 mM phenylmethyl sulphonyl fluoride (PMSF), 5 µg/ml aprotinin, protease inhibitor cocktail (Sigma), 2 mM sodium fluoride (NaF), 0.2 mM sodium orthovanadate (Na_3VO_4), 2 mM β-glycerophosphate]. Cells were broken with glass beads in a BioSpec Minibeadbeater.

For TCA extracts *S. pombe* cultures (5 ml) at an OD_{600} of 0.4 were pelleted just after the addition of 100% trichloroacetic acid (TCA) to a final concentration of 10% and washed in 20% TCA. The pellets were lysed by vortexing after the addition of glass beads and 12.5% TCA. Cell lysates were pelleted, washed in acetone, and dried. Alkylation of free thiols was accomplished by resuspension of the pellets in 50 µl of a solution containing 75 mM iodoacetamide, 1% SDS, 100 mM Tris·HCl (pH 8), 1 mM EDTA, and incubation at 25°C for 15 min.

Immunoprecipitations (1 to 10 mg of whole-cell lysate) were performed with 10 µl of prot. G separose, previously crosslinked with α-HA monoclonal antibody. Immunoprecipitates were washed after 1 hour of incubation three times with NET-N buffer and resolved in SDS-PAGE.

***In vitro* kinase assay.** Substrates were prepared as GST fusion proteins in *E. coli* as described (Dutta et al, 2008). For Cdk protein extracts (300 µg) from asynchronous cultures of strains with HA-tagged Cdc2, GFP-tagged Cdc13 were immunoprecipitated as described (Ayte et al, 2001), followed by three washes with NET-N buffer and one wash with CDK kinase buffer (10mM Hepes pH7.5, 20mM $MgCl_2$, 4mM EGTA, 2mM DTT). For Chk1 protein extracts (1mg) from MMS-treated cultures of a strain with HA-tagged Chk1 were immunoprecipitated as described (Ayte et al, 2001), followed by three washes with NET-N buffer and one wash with Chk1 kinase buffer (20mM Hepes pH7.5, 5mM $MgCl_2$, 0.5mM

$MnCl_2$, 50mM KCl, 1mM DTT, glycerol 15%). Immunoprecipitates were incubated in kinase buffer containing 5µg of substrate and 10µCi of [γ-^{32}P]ATP for 30 min at 30°C. Labeled proteins were resolved in SDS-PAGE and detected by autoradiography.

Gene expression analysis. RNA extraction was performed as described (Moldon et al, 2008) and 10 µg of extracted RNA were loaded on agarose gels and analyzed by northern blot. *cdc18*, *cdc22*, *tfb2* and actin probes contained the complete ORFs of the genes.

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

Table of strains used in this work

Strain	Genotype
972	h-
JA808	res1::ura4 ura4-D18 h?
JA809	res2::ura4 ura4-D18 h?
JA974	max1S114-115A-13Myc-kan h-
JA770	cdc10-HA::Nat+ leu1-32 h
JA1078	cdc10-HA-Nat+ yox1-114A-115A h+
JA1070	cdc10-HA::Nat+ cds1::Kan+ leu1-32 h-
JA1069	cdc10-HA::Kan+ rad3::Nat+ h-
JA1090	cdc10-HA-Nat+ chk1::ura4 ura4-D18 h?
JA1089	cdc10-HA-Nat+ cds1::Kan chk1::ura4 ura4-D18 h?
JA1160	chk1::ura4+ max1S114-115A-13Myc-kan ura4-D18 h?
JA1015	max1.S114-115A-13Myc-kan h+
JA1417	rad3::nat max1S114-115A-13Myc-kan h?
JA1269	Cdc10-Kan h-
JA977	max1-13Myc-kan nrm1-HA-Nat h+
JA803	cdc10-C4 h+
JA805	cds1::kan h-
JA795	max1::kan h-
JA672	chk1::ura4 ura4-D18 h+
JA1269	Cdc10-Kan h-
JA1270	Cdc10-S3-Kan h-
JA1271	Cdc10-S5-Kan h-
JA1272	Cdc10-S3,S5-Kan h-
JA1412	max1::Pheo nrm1::Nat Cdc10-Kan h+
JA1413	max1::Pheo nrm1::Nat Cdc10-S3A-Kan h-
JA1414	max1::Pheo nrm1::Nat Cdc10-S5-Kan h-
JA1415	max1::Pheo nrm1::Nat Cdc10-S3,S5-Kan h-
JA1416	max1::Pheo nrm1::Nat Cdc10-8A-Kan h-
JA780	cdc25-22 max1-13Myc-kan h?
JA1173	cdc25-22 Nrm1-HA-Nat leu1-32 h-
JA958	cdc2-L7::cdc2-HA(ura4+) ura4-D18 leu1-32 h-
JA610	cdc13-GFP in 972 h- background h-
JA1081	max1-13Myc-kan nrm1-HA-Nat h-
JA1196	mts2-1 Nrm1-HA-Kn h+
JA1155	mts3-1 nrm1-HA-Nat h+
JA1241	nrm1-HA-NatR skp1-A7 h-
JA1154	slp1-362 nrm1-HA-Nat leu1-32 h-

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