INO80 complex regulates the G1-to-S transcriptional wave through MBF

Iva Knezevic

Memòria presentada per optar al títol de Doctor per la Universitat Pompeu Fabra (UPF)

Barcelona, Juny de 2015

Treball dirigit pel Dr. José Ayté del Olmo

Oxidative Stress and Cell Cycle Group

Departament de Ciències Experimentals i de la Salut

Programa de Doctorat en Ciències de la Salut i de la Vida de la Universitat Pompeu Fabra

AGRADECIMIENTOS

To my parents and my sisters, Anja and Milica. You have always been a port in a storm. Thank you for unconditional love and support, advices and silence. For letting me be, for letting me choose and decide on my own, an being there if I ever fall. You make me feel safe and secure. Sticks in a bundle are unbreakable. I am grateful to you more that you know.

To Jose and Elena, who guided me through my thesis. For giving me an opportunity to do this thesis in your laboratory, for the opportunity to learn and grow. Thank you for forming me, for all the knowledge that you gave me. Jose, thank you for all your advices, ideas, conversations, patience and support. For being available to ask any question at any time. Elena, thank you for your enthusiasm, fresh views, new ideas on the project and inspirational discussions on my lab meetings. The last year was especially difficult for me, and thank you so much for all the help, support and concerns that you had for me.

To every person who was a part of 383 during this five years. For the great moments inside and for unforgettable ones outside the lab. For sharing science and life issues. I know I have made friends for a lifetime. There are to many memories to mention them here, but when I think of our lab I want to remember Friday's afternoons of Disney music with Esther,

but we would have to wait for Merce to leave, because she couldn’t stand it. Thank you all. It was honor working with you.

To my friends here and there. For making little moments so special, for making me feel at home, for waiting me back at home, for visiting. To Jelena, together we are stronger. To Vanessa that literally watches by back, thank you so much.

To Lula, my one and only, and his family. I know that sometimes was more than tough, but you stood by me. And I am always on your side too. Thank you for your immense patience. Thank you for choosing me, for believing in me, for reminding me of who I am, and how I can be. For waiting, for comforting. You are my best friend, and we are a great team!

SUMMARY

Summary

In eukaryotic cells a key regulatory step of cell cycle occurs in late G1, which has been termed “Start” in yeast cells. At this point, cells determine whether they will go to through a new round of proliferation, or choose alternative pathways; cell cycle arrest or sexual differentiation. In fission yeast, the MBF complex controls the transcriptional activation of some genes of G1/S transcriptional wave, including *cdc18*, *cdt1* (necessary to prevent onset of mitosis before of completion of DNA synthesis), and *cdc22* (ribonucleotide reductase). Control of MBF activity is essential for normal cell cycle progression. It has been found that MBF complex is bound to the promoter of its target genes throughout the cell cycle, implicating that MBF activity is not regulated by pure binding to DNA. We purified novel inetractor of MBF complex, INO80 complex. Here we demonstrate that INO80 regulates cell cycle genes through MBF, and that proper acetylation of histone variant H2A.Z is crucial for MBF dependent transcription.

Resumen

En las células eucariotas el paso clave en la regulación de ciclo celular ocurre en el final de la fase G1, nombrado como “Start” en levadura. En este punto, las células determinan si pasaran por nueva ronda de la proliferación, o elegirán vías alternativas: parada del ciclo celular o diferenciación sexual. En *S. pombe,* el complejo MBF controla la activación de la transcripción de algunos genes necesarios para la transición G1 / S, incluyendo *cdc18, cdt1* (necesario para evitar el inicio de la mitosis antes de la finalización de la síntesis de ADN), y *cdc22* (ribonucleótido reductasa). El control de la actividad MBF es esencial para la progresión normal del ciclo celular. El complejo MBF se une a los promotores de los genes, a lo largo del ciclo celular, implicando que la actividad de MBF no esta regulada por el simple hecho de unión al DNA. Hemos purificado un interactor nuevo de MBF, el complejo INO80. En esta tesis demostramos que INO80 regula los genes del ciclo a través de MBF, y que la acetilación adecuada de la histona H2A.Z es crucial para la transcripción de los genes MBF.

INDEX

INTRODUCTION

INTRODUCTION

1. Schizosaccharomyces pombe

Schizosaccharomyces pombe (also called fission yeast) is a unicellular eukaryote with a well defined genome, with 5036 genes on 3 chromosomes and it can proliferate in a haploid state. It has one single copy of the genome, which facilitates simple gene function analysis working with mutations and deletions. It has been used for cell-cycle investigations since the 1950s, and it is widely accepted as an excellent model organism for cell cycle research.

A major reason for using *S. pombe* to study fundamental biological problems is to exploit the acquired knowledge to understand more complex organisms, where molecular processes are so complicated that it is impossible to unravel and understand them, without prior knowledge from more simple systems. 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.

S. pombe grows by length in two cells which continues until the onset of mitosis, and divides by formation of septum. This characteristic growth allows positioning of a specific cell in a cell cycle by simple microscopic observation.

2. Mitotic cell cycle

The cell cycle is an ordered set of events, culminating in cell growth and division in two daughter cells. Cell cycle control in eukaryotic cells depends on precise regulatory machinery that ensures that those events will occur in the correct order. The main events to be regulated are the duplication of genetic content and the distribution of those components into two identical daughter cells. Defects in the process are the basis for many diseases.

Traditionally, eukaryotic cell cycle is divided in four separate, consecutive stages: S phase (DNA synthesis) and M phase (chromosome segregation) with these two separated by gap phases G1 and G2. Gap phases are important for cell cycle regulation, to control the progression to the next phase. Cell division is controlled in many ways and at many levels, including regulation of gene expression and protein activity, stability and distribution. All the steps of regulation take place at particular moments of the cell cycle named checkpoints. There are two major points of cell cycle control. One occurs in late G1 phase, checkpoint known as “Start” in yeast (“Restriction point” in mammals), and second one in G2/M transition.

Cell cycle control mechanisms ensure 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.

To maintain cell size, control of a cell growth is also important. Anytime when in a checkpoint control a problem in accomplishment of one of the phases is detected, cell cycle progression is delayed until the problem is solved.

2.1 Cell cycle in fission yeast

Mitotic cell cycle consists of a short G1 phase, S phase where DNA is duplicated, long G2, followed by M phase were chromosomes are segregated, and cell is divided in two daughter cells. Curiously, formation of septum at the central position in the cell coincides with S phase. Because of temporal difference in cariokinesis and citokinesis, *S. pombe* cells have DNA content of 2C throughout the cycle, albeit being a haploid organism. Asynchronous growing cultures shows a peculiar flow cytometry profile compared to other eukaryotes; a single peak of 2C DNA content.

S. pombe grows by length extension which continues until the onset of mitosis, and divides by formation of septum. This characteristic growth allows positioning of a specific cell

in a cell cycle by simple microscopic observation, and allowed the scientists in the 70s to isolate strains defective in cell cycle growth. Many key regulators of mitotic cell cycle were identified, and the genes were named *cdc* genes (cell division cycle). Some of the strains showed an elongated phenotype, whereas other mutations caused a reduction in cell size. Since many of those genes are essential, they were isolated as temperature sensitive strains (conditional 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. This characteristic was used as a powerful tool to synchronize cells. One of those strains is a Cdc25 phosphatase mutant, *cdc25-22*. This mutant has elongated shape due to longer G2, since cell are compromised to enter M phase, and get arrested in G2/M transition.

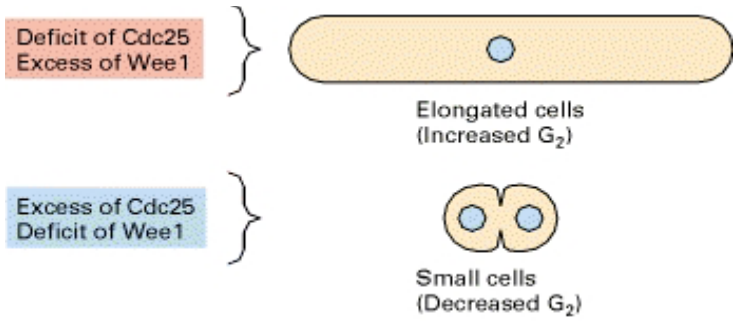


Fig 1. | Schematic representation of the *cdc25-22* and the *wee* phenotypes (From Molecular Cell Biology, Lodish, Darnell et al.).

Opposite phenotype was observed in Wee kinase mutant; small cells as they enter rapidly in M phase, shortening the G2. 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.

2.2 CDK/Cyclin complexes

Cell cycle is mainly controlled at the onset of M and S phase, ensuring that these two events occur in the correct order, and that there is 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.

Cyclins are a group of proteins that that control the progression of cells through the cell cycle by activating CDKs. Cylins were name that since they show typical protein periodicity along the cell cycle. They are regulated by several mechanisms to achieve the activation of the corresponding CDK/cyclin complex at the proper time; at the level of gene expression, and also at the level of degradation. These two mechanisms allow the oscillations in the protein levels.

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. 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.

In *S. pombe* a master regulator of cell cycle is Cdc2, essential for both DNA replication and entry into mitosis. (Fisher et al., 1996) Protein levels of Cdc2 are constant through the cell cycle, but its activity fluctuates, with the maximum activity at the onset of mitosis. Control of activity is achieved by binding to cyclins (Hayles et al., 1994), such as Cdc13, Cig2, Cig1 and Puc1.

Cdc13 is the single essential cyclin in *S. pombe*, and it is required for entry into mitosis (Booher et al., 1989). Its transcription is not cell cycle dependent, but the protein levels fluctuate, increasing in G2, and decreasing in

anaphase due to ubiquitin-mediated proteolysis (Creanor and Mitchison, 1996). Δ*cdc13* cells undergo multiple rounds of DNA replication without the subsequent mitosis (Hayles et al., 1994).

Cig2 is also a B type cyclin. Although initially it was thought to have a role in mitosis (Bueno and Russell, 1993), its main function is in the onset of S phase (Connolly and Beach, 1994a; Mondesert et al., 1996). Deletion of *cig2* does not have an effect on cell cycle or in cell viability, but Δ*cig2* cells show increased ability to enter the sexual cycle (Connolly and Beach, 1994b). Cig2 has a role in the regulation of the S phase, and among the substrates of the Cdc2/Cig2 CDK complex there are several proteins from the replication machinery, like Cdc18, that is inhibited when is phosphorylated by the complex (Lopez-Girona et al., 1998).

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

Puc1 has certain similarity to the G1 cyclins of *S. cerevisiae*. It was described to have a possible role in G1 (Forsburg and Nurse, 1994) but its function remains unclear. It was

described to regulate the length of G1, coupling it to the achievement of a critical cell size (Martin-Castellanos et al., 2000).

Among all the cyclins, only Cdc13 is essential and it can substitute any other cyclin in the different phases of the cell cycle (Mondesert et al., 1996, 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 Cdc2/Cdc13 (Broek et al., 1991; Fisher and Nurse, 1996).

2.3 G2/M transition

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.

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. 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. Degradation of the cyclins ensures the irreversibility of the process: CDK complex is inactivated, and the subsequent dephosphorylation of its

substrates avoids re-entry into early mitotic events, leading to the mitotic exit.

2.4 START

A key regulatory step of cell cycle control occurs at late G1, known as “START” in yeast cells and “Restriction point” in higher eukaryotes. At this point, cell determines whether it will commit to mitosis, or choose alternative pathways leading to arrest and sexual differentiation. After the passage through START, cells are committed irreversibly to complete the subsequent mitotic cycle, completing chromosome replication in S phase. Progression through Start requires the activity of the single fission yeast cyclin-dependent kinase Cdc2 and transcriptional activation of the G1/S transcriptional program, which depend on the MBF complex (Simanis et al. 1987), (discussed in Chapter 3).

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. Loosing the control at the restriction point in higher eukaryotes can lead to a misregulation in cell proliferation and is frequently associated to cancer (Pardee 1989).

2.5 DNA replication and S phase

DNA replication occurs in S phase. Replications starts from so-called origins, specific regions on the chromosome, and replication machinery moves bidirectionally from them until chromosomes are completely duplicated. In a process called origin licensing, in early G1 phase, pre-replicative complexes (pre-RC) start assembling at origins, preparing them for the future origine firing. 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). Although it is not well established how the ORC recognizes the origin sites at DNA, it seems to depend on specific DNA sequences and on chromatin structure. These DNA sequences are well defined in *S. cerevisiae* and less conserved in other eukaryotes (Stillman 1993; Antequera

2004). Second, 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 activity 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 and DNA polymerases, is recruited later onto the origins, originating the replication forks. The process of starting replication is called origin firing. 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 are early and other late origins.

Once replication begins, it proceeds until its completion. There are two main features of DNA replication that are essential to maintain genome integrity and to avoid problems later in the cell cycle in chromosome segregation: (1) cells ensure that each chromosome duplicates only once per cycle, (2) when one origin has been activated, firing will not occur in the same origin until the next cell cycle. CDK machinery is in charge to regulate the process; for example regulating the degradation of the components of the pre-RC once replication has been initiated, to avoid new origin recognition (Diffley 2004).

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

3. Transcriptional program at the G1/S transition

In *S. pombe* four waves of gene expression along the cell cycle have been described: G1/S, S phase, G2/M, and M/G1 transcriptional programs.

a) G1/S wave was first to indentified and it si well characterized. The specific transcription of around 20 genes whose encoded products are essential for DNA synthesis is induced during the G1/S transition. All these genes share a DNA motif in their promoter called MCB (Mlu1 cell cycle box), which is recognized by the specific transcriptional factor MBF (Mlu1 cell cycle box binding factor) (Lowndes et al. 1992).

b) During the S phase a number of genes encoding histones are expressed (Matsumoto and Yanagida 1985).

c) Although G2 contributes to a significant proportion of the mitotic cell cycle in fission yeast, and G2/M being one of two major regulatory points, only a smaller group of weakly induced genes at this cell cycle phase have been identified. These include genes with a putative common promoter UAS (Rustici et al. 2004).

d) In M/G1 at least 20 genes are transcribed. 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), including two forkhead-like transcription factors, Fkh2 and Sep1 (Zilahi et al. 2000; Buck et al. 2004)

3.1 *S. pombe*: MBF

MBF (Mlu1 cell-cycle-box binding factor) is a high molecular weight transcriptional factor complex, with a number of components identified. It plays an important role in cell cycle regulation, since its activity contribute to the timely expression of G1/S transition genes. 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 comprised by Cdc10, Res1, Res2, and few other regulatory components. Cdc10, Res1 and Res2 have constant protein levels over the cell cycle (Simanis and Nurse 1989; Whitehall et al. 1999). Cdc10 has been found bound to its target promoters throughout the cell cycle (Wuarin et al. 2002), indicating that the regulation of MBF

dependent transcription is not achieved by simple modulation of DNA binding activity of the complex. The exact mechanism of how MBF complex is being activated at M phase in not clear, but so far there are evidences that MBF is regulated by posttranslational modifications and by other regulatory subunits (Gomez-Escoda et al. 2011).

3.1.1 Cdc10

One of the first components of MBF complex to be identified was Cdc10, considered to be the activating component of the complex. This gene is absolutely necessary for cell viability. Cdc10 is not binding directly to DNA, rather through its partners Res1 and Res2. The carboxi-terminus of Cdc10 is important for the regulation of MBF function, and seems to be critical for the formation of the complex. (Reymond and Simanis 1993). It has a region with ankyrin repeats, motifs present in a large number of functionally diverse proteins and considered sites for protein protein interaction. The ankyrin motifs are a conserved sequence of about 30 amino acids repeated four or more times, and it allows Cdc10 to interact with its MBF partners Res1 and Res2. 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; Whitehall et al. 1999).

A truncated form of the protein (Cdc10-C4) has been widely used to understand regulation of MBF. It lacks the 61 amino

acids in its C terminus, leading to a highly induced transcription of MBF genes throughout cell cycle. Hence, the C terminus of Cdc10 is important for the regulation of MBF function (McInerny et al., 1995).

On the other hand, overexpression of Cdc10 under a strong inducible promoter (pREP1) does not affect periodic transcription of MBF dependent genes (White et al. 2001) and regulation is maintained despite this overexpression. This results reinforce the idea that other regulators, rather than the amount of protein, control the activity of Cdc10/MBF complex.

3.1.2 Res1 and Res2

Res1 and Res2 are the DNA binding subunits of the complex. They are higly homolog to each other and they bind DNA through a homologous N terminal domain. They also have ankyrin repeats domains in their C terminus part. Despite their stuctural similarity both proteins have different functions.

Res1 was isolated as a suppressor of *cdc10* (Tanaka et al. 1992). Overexpression of Res1 rescues 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). However, overexpression of Res1 in a wild type induces

arrest in G1. This arrest is not due to overexpression of MBF dependent genes. One explanation could be that occurs an aberrant transactivation of genes that are not normally MBF dependent, or maybe overexpression of Res1 might behave as a dominant negative mutant by sequestering other MBF components (Ayte et al. 1995).

∆*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).

Res2 is required for the initiation of mitotic and premeiotic DNA synthesis. The main role of Res2 is in meiotic MBF (Ayte et al. 1997). Its expression is induced in premeiotic DNA synthesis, and ∆*res2* cells have severe defects in meiotic DNA synthesis (Miyamoto et al. 1994). But Res2 is also involved 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 ∆*res1* defects (Miyamoto et al. 1994). There is a general derepression of MBF-dependent transcription in ∆*res2* cells (Baum et al. 1997). Although it was thought that the phenotype of the *cdc10*-C4 mutant was due to loss of interaction with Res2, it was 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 switching from Res1 to Res2 to form an inactive MBF complex, since both components remain in the complex together with Cdc10 throughout the mitotic cycle (Whitehall et al. 1999). Also, microarray data (Dutta et al. 2008) indicate that both, Res1 and Res2, can act as repressors and activators, but in different subset of genes. ∆*res2* cells show constitutive derepression of most MBF dependent genes, except for *max1, cig2*, and *mik1*, which have wild type levels of expression, while c*dc22* is induced. ∆*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.

3.1.3 Other interactors of MBF

Other components of MBF complex include Rep1, Rep2, Cig2, Nrm1 and Yox1. Rep1 was initially described as a component of meiotic MBF, controlling the onset of premeiotic DNA synthesis by regulating Res2 in a Mei2 independent cascade (Sugiyama et al. 1994). However, overexpression of Rep1 in mitotic cycle results in deregulation of MBF genes, which becomes constitutively transcribed throughout the cell cycle (White et al. 2001). This

is why Rep1 is considered as a possible activator of MBF complex.

Little is known about Rep2, but it is postulated to be co-activator of MBF complex during mitotic cycle (Tahara et al. 1998). Overexpression of Rep2 also leads to constitutive derepression of MBF genes (White et al. 2001). Another study shows that Rep2 is degraded through ubiquitin-mediated proteolysis, and that that S-phase checkpoint kinase Cds1 activates the MBF transcriptional activity through the inhibition of APC/C-Ste9 (Chu et al., 2009).

The mitotic cyclin Cig2 is the product of one of the genes regulated by MBF. Forms a feedback-inhibition loop with MBF which is important for normal regulation of the cell cycle. This was the first evidence of a direct regulation of MBF transcription by CDKs in fission yeast (Ayte et al. 2001). 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.

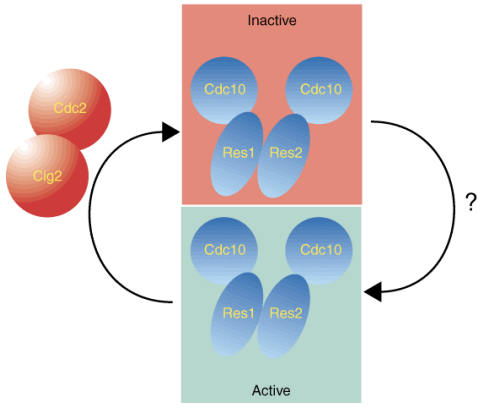


Fig 2. | Negative regulation of MBF by Cdc2/Cig2 phosphorylation (Ayte et al. 2001)

Another two negative regulators of the complex are Nrm1 and Yox1, also implicated in negative feedback loop (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 (Gomez-Escoda et al. 2011). This leads to a conclusion that both Yox1 and Nrm1 are necessary for inactivating MBF, and that are acting as corepressors. While Yox1 acts as a real repressor, it needs Nrm1 to load on 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.

3.2 *S. cerevisiae*, MBF/SBF

Compared to *S. pombe*, in *S. cerevisiae* transcriptioanal program of G1/S wave depends on two transcription 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 the MCB box (MluI cell cycle box, ACGCGTNA), a specific DNA element, present in the regulatory region of genes. Gene regulated by MBF are coding proteins with a role in DNA synthesis (POL1, POL2), 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, comprised by two homologous components of MBF, Swi6 and Swi4, 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 binds MCB boxes as well (Partridge et al. 1997). It is required for passage through START, activating transcription of genes required for spindle pole body duplication, budding and cell morphogenesis.

There is some overlap in the role of both transcription factors. Their sequence requirement to bind DNA is 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). 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, and that their function may be redundant.

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 both complexes (Dirick et al. 1992).

Although MBF and SBF are the main regulators of START, 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, and they

bind to promoters of other transcription factors (Horak et al. 2002).

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

3.3 Metazoans: E2F/DP

E2F/DP is the functional 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, this family is composed of at least eight E2F and two DP subunits; eight E2F genes (E2F1–8), two DP (DP1 and DP2) genes and three genes encoding RB (Retinoblastoma) related proteins (pRB, p107 and p130). The study of E2F began in the mid 1980s when it was identified as a transcription activator of the adenoviral *E2* gene promoter (Kovesdi et al. 1986).

While E2F7 and E2F8 bind as homodimers, they have two DNA-binding domains and do not require a DP partner to bind to DNA, E2Fs 1–6 require dimerization with a DP family member, which are essential for the DNA binding of E2F (Trimarchi and Lees 2002). The E2F family members can be also distinguish based on 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, since they lack transactivation domain. (Attwooll et al.2004). E2F6, E2F7, and E2F8 are classified as transcriptional inhibitors, but they function independently of the RB family (DeGregori and Johnson 2006).

E2F1 protein has been most studied, with its role in G1/S well established. E2F2-E2F5 have a well characterized role in regulating the G1/S transcriptional program, also. 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.

Transcriptional activation of G1/S genes depends 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. Other biological function of E2F apart from G1/S control, are in DNA repair (Ishida et al., 2001), apoptosis (Asano et al., 1996; Du et al., 1996b), differentiation and development (Field et al., 1996; Yamasaki et al., 1996, Page et al., 2001).

3.4 G1/S gene expression regulation

MBF, SBF and E2F 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 from yeast to metazoans.

In *S. pombe*, MBF dependent transcription is constrained to M, G1, and S phases by inactivation of the complex as cells exit S phase. Inactivation is achieved by double negative feedback loop; 1) Inactivation by phosphorylation by Cdc2/Cig2 complex, 2) Binding of corepressors Nrm1/Yox1. 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; Wagner et al., 2009). However, when phosphorylation mutants of Whi5 were tested, there was not any effect on transcription.

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. Recently, another negative regulator of START was described, Whi7 Galal Yahya et al., 2013). Whi7 is also phosphorylated when cells execute the G1/S transition, or after induction of Cln3

expression. Phosphorylated forms of Whi7 display an increased affinity for Cks1, which suggests that Whi7, in a similar manner to Whi5, could be regulated by Cdc28-dependent hyperphosphorylation during cell-cycle entry. Whi7 acts as an inhibitor of Start and, at the same time, is also a target of the Cdk under its regulation, which suggests that Whi7 could be a central component of a positive feedback loop in releasing the G1 Cdk-cyclin complex in late G1(Galal Yahya et al., 2013)

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 one 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, 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. Furthermore, Nrm1 appears to be stabilized via phosphorylation, carried out by Cdc28. Initial drop of Cdc28 activity in early G1 leads to Nrm1 dephosphorylation, that results in Nrm1 degradation, in APC dependent manner (Ostapenko et al,. 2011). 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).

The Rb gene is an archetypal tumor suppressor gene that was first identified in a malignant tumor of the retina known as retinoblastoma. Several human tumors show mutations and deletions of the Rb gene, and inherited allelic loss of Rb confers increased susceptibility to cancer formation (Dunn et al., 1988). It has been shown that Rb protein (pRb) is responsible for a major G1 checkpoint (restriction point) blocking S-phase entry and cell growth, promoting terminal differentiation by inducing both cell cycle exit and tissue-specific gene expression (Weinberg, 1995).

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. 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. pRb is hypophosphorylated in resting G0 cells. When in its actively growth-suppressing hypophosphorylated state, pRb physically associates with E2F factors and blocks their ability to activate expression of genes that encode products necessary for S-phase progression.Upon mitogenic signals, pRb is getting phosphorylated, through G1 that results in and dissociation form E2F, and is maintained in a hyperphosphorylated state until late mitosis (Weinberg, 1995; Claudio et al., 1996, Claudio et al., 2002). The switch that allows cells to entry into cell cycle from quiescent state is the due to activity of CDK in response to external signals.

Rb/E2F and cancer

Loss of pocket protein functions may induce cell cycle deregulation and lead to a malignant phenotype. Rb protein can be functionally inactivated by phosphorylation, mutations or viral oncoprotein binding. As a direct consequence, E2F transcription factors are liberated by control of Rb protein and induce deregulation of the cell cycle (Giacint et al., 2006).

It is believed that pRB has a role, directly or indirectly, in nearly all the human cancers (Burkhart and Sage 2008).

Gene inactivation through chromosomal mutations is one of the principal reasons for retinoblastoma loss of function in cancer. Inappropriate pRb phosphorylation due to rearrangement and overexpression of cyclin D1 also contributes to the development of several types of human tumors, including parathyroid adenomas, B-cell lymphomas and squamous cell carcinomas (Hunter et al., 1994). The main role of RB as a tumour suppressor is 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. The overproduction of cyclin D1 and Cdk4 leads to a constitutive phosphorylation of Rb proteins and to a deregulation of E2F transcriptional activity. It has also been reported that deregulation of other G1 cyclins (D2, D3 and E) is associated with tumorigenesis (Keyomarsi et al., 1995).

Anyhow, breakdown of cell cycle, owing to products of oncogenes (cyclins, cdks and oncovirus) and tumor suppressor genes (Rb proteins and CKI) whose functions converge on alteration of E2F genes, leads to a cancer phenotype.

4. DNA damage and DNA checkpoints

The main objective for every life-form is to deliver its genetic material, intact and unchanged, to the next generation. This must be achieved despite constant assaults by endogenous and environmental agents on the DNA. 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. To combat threats posed by DNA damage, cells have evolved mechanisms – collectively termed the DNA-damage response (DDR) – to detect DNA lesions, signal their presence and promote their repair. DNA replication checkpoints slow down or arrests cell-cycle progression, which is thought to increase the time available for DNA repair before replication or mitosis ensues.

4.1 Endogenous sources of DNA damage

DNA damage can be generated spontaneously during DNA metabolism. Endogenous DNA damage, produced by normal cellular processes, occurs at a high frequency compared with exogenous damage. 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.

Replication arrest is a recognized source of genetic instability in all organisms. Proteins that protect, process, and restart arrested replication forks have been identified, and in eukaryotes their action is coordinated with the induction of a check-point response to prevent cell cycle progression until replication resumes (Branzei et al., 2007, Gabbai et al., 2010). 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. Also, the DNA damage checkpoints monitor the proper activity of these enzymes to ensure a normal chromosome segregation and chromosome stability (Nitiss 2009).

Another endogenus DNA damage occurs during transcription. 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).

4.2 Exogenous sources of DNA damage

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). The most pervasive environmental DNA-damaging agent is ultraviolet light (UV). 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. Many of those compounds and their analogues are isolated in anticancer drug screening, and are used in treatments of various tumours.

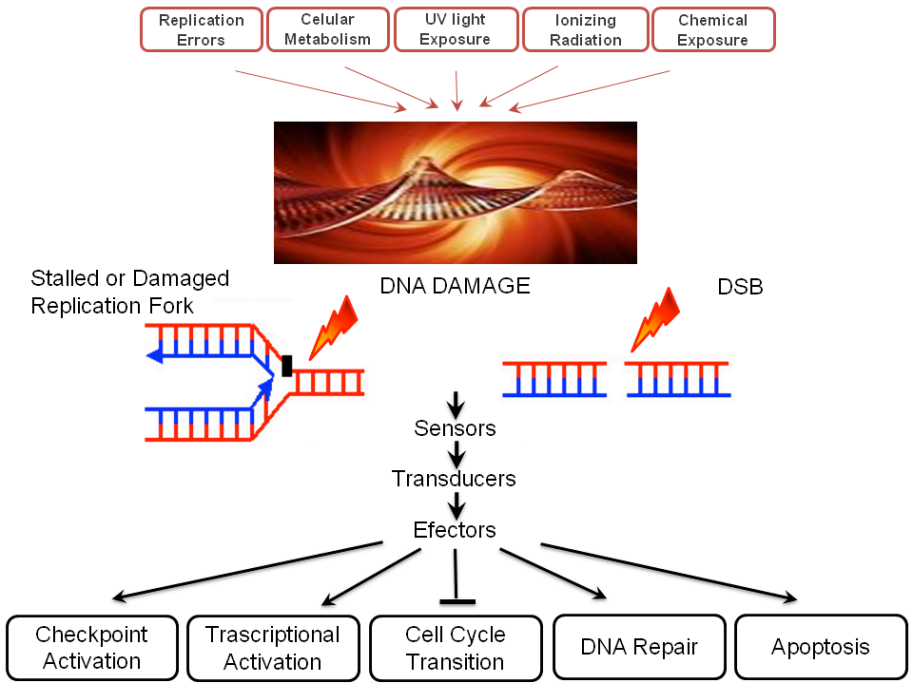


Fig 3. | Schematic representation of DNA damage: sources, types and cell effects

4.3 DNA damage response

The DNA damage response (DDR) is a network of cellular pathways that sense, signal and repair DNA lesions. Surveillance proteins that monitor DNA integrity can activate cell cycle checkpoints and DNA repair pathways in response to DNA damage, to prevent the generation of potentially deleterious mutations. 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. 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).

4.3.1 Sensors

Key players in the checkpoint response are phosphatidylinositol 3-kinase related protein kinases, such as mammalian ATM (ataxia-telangiectasia-mutated) and ATR (ATM- and Rad3-related), *Saccharomyces cerevisiae* Tel1 and Mec1, and *Schizosaccharomyces pombe* Tel1 and Rad3.

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).

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 Tel1ATM 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

Rad3ATR/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.

When DNA damage is detected, chromatin that flanks this damage is marked by the DDR. 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, is subjected to a variety of post-translational modifications that impact on genome function by either directly affecting nucleosome stability or providing a docking site for distinct regulatory proteins.

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). ATM-dependent phosphorylation of H2AX. MDC1, a BRCT-domain containing protein needed for ATM activation, binds to γH2AX 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.

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). 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.

4.3.2 Transducers

Once DNA damage is sensed, the cell must transduce this signal down to its appropriate effector. Among the complexes recruited to the damage sites, there are some multi-protein complexes such as the Mre11-Rad50-Nbs1Xrs2 (MRN) protein complex which directly binds DSB ends and thus allows Tel1ATM association. The Rad family members: Rad1, Rad9, Rad17, Rad26, and Hus1; where Rad26 (ATRIP in mammals) is a partner protein interacting with Rad3ATR; 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).

In fission yeast the functions of ATM and ATR orthologs are intimately linked 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).

4.3.3 Effectors kinases and DNA replication checkpoint

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. In fission yeast, Cds1 is the effector of the replication checkpoint and Chk1 mediates the G2–M DNA damage checkpoint. Chk1 was originally identified in fission yeast as a kinase required for the DNA damage checkpoint but not the replication checkpoint (Walworth et al., 1993). Chk1 is the most downstream member in the DNA damage checkpoint pathway and specifically downstream of Rad3 (Walworth and Bernards, 1996).

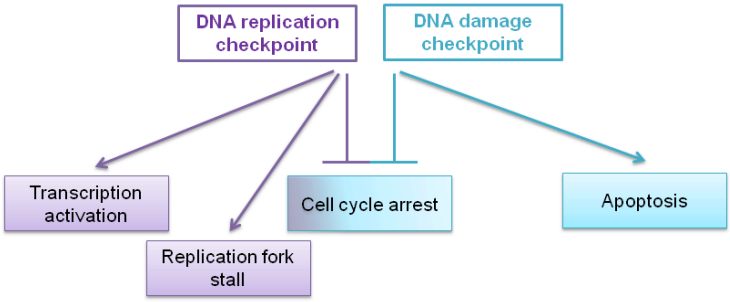


Fig.4 | DNA damage effector responses

The DNA replication checkpoint response regulates cell cycle arrest due to 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: 1) Cell cycle arrest; 2) Stabilization of stalled replication forks; 3) Activation of a transcriptional response.

4.3.4 Cell cycle arrest

In most eukaryotes, the replication checkpoint maintains Cdc2 at an interphase level of activity, thus preventing cell cycle progression and entry into mitosis. 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 its 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 damage is detected in S and in G2 phases, the entry into mitosis is blocked, to avoid segregation of damaged chromosomes.

In fission yeast, Chk1 is the effector of the DNA damage checkpoint pathway (Walworth et al., 1993). Chk1 is phosphorylated in a Rad3-dependent manner in response to activation of the DNA damage checkpoint, but not the replication checkpoint, and this phosphorylation correlates with its ability to arrest cells in G2 (Walworth and Bernards, 1996). The activity of Cdc2 is attenuated by phosphorylation of a critical tyrosine residue, tyrosine 15. This inhibitory phosphorylation is carried out by the tyrosine kinases Wee1 and Mik1. Cdc2 is activated by dephosphorylation of Y15, which is performed by the phosphatase Cdc25. This inhibition is presumed to be due to direct regulation by Chk1, which binds to Cdc25 in vivo and phosphorylates it in vitro (Furnari et al., 1997; Zeng et al., 1998); phosphorylated Cdc25 I retained in the cytosol, and cannot dephoshorylate Cdc2, leaving it in inactive form.

Cds1 is activated by the checkpoint and is required for cells to survive treatments that block replication (Boddy et al., 1998; Lindsay et al., 1998). However, in the absence of Cds1, Chk1 can act to impose a checkpoint delay (Boddy et

al., 1998). Cds1 seems to regulate Cdc25 in a similar manner to Chk1. Cds1 phosphorylates Cdc25 in vitro on sites similar to Chk1, and inhibits Cdc25 in vitro (Zeng et al., 1998; Furnari et al., 1999). In addition to Cdc25, Mik1 and Wee1 are also an important target of Cds. Mik1 accumulates in replication checkpoint arrested cells in Cds1 dependent manner. (Boddy et al.,1998; Murakami and Okayama 1995). The accumulation of Mik1 correlates with the accumulation of its mRNA. The upregulation of several other S-phase-specific transcripts in response to activation of the replication checkpoint requires the MBF-dependent S-phase transcription factor (Baum et al., 1997). It seems that Cds1 acts through MBFmachinery to maintain the S-phase transcription program during a replication checkpoint arrest.

Cds1 is also involved in the stabilization of stalled replication forks, to avoid lethal fork collapse. 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).

4.3.5 Activation of a transcriptional response

Upon replication stress the transcription of genes with role in DNA repair and replication is highly induced. 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. *Δcds1* and *Δrad3* 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. Under replicative stress, the activation of MBF-dependent transcription is a consequence of phosphorylation of several components of the MBF complex, including Cdc10 (Dutta et al., 2008), the corepressor Nrm1 (de Bruin et al., 2008), the repressor Max1 (Gomez-Escoda et al., 2011; Purtill et al.,) and the coactivator Rep2 (Nakashima et al., 1995). Nrm1 was also described to play an important role in DNA replication checkpoint response (de Bruin et al. 2008). 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.

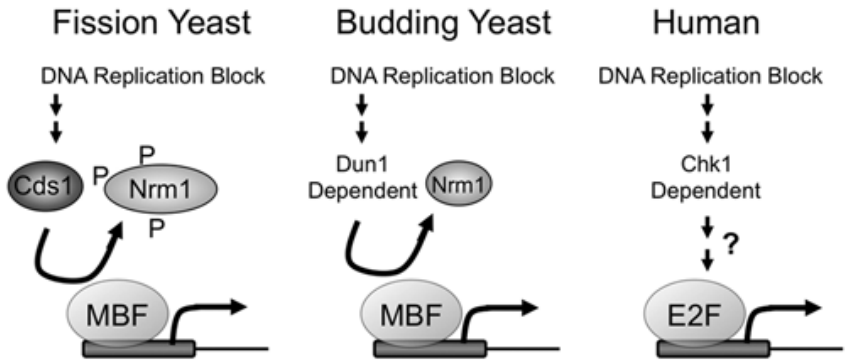


Fig. 5 | The DNA replication checkpoint promotes persistent expression of cell cycle regulated transcripts in eukaryotes (de Bruin and Wittenberg 2009)

Recently it was reported DNA damage checkpoint exerts a new layer of control on the MBF complex, inactivating MBF-dependent transcription. This is achieved by direct phosphorylation of Cdc10 by Chk1, at two different sites on its carboxy-terminal domain. This phosphorylation induces the exit of Cdc10 from the chromatin and thus the repression of the transcription of the MBF-dependent genes (Ivanova et al., 2013).

5. INO80 complex

The INO80 chromatin remodeling complex functions in transcriptional regulation, DNA repair, and replication. The Ino80 ATPase is a member of the SNF2 family of ATPases and functions as an integral component of a multisubunit ATP-dependent chromatin remodeling complex. Ino80 was first isolated in Saccharomyces cerevisiae, in a genetic screen for yeast defective in activating transcription in response to inositol depletion (Ebbert et al., 1999).

5.1 Subunits and organization

INO80 complexes purified from budding yeast, fission yeast and mammalian cells contain core subunits, which are conserved across species, as well as species-specific proteins. There are nine subunits that are conserved among all tree species: Ino80, Rvb1, Rvb2, Arp5, Arp8, Alp5, Ies2, Ies6 and Act1 (Jin et al., 2005, Shen et al., 1986, Hogan et al., 2009). Fission and budding yeast share the HMG-box protein, nht1 (NHP10). On the other hand. Iec1, Iec3, Ies4 and Ies5 although wear same name are not yet confirmed homologues.

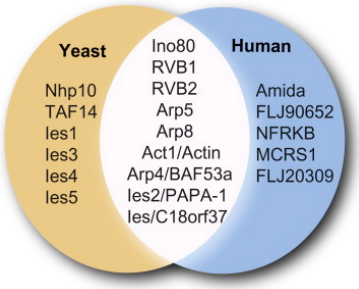


Fig 6. | INO80 homologue and nonhomolouge subunits

Ino80, Rvb1, Rvb2, Ies2

Ino80p ATPase is thought to provide the catalytic activity for chromatin remodeling by the INO80 complex because Ino80p ATPase activity is required for its function in cells (Ebbert et al., 1999) and for ATP-dependent nucleosome sliding in vitro (Shen et al., 2000). Ino80 provides docking sites for several core subunits including other two ATPases, Rvb1 and Rvb2. Rvb1/2 proteins form a hexameric AAA+ ATPase, related to the microbial RuvB, which facilitates the migration of strand exchange structures during recombination in bacteria (Jha et al., 2009). These two proteins are not unique for INO80-C, they are shared with another chromatin remodeling complex SWR-C (Wu et al., 2005, Chen et al., 2011). The Ino80 complex has both Rvb1 and Rvb2 in a 6:1 stoichiometry relative to the other subunits, consistent with the double-hexameric structure of RVBs. In vitro assays show that RVBs are essential for Ino80-dependent chromatin-remodeling activity, but not for the binding of Ino80 complex to the promoters (Jonsson et al., 2004). Rvb1/Rvb2 form so-called head module of INO80 complex (Tosi et al., 2013).

Ies2 is another conserved subunit of INO80-C among eukaryotes. In budding yeast was described as chromatin remodeling factor involved in telomere silencing (Benbow et al., 2008). Ies2 has a special structural role. Interlinks with its PAPA-1 domain to both Rvb1 and Rvb2, but also to Ies3 of the Nhp10-module and to regions along Ino80's entire polypeptide chain (Tosi et al., 2013). In humans is found that Ies2 is required for maximal ATPase and nucleosome remodeling. The efficiency of nucleosome remodeling by Ino80ΔN core complexes was stimulated by addition of recombinant Ies2 (Chen et al., 2013).

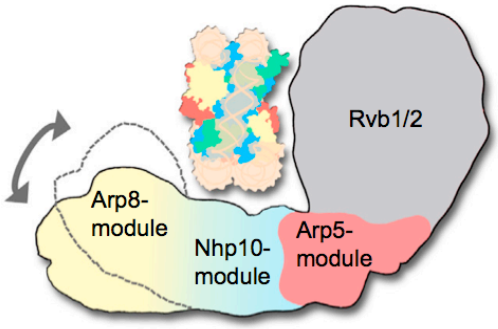


Fig 7. | INO80 moduls and nucleosome remodeling mechanism (Tosi et al., 2013)

Arp5, Ies6

Arp5 and Ies6 are forming neck module. Ies6 is exclusively crosslinked to domain 2 of Rvb2, whereas Arp5 crosslinked exclusively to Ies6 (Tosi et al., 2013). Ies6 and/or Arp5 are needed for optimal binding to nucleosomes (Chen et al., 2013). Arp5–Ies6 recruitment to the neck of INO80-C requires RvB proteins (Jonsson et al., 2004). This subcomplex is essential for nucleosome remodeling (Tosi et al 2013) ,although they may bind to chromatin independently of INO80-C (Yen et al., 2012).

In budding yeast IES6 is phenotypically simiral to ino80 mutant strain. Loss of ies6 results in increase ploidy, and it is believed that function to maintain chromatin structure at centromeres (Chambers at al., 2012). Actin related protein Arp5 also mimics ino80 deletion in budding yeast and it is required of INO80 ATPase activity. (Shen et al., 2003). Taken together, these data suggest that both ies6 and arp5 contribute to nucleosome recognition and are both essential for proper functioning of INO80 complex.

Nht1, Iec1, Iec3, Ies5

In *S. Cerevisiae* these subunits form body module and it is least conserved part of INO80-C. Nhp10 module of INO80 is a high-affinity nucleosome- binding module. Nhp10 is an HMG2-box protein known to bind distorted DNA (Ray at al.,

2009). It is thus plausible that the Nhp10 module interacts with nucleosomal DNA. Reduced affinity to nucleosomes and attenuation of remodeling, but no impact on the ATP hydrolysis, raises the possibility that the Nhp10 module facilitates nucleosome sliding by binding to reaction intermediate states (Tosi et al., 2013).

In *S. Pombe*, zinc-finger protein Iec1 was described as a component of INO80-C in 2009. Involved in replication and DNA damage response, but also linked to phosphate and nucleotide metabolism. Required for the binding of the Ino80 complex to target genes, and important for the expression of *cdc22* gene (Hogan et al., 2009), a gene that is also under MBF regulation.

There are almost no data on iec3 and iec5 neither in fission or budding yeast.

Arp8, Arp4, Ies4, Act1

The Arp8 module is a nucleosome-binding module and is located in the foot of the INO80-C complex. The Arp8 module in budding yeast comprises the evolutionary conserved subunits Act1, Arp4, and Arp8, Ies4 and Taf14 (Tosi et al., 2013). Yeast Arp4p and Arp8p have been reported to bind core histones.

Arp8 Binds weakly to dsDNA, H2A–H2B dimers, H3–H4 tetramers, or whole nucleosomes, possibly as an Arp8

homodimer (Gerlhod et al., 2012). When the INO80 complex binds to chromatin, a nucleosome is captured and placed between the Arp8 module and the head module. As the link between the Arp8 module and body module is flexible, it allows the Arp8 module to fold back and stabilize the nucleosome.

Actin (Act1) Actin has well-established functions in the cytoplasm, but the presence and potential functions of nuclear actin have been debated over several decades. Early observations of biochemical co-purifications of actin with nuclear proteins were dismissed as contaminations of cytoplasmic actin, which is a major protein component in the cytosol. While in cytoplasm forms filaments, in nucleus is monomeric, at it is a part of not only INO80-C but also SWR-C and NuA4 (Kapoor et al., 2013).

Arp4 is also a subunit of SWR-C and NuA4 complexes. Arp4 was also found to copurify with histone H2A from yeast extracts and interact with histones in two-hybrid assay (Harata et al., 1999), and binds to phosphorylated H2A histone upon DNA damage (Downs et al., 2004). In fission yeast is reported that Arp4 forms a complex with Mst1 acetyltransferase, and required for histone H4 acetylation, kinetochore-spindle attachment, and gene silencing at centromere (Minota et al., 2005).

Ies4 has no chromating binding role. It is phospshorylated upon DNA damage, and is target of Mec1/Tel1 kinases. Phosphorylation of Ies4 regulates the involvement of INO80 in checkpoint pathways that are initiated by replication stress. it is possible that the phosphorylation status of Ies4 in the INO80 complex may influence this chromatin remodeling process. Alternatively, it is also possible that Ies4 phosphorylation status modulates potential direct interactions between INO80 and checkpoint factors (Morrison et al., 2007).

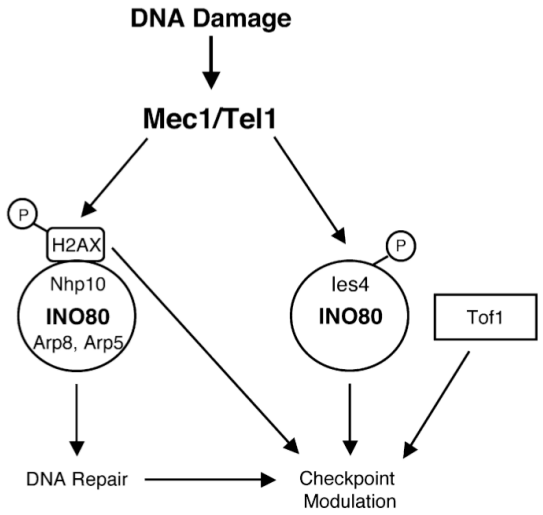


Fig 8. | Involment of Ies-4 in DNA damage reponse (Morrison et al., 2007)

5.2 H2A.Z Histone and +1 Nucleosome

A hallmark of eukaryotic genomes is the organization of DNA in nucleosomes. Nucleosomes are composed of octamers of histone proteins designated as H2A, H2B, H3, and H4 that wrap approximately 146 bp of DNA (Arents et al., 1991). Chromatin is generally highly repressive to cellular processes that involve DNA transactions such as gene transcription. In order to overcome this nucleosomal barrier cells have several mechanism; the first involves ATP hydrolysis to mechanically displace nucleosomes, the second consists of chemically modifying the tails of histones, such as by acetylation, phosphorylation, methylation, and ubiquitination, and third is to alter the composition of nucleosomes through the incorporation of histone variants that can directly or indirectly alter the permissiveness of chromatin to gene expression.

The bulk of the nucleosomes in chromatin contain two each of four major histones, H2A, H2B, H3 and H4; however, certain regions of chromatin are marked with nucleosomes containing minor histone variants. Histone H2A has the largest number of variant isoforms, but only one isoform, H2A.Z, is conserved in all eukaryotes. Studies in budding yeast show that H2A.Z is important for regulation of gene expression (Kobor et al., 2004; Mizuguchi et al., 2003), gene silencing (Meneghini et al., 2003), DNA repair (Kalocsay et

al., 2009), cell cycle progression (Dhillon et al., 2006) and chromosome stability (Krogan et al., 2004).

Histone H2A.Z is a stereotypic component of the chromatin landscape at eukaryotic promoters. H2A.Z is expressed throughout the cell cycle, and it can be incorporated into chromatin in the absence of DNA replication. Most yeast promoters have a DNase I hypersensitive site relatively depleted or free of nucleosomes (NDR), interrupting the nucleosome array; the two nucleosomes flanking the NFR are referred to as nucleosome −1 and +1. In all eukaryotes, the +1 nucleosome and a few downstream nucleosomes of active genes are enriched for the histone variant H2A.Z. The first (+1) nucleosome of an array engages the transcription machinery and so is potentially subjected to extensive regulation. Nucleosomes present a physical barrier that causes backtracking/arrest of RNAPII (RNA polymerase II), and this barrier cannot be efficiently overcome unless the nucleosome is destabilized. The main difference between H2A and H2A.Z nucleosome is in their stability. Incorporation H2A.Z into nucleosomal arrays alters their biophysical properties (Fan et al., 2002), potentially creating distinct chromatin structures. The extent of the +1 nucleosome barrier correlates with nucleosome occupancy but anti correlates with enrichment of histone variant H2A.Z. Importantly, depletion of H2A.Z from a nucleosome position results in a higher barrier to RNAPII. Nucleosomes present

significant, context-specific barriers to RNAPII in vivo that can be tuned by the incorporation of H2A.Z (Weber et al., 2014).

Genome wide location assays in budding yeast showed that H2A.Z is mainly localized in promoter of inactive genes. There is an inverse correlation between the transcription rate and H2A.Z occupancy. Housekeeping genes are by definition transcribed in most, if not all, conditions, and since H2A.Z is present at promoters of inactive genes, H2A.Z might be more important for the regulation of nonhousekeeping genes that are generally repressed when grown in rich medium but are strongly induced under specific growth conditions (Guillemette et al., 2005). These findings may account for a mechanism by which H2A.Z regulates transcription, since the absence of H2A.Z prevents RNA polymerase II and TBP from being efficiently recruited to specific yeast promoters (Adam et al., 2001).

In fission yeast it has been reported that H2A.Z mediates suppression of antisense transcripts (Zofall et al., 2010), is critical factor for modulating cohesin dynamics (Tapia-Alveal et al., 2014), regulates centromere silencing and chromosome segregation (Hou et al., 2010).

H2A.Z is incorporated into chromatin by SWR-C and is removed by INO80-C (Mizuguchi et al., 2004). Experiments in budding yeast shows that INO80 does not impact the total

amount of H2A.Z that is incorporated into chromatin, but rather there is an extensive reorganization of nucleosomal H2A.Z across the genome in the absence of Ino80. In the ino80Δ mutant H2A.Z becomes globally mislocalized, that support a role for the INO80 complex in regulating proper genomewide H2A.Z localization. Also, H2A.Z genomic occupancy is altered between the two cell cycle phases G1 and G2/M, in the wild type strain. Furthermore, H2A.Z was depleted during transcriptional activation of a cell cycle regulated gene in G1 cells. In contrast, H2A.Z levels in the *ino80Δ* mutant remain high and similar to the repressed level in both G1 and G2/M phases (Papamichos-Chronakis et al., 2011).

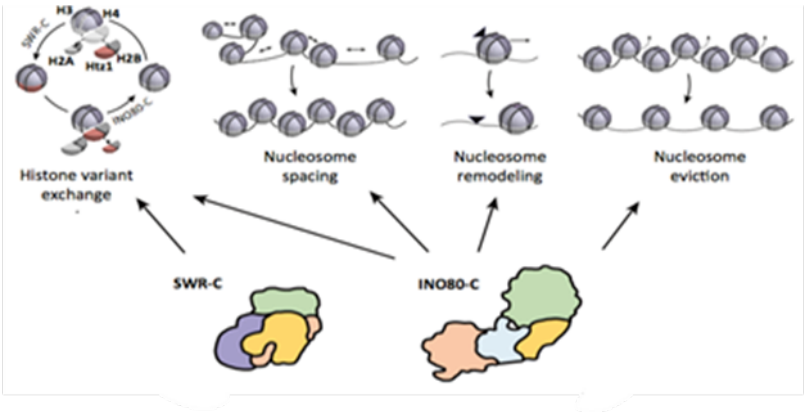


Fig 9. | SWR-C and INO80 role in nucleosome remodeling

Analyses of Ino80p and Arp5p chromosome-wide distribution by chromatin immunoprecipitation indicate that as many as two-thirds of the yeast genes in which transcription is affected by *ino80* mutations might be directly regulated by

the INO80 complex (Shimada et al., 2008). Depletion of Ies2 or Ies6 blocks activation of at least two well-characterized YY1 human transcription factor activated genes, cell division cycle 6 (CDC6) and glucose-regulated protein of 78 kDa (GRP78), indicating that the human INO80 complex can function as a YY1 coactivator (Affar et al 2006).

Finally in Ino80s mutant H2A.Z is also deacetylated apart from being mislocalized, and panacetylated mimetics repress i*no80* phenotypes. Acetylated H2A.Z is enriched at transcriptional active promoters where H2A.Z is preferentially evicted, and it has been suggested that H2A.Z acetylation may facilitate re-assembly of H2A.Z nucleosomes during gene repression (Millar et al., 2006). Hence, mislocalization of unacetylated H2A.Z is an inhibitor of genome stability that must either be acetylated or be removed by INO80 (Papamichos-Chronakis et al., 2011).

Results of ChIP experiments revealed that the yeast INO80 complex is recruited to origins of replication as cells enter S phase under normal, non-stress conditions and to stalled replication forks and unfired origins of replication in cells stressed by growth in HU (Shimada et al., 2008, Vincent et al., 2008). It is believed that INO80 helps progression and stability of replication forks, promoting fork migration by remodeling or removing nucleosomes in the path of the replication fork and perhaps by helping in the reassembly of nucleosomes behind the fork.

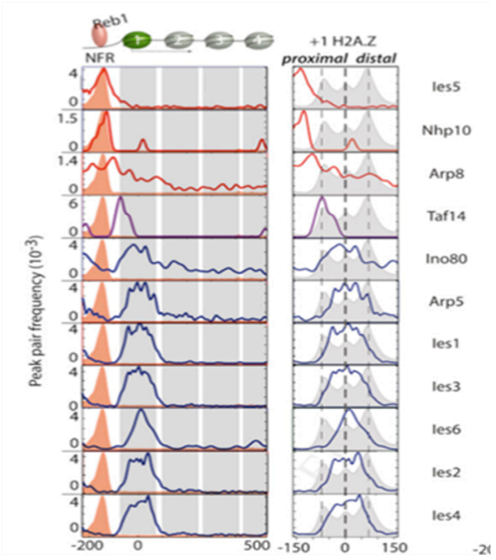


Fig 10. | Subnucleosomal detection of INO80 subunits across +1 nucleosome (Yen et al., 2013)

Although it has become clear that the INO80 complex and other chromatin remodeling complexes contribute to processes from transcription and DNA replication and repair to regulation of cell-cycle checkpoints, little is known about INO80 in *S. pombe*. Here we try to unreveal how MBF and INO80 complexes co-work together for a proper activation of MBF target genes and cell cycle progression.

OBJECTIVES

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

1. **To identify new MBF interactors.**
2. **To better understand how G1/S genes controlled by MBF are being activated.**

RESULTS

1. Identification of MBF interactors from previous purifications

Previously one-step immunopurification of MBF complex was performed. Cdc10 tagged in its own locus with HA was used as bait, and purification was sent to mass spectrometry.

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

A total of 2046 peptides, were identified. Few peptides were overrepresented in sample compared to the control sample. It is not possible to establish the threshold to consider any given peptide as clearly overrepresented in one sample, but the higher the iTRAQ ratio is, higher is the specificity of the purification.

From this purification Yox1 was identified as a new repressor of MBF activity (Gomez-Escoda et al., 2010). On this project we focused to characterize several other proteins from this purification as possible MBF interactors, as well as establishing and performing new immunopurification of Cdc10 under different conditions.

1.1 HMG proteins

A side from components of MBF and Yox1, there were no other proteins with that high ratio of purification. Yet, our attention was brought to two proteins with ratios around 1.3 that both contained High Mobility Group (HMG).

High mobility group (HMG) proteins are the most abundant non-histone chromatin associated proteins, named so because of their high mobility in acrylamid gel. Although having the ability to modulate transcription of their target genes by altering the chromatin structure, they do not possess intrinsic transcriptional activity, thus are called architectural transcription factors. This led us to investigate these proteins as possible modulators of MBF activity.

SPBC28F2.11 and SPAC57A10.09c were two proteins from the list and with another HMG protein that we had in our mutant collection (SPBC19G7.04) were checked for possible phenotypes.

Survival assay

We first checked survival upon hydroxyurea treatment of cells lacking genes decoding for HMG proteins (Fig. 1). HU is drug that directly inhibits Cdc22 protein that is under control of MBF. This inhibition leads to upregulation of all MBF dependent genes and activation of checkpoints. If HMG proteins are involved in any way in this particular regulation, when corresponding genes deleted, cells would have a defect in growth. Anyhow, no mutant showed defect in HU plates different from the wild type cells.

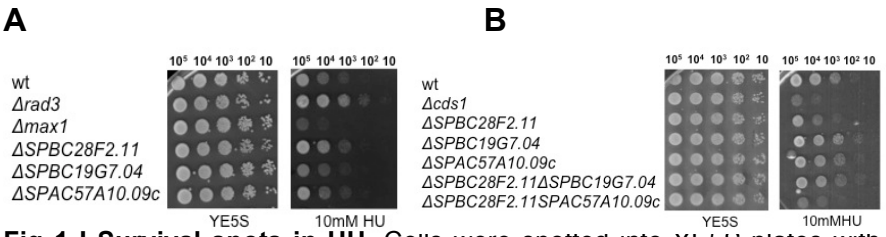


Fig 1 I Survival spots in HU. Cells were spotted into YE5S plates with HU drug at the indicated concentrations and incubated at 30°C for 3 to 4 days. **A)** Individual deletions of HMG protein do not show sensibility in HU plates **B)** Double deletions of HMG proteins do not show sensibility in HU plates.

We were wondering is this result was due to redundancy of HMG proteins, so we made double deletion and repeated the assay. Unfortunately, we did not observe any obvious phenotype neither.

Induction of MBF dependent genes is not impaired

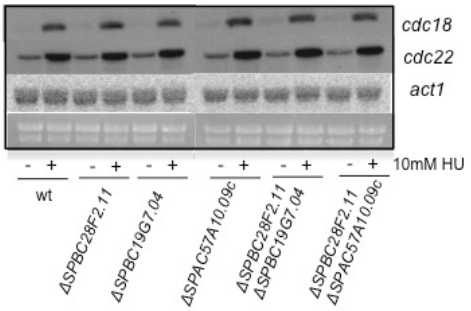


Fig 2. I mRNA expression of HMG proteins upon HU. Total RNA was prepared from untreated (-) or hydroxyurea-treated (+) cultures (3 hours at 30°C), and analyzed by hybridization to the probes indicated on the left. *act1* probe was used as a loading control.

At the same time we checked the induction of MBF genes upon HU in various mutations of HMG proteins. We could conclude that there was no clear difference compared to wild type strain (Fig. 2).

SPBC28F2.11 expression is cell cycle dependent

While investigating the literature for HMG proteins, we noticed that *SPBC28F2.11* is annotated as cell cycle regulated, peaking at late G1 phase. This led us to think that it could be important for events in S phase. Contrary to MBF dependent genes, upon HU treatment *SPBC28F2.11* is downregulated (Fig. 3, A). Protein levels follows mRNA levels; hence there was also less protein product upon same conditions (Fig. 3, B).

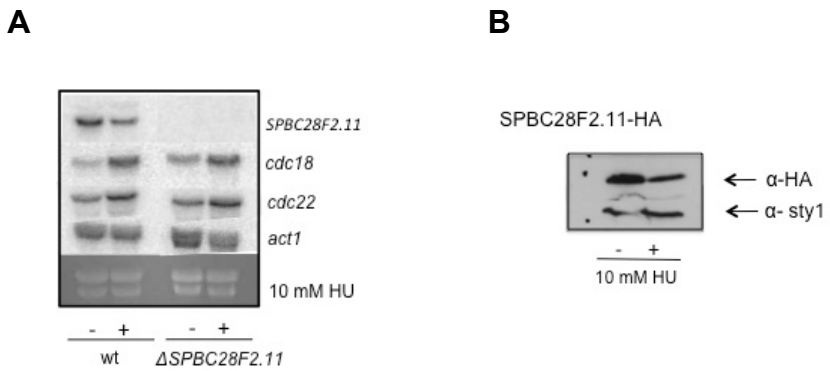


Fig 3. I SPBC28F2.11 is downregulated upon HU. A) mRNA expression of SPBC28F2.11. Total RNA was prepared from untreated (-) or hydroxyurea-treated (+) cultures (3 hours at 30°C) and analyzed by hybridization to the probes indicated on the left. *act1* probe was used as a loading control. **B)** Native extracts were analyzed by Western blot with anti-HA and anti-Sty1.

Since HU provokes activations of checkpoints and arrests cells in S phase, and we assumed that HMG protein is important for what is coming alter START, downregulation of SPBC28F2.11 would be expected.

We tested if this could be due to the activation of S phase checkpoint kinase Cds1, so we put HA tagged SPBC28F2.11 in *Δcds1*, and in its upstream kinase *Δrad3*. Anyhow, protein levels changed as in wild type (Fig. 4).

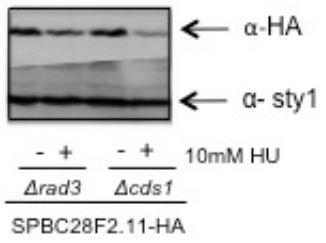


Fig 4. I Change in protein level upon HU is not due to checkpoint kinases Rad3 or Cds1. Native extracts were analyzed by Western blot with anti-HA and anti-Sty1.

We also checked the level of SPBC28F2.11 expression throughout cell cycle. To do that we synchronized the culture using *cdc25-22* strain, that is temperature sensitive. After four hours at non permissive temperature cells are blocked at G2/M, and release them at permissive temperature would synchronized them in cell cycle, entering mitosis after about 20 minutes.

The results showed that SPBC28F2.11 actually cycles, with the peak around G1/S transition (Fig. 5).

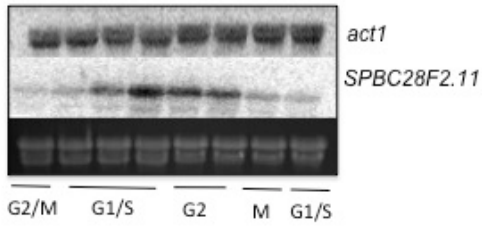


Fig 5 I SPBC28F2.11 is cell cycle regulated gene. Total RNA from *cdc225-22* synchronized culture was obtained every 20 minutes and analyzed by Northern blot, and hybridized with *act1* and *SPBC28F2.11* probe.

When we checked the protein level in cell cycle experiment, we also observed the accumulation of protein as cells were passing through S phase (Fig 6). Difference to mRNA levels that decay at late G2 and M phase, protein is accumulated throughout G2 and gets rapidly depredated in M phase.

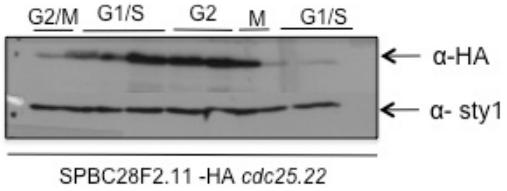


Fig 6. I SPBC28F2.11 protein level is cell cycle dependent. Native extracts were obtained every 20 minutes and analyzed by Western blot with anti-HA to detect SPBC28F2.11-HA and anti-Sty1 as a control of protein quantity.

SPBC28F2.11 is found in MBF promoters

To test whether SPBC28F2.11 could modulate MBF dependent transcription as architectural protein, we performed Chromatin immunopercipitation (Fig. 7).

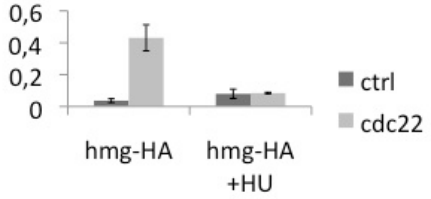


Fig 7. I ChIP data for SPBC28F2.11 occupancy at MBF genes. Data was obtained from three independent experiments and are expressed as mean ± SD.

We found that SPBC28F2.11 is bound to MBF promoters, but is evicted upon HU. This eviction could be real or due to disregulation of SPBC28F2.11 in HU conditions.

Deletion if SPBC28F2.11 has no effect on MBF binding or MBF dependent transcription

Although it was clear that SPBC28F2.11 because of its periodicity would be involved at some point in cell cycle regulation, and because HU was evidently affecting its transcription would be important for checkpoint signalling, we could not find connection between SPBC28F2.11 and MBF complex. Since there was no difference compared to wild type of MBF dependent genes in asynchronous cultures or upon HU treatment, we decided to test transcription in a *cdc225-22* strain, where any difference would be more obvious (Fig. 8, A).

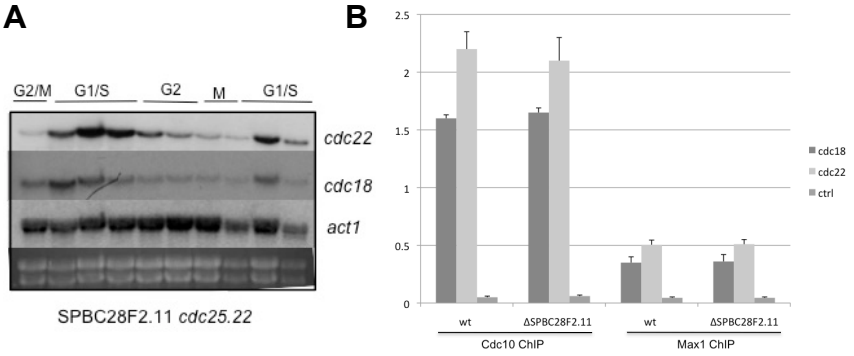


Fig 8. I A) Total RNA from SPBC28F2.11 *cdc25-22* synchronized culture was obtained every 20 minutes and analyzed by Northern blot, and hybridized with *cdc22, cdc18* and *act1*probe **B)** ChIP data for Cdc10 and Yox1 occupancy at MBF genes in wild type and *ΔSPBC28F2.11* strain.

Anyhow, not even under these conditions we observed a clear difference in *ΔSPBC28F2.11* compared to the wild type. Furthermore, binding of Cdc10 and Yox1 was not impaired when *SPBC28F2.11* deleted (Fig. 8, B). Taken together all

this results, and having other candidates and new purification of MBF, we decided to move over, leaving the possible role of HMG proteins as modulators of MBF activity unclear.

1.2 Mbf1– Multiprotein Bridging Factor

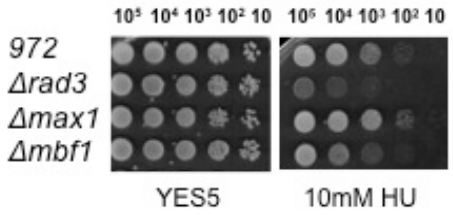


Fig. 9 I Survival spots in HU. ***Δmbf1* does not show difference compared to the wild type.** Cells were grown in YE5S and were spotted from 10 to 10 in YE5S plates containing HU at the indicated concentrations and incubated at 30°C for 3 to 4 days.

Another protein that seemed interesting as a possible modulator of MBF activity was multiprotein bridging factor – Mbf1, annotated as transcriptional coactivator, and with iTRAQ ratio of 1.2.

First we checked for the phenotype in HU plates. We found that *Δmbf1* is no different than the wild type (Fig. 9).

Mbf1 localizes in nucleus and co-precipitates with MBF complex

In order to find out the localization of Mbf1 and possible interactions with MBF complex, we tagged Mbf1 protein with HA and GFP tags on its own locus at the carboxi terminus. We used Mbf1-GPF for microscopy and we also crossed it

with Cdc10-HA, for Immunopercipitation experiments, as Mbf1-HA was crossed with Yox1-Myc for same reasons. We verified *in vivo* interaction of Mbf1 with Cdc10 (Fig.10, A), but also with the repressor of MBF complex Yox1 (Fig. 10, B).

A **B**

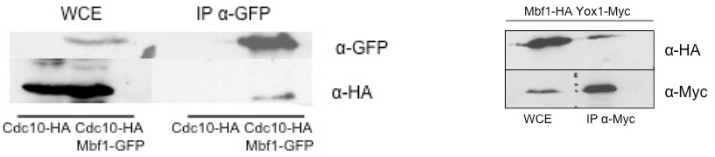


Fig. 10 l Mbf1 interacts with A) Cdc10 an B) Yox1. Native extracts were obtained, immunoprecipitated 2mg of protein with corresponding antibody and detected by western blot with indicated antibodies.

When observed under microscope, Mbf1-GFP is localized in nucleus as well as in cytoplasm. We decided to put Mbf1-GFP in *cdc25-22* background that allows us to synchronize cells, so we could test if localization changes through out cell cycle. What we observed was that Mbf1 was starting to accumulate to nucleus upon release, reaching maximum of accumulation after about one hour, that corresponds to peak of S phase (Fig. 11).

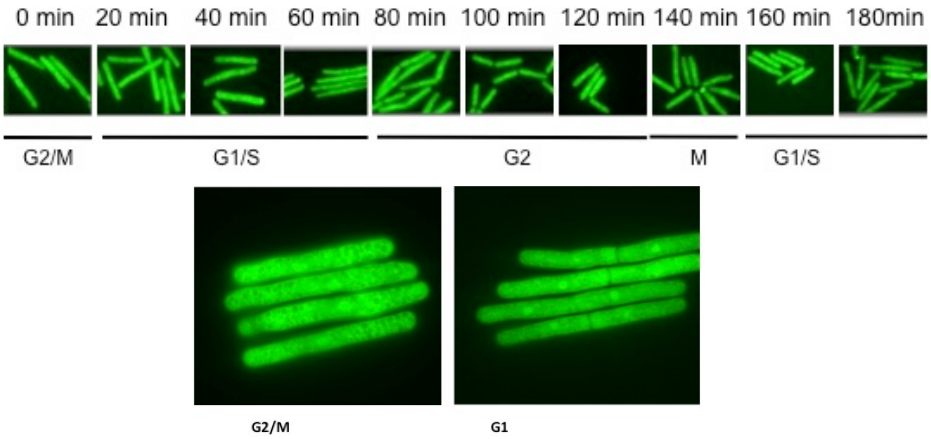


Fig 11. I Accumulation of Mbf1 is cell cycle dependent. Mbf1-GFP *Cdc25-22* was synchronized by blocking for four hours at non-permissive temperature, after it was released and samples was obtained every 20 minutes for microscopy.

Levels of Mbf1 protein change through out cell cycle

Since we saw accumulation of Mbf1-GFP in nucleus upon release, we also wanted to check protein levels under same condition. We used Mbf1-HA in *cdc25-22* background and analyzed by western blot.

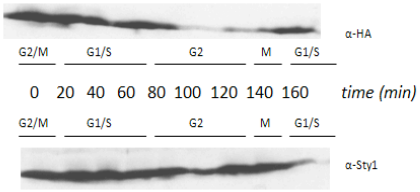


Fig 12. I Level of Mbf1 is cell cycle dependent. Mbf1-HA *cdc25-22* was synchronized by blocking for four hours at non-permissive temperature, alter it was released and samples was obtained every 20 minutes for western blot analysis

Protein levels seemed to be constant after release and until end of the S phase, in G2 and M phase protein was downregulated, and started to accumulate again at the beginning of the G1 phase (Fig. 12).

Mbf1 does not affect periodicity of MBF genes or induction upon HU treatment

Although we could confirm interaction between Mbf1 and MBF complex, we could not see how Mbf1 could affect MBF transcription. When deleting *mbf1,* transcription of MBF dependent genes was not impaired. Further more, the induction was changed upon HU, and cyclic nature of those genes was not changed when deleting *mbf1* in *cdc25-22* background (Fig. 13, A).

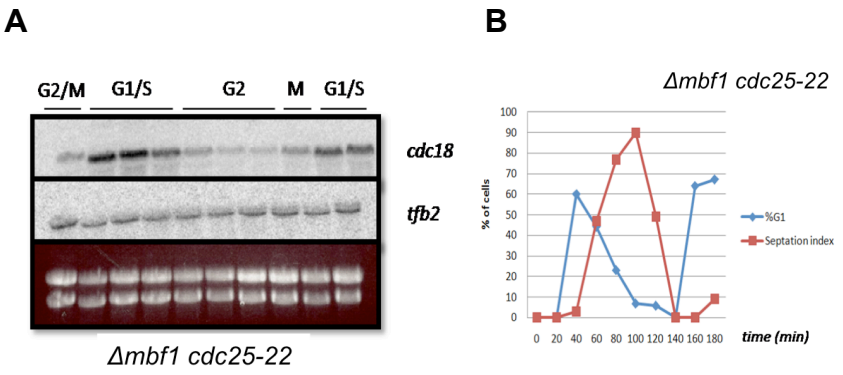


Fig 13. I A) Total RNA from Δ*mbf1 cdc25-22* synchronized culture was obtained every 20 minutes and analyzed by Northern blot, and hybridized with *cdc18* and *tbf2* probe. **B)** Synchronicity of a cell cycle was measuried by counting 2 nuclei cells and septation.

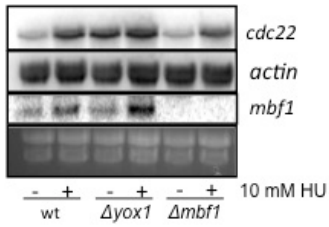


Fig 14. I *mbf1* is upregulated upon HU. Total RNA from Δ*mbf1,* Δ*yox1* and wild type cultures HU treated and nontreated was obtained and analyzed by Northern blot, hybridized with *cdc22, mbf1* and *actin* probe.

Nevertheless, deletion of *mbf1* was speeding up the cell cycle for about twenty minutes (Fig. 13, B). This does not affect the peak of MBF transcription in G1/S. One interesting thing was observed when checking HU induced transcription in Δ*mbf1* (Fig. 14). Although in Δ*mbf1* HU induced transcription is just as wild type, *mbf1* gene was also induces upon HU, just as *cdc22*. This induction is not MBF dependent, because in Δ*yox1 mbf1* is not induced like others MBF dependent genes.

Since we could not observe clear affect of Mbf1 on MBF we decided to move forward, while preparing new purification.

2. Immunopurification of MBF complex

Previous immunoprecipitation of Cdc10-HA was performed in asynchronous culture, where majority of cells are in G2, and MBF complex mostly repressed. In this conditions repressors are bound to the complex. Little is known how MBF is being activated and who are the activators if any. Since at the moment two corepressors were well characterized, and we

were more interested in possible ways of positive regulation of MBF we had to find conditions of purification where MBF transcription is fully ON, and possible activators are interacting with the complex.

In order to do so, our original idea was putting Cdc10-HA strain in *cdc25-22* background. This would allow us to synchronize a culture and obtain protein extract from a specific cell cycle phase, G1/S where MBF transcription is fully induced.

In *cdc25-22* strain after four hours of blocking cells at 37° C, a non-permissive temperature, cells are blocked in late G2. Realising the culture at 25° C, cells rapidly enter mitosis, and after about 30 minutes MBF dependent genes are fully induced. In this moment if any, activators of MBF would be present. Then we could pellet cells at this moment and immunoprecipitate Cdc10-HA. In order to set up an experiment, once we had tagged Cdc10 in *cdc25-22* we had to check cell cycle and do the experiment on a small scale. We did a block and release experiment of Cdc10-HA *cdc25-22*, together with *cdc25-22* strain as a control.

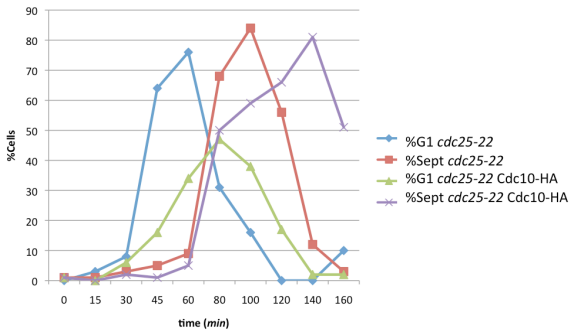


Fig 15. I Tagging Cdc10 in *cdc25-22* background impair cell cycle. Samples of *cdc25-22* and *cdc25-22* and Cdc10-HA were taken during cell cycle for DAPI staining, and microscopy analysis for cell cycle phases.

What we did not expect was that Cdc10-HA *cdc25-22* had a huge delay entering cell cycle after release, for about 40 minutes (Fig. 15). The experiment was repeated several times to be sure that is not a question of experimental conditions or artefact, but every time we could conclude the same. One possibility was to take the samples later after release, to coincide with the peak of G1 of this delayed enter, but we could not be sure at all what possibly happens with MBF in a strain with such delay.

We decided to check MBF transcription of this tagged strain, compared to *cdc25-22* only. We saw that although *cdc18* gene, that we use as one of standard genes to check MBF activity and regulation, was behaving as in wild type conditions, *cig2* (gene that encodes for Cig2, cyclin necessary for proper progression of a cell cycle and at the

same time is MBF dependent and inhibits MBF complex in a negative feed back loop manner), was misregulated (Fig. 16).

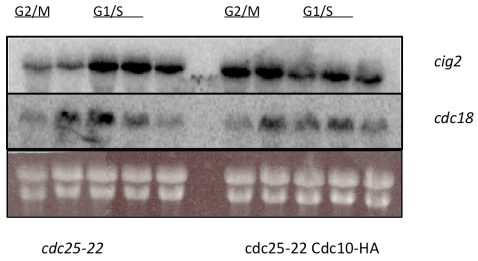


Fig 16. l MBF genes in *cdc25-22* Cdc10-HA are misregulated. Total RNA from *cdc25-22* and *cdc25-22* Cdc10-HA synchronized culture was obtained every 20 minutes and analyzed by Northern blot, and hybridized with *cdc18* and *cig2* probe.

We do not understand why Cdc10-HA strain that behaves as wild type in a *cdc25-22* background has this phenotype. Still, carboxi terminal of Cdc10 in essential for binding to its regulators and for phosphorylation. Tagging Cdc10 at carboxi terminal with HA tag although a small one could impair Cdc10 fine regulation. This may not been seen in asynchronous culture, but in a *cdc25-22* background where Cdc25 phosphatase is never fully active, this phenotype of Cdc10-HA could be observed. These reasons made us leave the possibility of working with this strain so we had to find another way of catching Cdc10 when MBF is fully active.

At the same time we manage to obtain polyclonal anti Cdc10 antibody (Fig. 17). This antibody was going to let us work with untagged *cdc25-22* strain, but we were facing another problem. In ideal settings we would use a control of purification. In a case of tagged strains, control would be an untagged one, but in case were we directly immuniprecipitate

with an antibody against protein epitope control would be *cdc25-22* Δ*cdc10.* Since this could not be, since Cdc10 is essential, we decided to use the same strain *cdc25-22* and precipitating with anti Cdc10 as sample and anti HA as control.

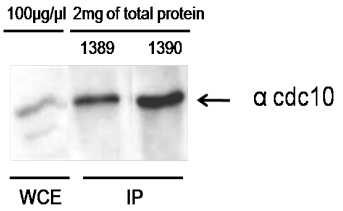


Fig. 17 I Cdc10 purification from *cdc25-22* strain. Cells were synchronized by shifting the temperature at 36° C, after 4 hours culture was release at permissive temperature, and protein extract were obtained after 30 minutes.

We have checked polyclonal antibody Cdc10 for immunoprecipitation, and performed the experiment. 3 liters of culture was synchronized by blocking cells at 36° C for four hours. Pellet was collected 30 minutes after release, at the onset of mitosis. Procedure of obtaining native extracts and immunoprecipitation is described in section Materials and Methods. The sample was split in half, one for anti Cdc10 and other for anti HA IP. The success of IP was checked by CoIP experiment between Res2 and Cdc10 (Fig. 18).

The samples were sent to Mass Spectrometry analysis. iTRAQ procedure is described in section Material and Methods. In order to obtain iTRAQ ratio, same protein has to be found in both purifications, meaning if some proteins are found exclusively in one sample, they would be left out from ratio ranking. Our bait protein Cdc10 was found only in one

immunoprecipitation, anti Cdc10, as many others. Among other HMG protein SPBC28F2.11 was precipitating with Cdc10 again, but our attention was brought to two other proteins Rvb1 and Ino80 helicase.

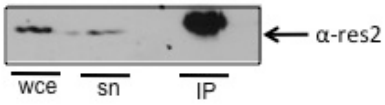


Fig. 18 I Cdc10 was precipitated with anti Cdc10, Western blot was performed and developed against anti Res2

This is the main limitation of MS method. To be sure that proteins co-immunoprecipitating with Cdc10 are actually specific for this conditions we have had to repeat the procedure. Yet, we have decided to change conditions and antibodies, leaving one condition that MBF gene must be fully induced.

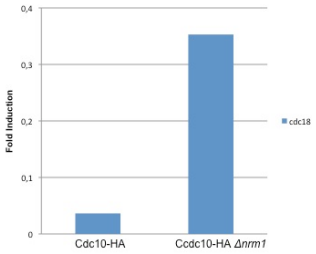


Fig. 19 I Total RNA was isolated from asynchronous Cdc10-HA and Cdc10-HA *Δnrm1* strains, and level of expression of *cdc18* gene was checked by RT-PCR method.

We were having two problems with this purification, Cdc10 antibody is not so strong for IPs, and using different antibodies for sample and control was also not proper. Instead, we decided to use *Δnrm1* strain where MBF genes

are induced and to tag Cdc10 with HA. To be sure that in this background HA is not influencing MBF transcription we have performed RT-PCR. After assuring that HA tag is not changing induction in *Δnrm1* mutant (Fig. 19), we decided to perform a new purification.

One liter of each culture was grown asynchronously. Native extract was obtained, and samples were immunoprecipitated with anti HA crosslinked with protein G, sepharose beads. Detailed procedure is described in Material and Methods section. We have checked success of IP performing Western Blot. We also checked how a random protein, that should not IP with Cdc10 behaved in this particular purification (Fig. 20).

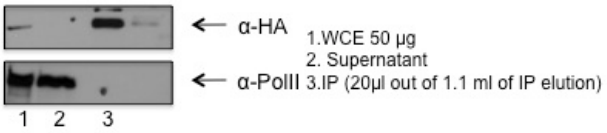


Fig. 20 I After purification Cdc10-HA was checked by Western Blot. To check specificity of purification western blot was checked for Pol II antibody also.

After assuring that IP was successful, at least in manner of IP of the bait Cdc10-HA, samples were sent to MS. The avoid the problem of ratios considering protein precipitation in one or other sample, Proteomic Unit decided to give provisional number to every protein in a sample that was not found, but did precipitated in one of them. Total of 389 proteins were found. Cdc10-HA once again precipitated only in main purification. We were also able to isolate Res2.

We where surprised to see that this time four different subunits of same complex INO80 was co-immunoprecipitating with Cdc10; Ies5, Alp5, Ies4, and Rvb1 (Fig. 21). This confirmed previous purification where main subunit Ino80 helicase, together with Rvb1 was precipitating with Cdc10. Next section describes characterization of INO80 complex, and its role in MBF dependent transcription.

Description	ΣCoverage	RATIO
Res2	1,67	21,74243755
Cdc10	10,17	21,44281659
Ies4	5,67	21,43502874
Ies5	15,97	20,86776469
Alp5	4,39	20,6674818
Rvb1	15,57	2,404240451

Fig. 21 I List of proteins isolated in purification. Four different subunits of INO80 complex were coimmunoprecipitated with Cdc10-HA

3. INO80 characterization

1.1 no80 and Ies4 interact with MBF

In order to determine whether subunits of INO80 complex interact with MBF, we have decided to tag Ies4 with Myc tag, Ino80 with FLAG tag in its own corresponding loci, at the carboxi terminal of the protein. We have constructed a strain that contained Cdc10-HA in either Ies4-Myc or Ino80-FLAG background, to perform co-immunoprecipitation experiments. We used native protein extracts, and antibodies against Myc, HA and FLAG.

We verified the *in vivo* interaction of both proteins with Cdc10- We discarded unspecific binding to the antibodies using strains carrying only one tag (Fig. 22 A, B).

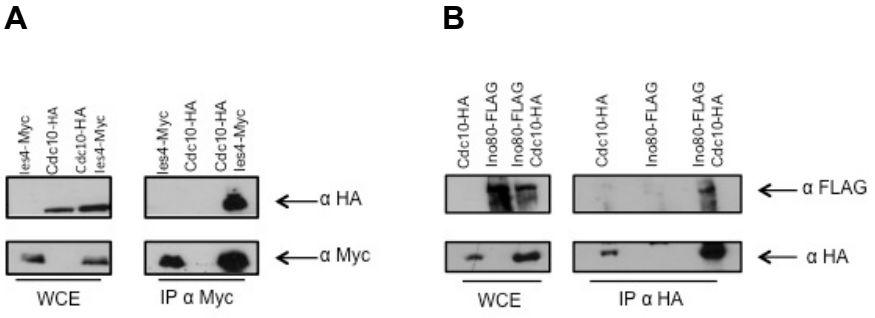


Fig. 22 I INO80 complex interacts with MBF. A) Ies4 CoIPs with Cdc10. **B)** Ino80 CoIPs with Cdc10. Extracts from strains expressing Ies4-13Myc, Cdc10-HA, Ino80-FLAF were immunoprecipitated (2 mg) with the indicated antibodies and proteins were detected by western blotting.

To further characterize interaction between INO80 and MBF complex, we analyzed binding of Ino80 and Ies4 to MBF dependents promoters (Fig. 23).

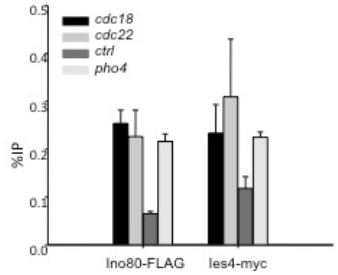


Fig. 23 I Representative ChIP data for Ino80 and Ies4 occupancy at MBF genes promoters. Ino80 and Ies4 are bound to the promoters of MBF genes. Occupancy at MBF promoters was measured using α-FLAG and α-HA antibodies. Data was obtained from three independent experiments and are expressed as mean ± SD. *pho4* was used as positive control of INO80 binding.

2.2 INO80 complex influence MBF transcription

After that we have confirmed that Ino80 and Ies4 interact with Cdc10 and that are bound to the promoters of MBF genes, we were wondering how deletion of different subunits of INO80 complex influences expression of MBF genes. Since, many of INO80 genes are essential, and deletions are unviable, we were not able to check all subunits, but we did check the viable ones. We also included a histone variant H2A.Z, *pht1*, a histone whose localization was regulation INO80 complex. From the experiment we could conclude that while deleting some subunits does not influence MBF dependent transcription, in many others transcription of *cdc18* and *cdc22* was downregulated.

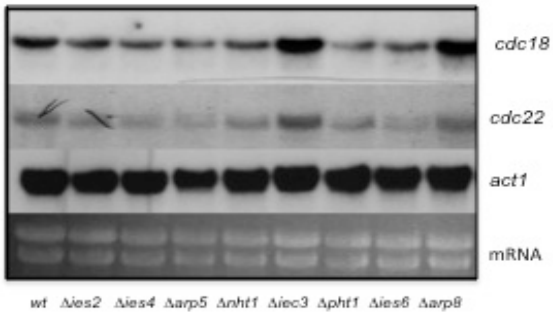


Fig. 23 I INO80 subunits regulate MBF-dependent transcription. Total RNA was prepared from asynchronous cultures, and analyzed by hybridization to *cdc18, cdc22* and *act1* probe.

We could see that *ies2, ies4, arp5, nht1, pht1* and *ies6* deletions led to downregulation of MBF transcription (Fig. 23). Still, *Δarp5* and *Δies6* had also other defects; slower cell growth and polyploidy. What we also saw was that *Δpht1* mutant had impaired MBF transcription, that was surprising. We decided to check also two histone mutants, which are mimicking acetylated Pht1-4KQ or nonacetylaed histone, Pht1-4KR.

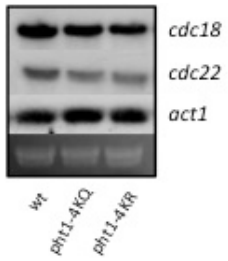


Fig. 24 I In Pht1-4KR mutant, MBF transcription is impaired. Total RNA was prepared from asynchronous cultures, and analyzed by hybridization to *cdc18, cdc22* and *act1* probe.

From that we could conclude that unacetylated mutant Pht1-4KR also had problem to fully induce MBF transcription (Fig. 24), indicating that proper acetylation of this histone variant is important for regulation of MBF dependent transcription. We

were also able to construct *ino80* under nmt, thiamine promoter. We have checked protein level of Ino80 after adding thiamine, and set up conditions where we could not see protein by Western Blot, to see how loss of Ino80 protein affects MBF transcription. After 5 hours of growing culture with thiamine Ino80 was not detected by Western Blot, and we performed Northern Blot experiment (Fig. 25).

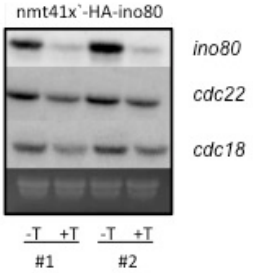


Fig. 25 l Ino80 affects MBF dependent transcription. . Total RNA was prepared from asynchronous cultures before and after 5 hours of thiamine treatment, of two clones, and analyzed by hybridization to *cdc18, cdc22* and *ino80* probe.

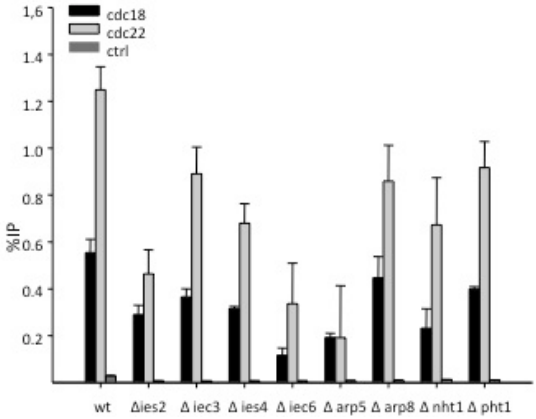


Fig. 26 l Representative ChIP data for Cdc10 occupancy at MBF genes promoters in different INO80 mutants. Occupancy at MBF promoters was measured using α-Cdc10 antibodies. Data was obtained from three independent experiments and are expressed as mean ± SD. *pho4* was used as positive control of INO80 binding.

We saw that under this conditions where Ino80 protein level was diminished MBF transcription was downregulated. When we checked binding of Cdc10 to its promoters in different INO80 mutants we saw that Cdc10 was binding to lower extent in several mutants compared to wild type (Fig. 26). Although the major effect we saw in *Δies6* and *Δarp5*, since these mutants have many other problems we could not trust results in these two particular mutants.

3.3 INO80 mutants rescue *Δcds1* phenotype in HU plates

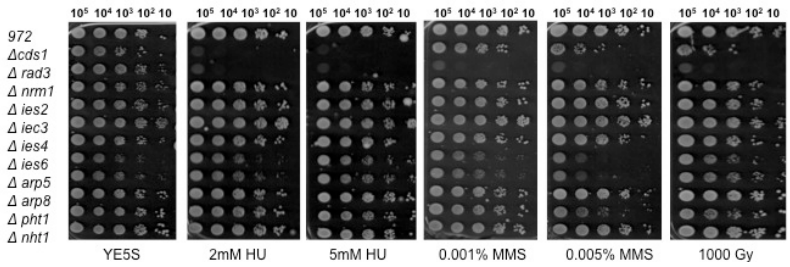


Fig. 27 I INO80 mutants phenotypes upon HU, MMS and radiation treatment. Cells were grown in YE5S and were spotted from 10 to 10^5 in YE5S plates containing HU or MMS at the indicated concentrations or treated with radiation with indicated grays. and incubated at 30°C for 3 to 4 days

We have also checked if mutants of INO80 would have any defect when growing in HU. We were expecting to see sensitivity phenotype, because of annotated roles of INO80 in checkpoints. Still, this was not the case. We have checked sensitivity of different INO80 deletions upon replicative stress (HU) and double break strain (MMS, radiation). While in HU plates we did not observed no phenotype, in MMS plates only *Δarp5* and *Δies6* showed sensitivity (Fig. 27). Once again,

these two mutants are the one with biggest problems considering cell growth.

We were very surprised to see that none of the mutants had no sensitivity HU plates, opposite of what was previously described for *S. cerevisiae.* We decided to put these mutants in *Δcds1* background, and to check HU sensitivity again.

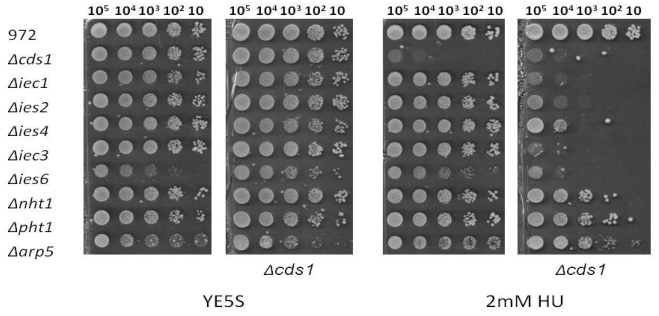


Fig. 28 I INO80 mutants reverted *Δcds1* phenotype. Cells were grown in YE5S and were spotted from 10 to 10^5 in YE5S plates containing HU concentrations and incubated at 30°C for 3 to 4 days

Many of these mutants were able to revert *Δcds1* phenotype, making it less sensitive upon HU treatment (Fig. 28). This could mean that INO80 is important in transducing the information of DNA damage, where when subunits are lost, an important message of blocking cell cycle was lost. To further test this we have performed to different experiments, Chromosome loss assay, and followed cell cycle upon HU by FACS.

3.4 INO80 mutants have genomic instability

If INO80 mutants had some aberrant S phase regulation, a different way to detect it would be to analyze the possible consequences of this misregulation. We tested for chromosomal instability of different INO80 strains. We constructed strains carrying an extra chromosome (minichromosome 16), that is an episomal plasmid that complements the *ade6*-M210 mutation in the *ade6* gene (required for the synthesis of adenine).

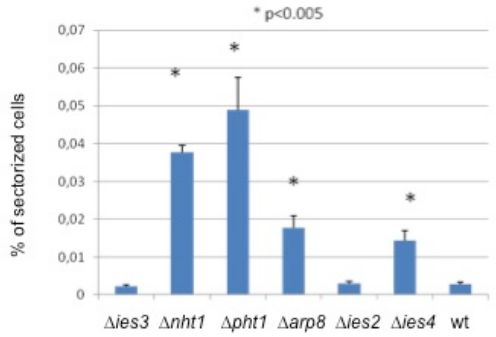


Fig. 29 I INO80 mutants show genomic instability. Strains carrying the minichromosome 16, (WT, Δ*ies2,* Δ*ies3,* Δ*ies4,* Δ*arp8,* Δ*nht1,* Δ*pht1*), were grown in YE5S till midlog phase and 500 cells were spotted into MM plates. Number of sectorized (white and pink) colonies was measured as a percentage of chromosome loss.

This minichromosome was transformed in a wild type *ade6*-M210 strain and in a Δ*ies2,* Δ*ies3,* Δ*ies4,* Δ*arp8,* Δ*nht1* and Δ*pht1 ade6*- M210 strain. The transformed strains are able to grow in media without adenine unless they loose the extra chromosome. If chromosome loss occurs, cells growing in media without adenine become pink as a consequence of the

accumulation of an intermediate product of the adenine biosynthetic pathway. Percentage of appearance of partially pink colonies (white colonies with pink sectors) is an index of chromosome loss and therefore indicates chromosomal instability. While Δ*ies3* did not show genomic instability, every other strain that we checked did. We were never able to obtain Δ*ies6* and Δ*arp5* with minichromosome, since both strains diploidize (Fig. 29).

3.5 INO80 mutants overcome HU arrest

To further test phenotypes of INO80 mutants upon S phase checkpoints we decided to analyze the cell cycle by FACS, upon HU treatment. After 3 hours of treating log phase growing culture with 10 mM HU, wild type cells blocks in S phase, with 1 C DNA content. After washing cells form HU, and releasing them from S phase block, cells will start to progress through cell cycle, and alter about one hour, synthesis had occurred, DNA content is duplicated, 2C, and cells progress further.

Δ*cds1* cells, which lack the effector kinase of the DNA synthesis checkpoint, are highly sensitive to HU, still they are able to release from HU block, and progress through cell cycle, although slower, but eventually die due to high sensitivity in HU.

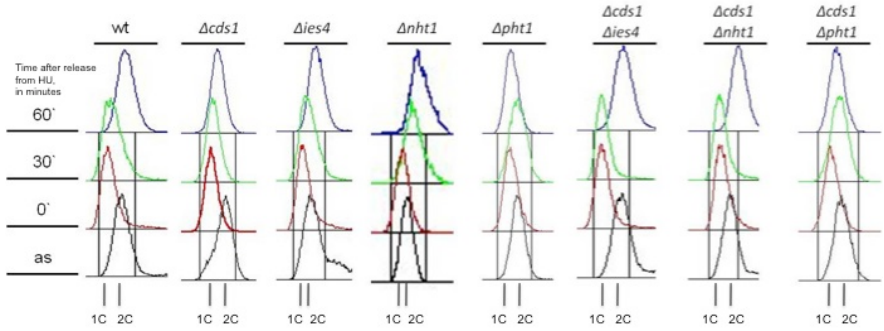


Fig. 30 I FACS profiles of cells after release from HU. Cells were treated with 10mM HU for 3 hours, after what cells were washed two times with YE5S, and then cell cycle was followed by FACS.

Since we saw genetic interaction between INO80 subunits and Cds1 in HU plates, we were wondering how those mutants progress through cell cycle upon release from HU. We saw that *Δnht1* and *Δpht1* progress faster through cell cycle after release from HU, and that in double mutants *Δcds1* also progress faster (Fig. 30). Taken all together, INO80 mutant are resistant to HU, release faster, but do have aberrant S phase regulation, since they exhibit genomic instability. All this could mean that some subunits of INO80 are important for S phase regulation and response.

3.6 Ies4 phosphorylates upon HU

Although we have seen phenotypes upon HU treatments, we did not see clear change in regulation of MBF dependent genes upon HU. We focused to further characterize Ies4 protein, since it was one of INO80 subunits that co-immunioprecipated with Cdc10.

We saw that upon HU treatment Ies4-Myc that in Western Blot show 2 bands, with prominent upper one, collapsed to lower band. This means that Ies4 has phosphorylated and nonphosphorylated form. Usually, phosphorylation of a protein leads to shift in acryl-amid gel, this phosphorylation normally leads to retarded band. Yet in our case, upon HU we observed that Ies4 migrates faster. Although not usual the case, phosphorylation could lead to faster migration, too. We checked for possible Cds1 and Rad3 conserved sites, and found that Ies4 has one conserved Cds1 phosphorylation site. Cds1 sites are LXRXXS/T (Fig. 31). Ies4 is a small protein that has 194 amino acids and molecular mass of 21,2 kDa.

MSETLVLHLKVPTERFREVLSSLKEKQNFTASPSSQPKPQERPFQMKKPRAPYGMG
PRAMKRREKAEKEKLGVVNDELAESSKPSSGAATPTRSAPKSSAGLINSGLRALDRS
GKPCRRWEKKPISI**RSIS**TIVWKLPLWIGTPDSIPNTPELPVKTTLDSVNEIAAALSTHA
ESSPMDA TSPVDSMPES ATGI

Fig. 31 I Cds1 phosphorylation site in Ies4 protein sequence. Phosphorylation site showed in red.

The next step was to determine if the change of mobility observed was checkpoint dependent. To answer this question, we checked if the phosphorylation shift band disappeared in cells deleted for the kinases of the replication checkpoint pathway. We tested both, Rad3, the upstream kinase, and Cds1, the effector kinase. We analyzed by western blot the mobility of Ies4-Myc in Δ*cds1* strains. The phosphorylation shift was not observed in strain carrying the deletion. To be sure that those bands are due to different

phosphorylation forms of protein, we also treated the samples with Alkaline Phosphatase (Fig. 32).

We could conclude that Ies4 was phosphorylated upon HU, and that phosphotylation was lost in ∆*cds1* strain, suggesting that Cds1 is phosphorylating Ies4. We also checked if this band shift could be seen upon MMS treatment, but no shift was observed. This could mean that Ies4 phosphorylation is specific for S phase regulation, but not for double strand break, and DNA damage. Our next idea was to mutate Cds1 site, and tag mutated protein with Myc for further confirmation. Although we were able to made punctual mutation of Ies4 S131A, we were never able to tag this protein with any tag that we had available in our lab. This final experiment that serves as confirmation that Cds1 is phosphorylating Ies4 upon HU has left undone.

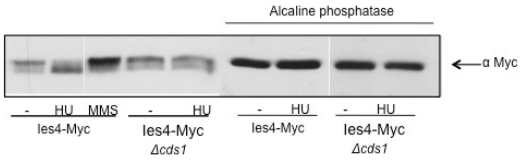


Fig. 32 I Ies4 Max1 is a substrate of the DNA replication checkpoint. Native extracts prepared from untreated (-) or 10mM hydroxyurea-treated (+) cultures of wild type, ∆*cds1* strains expressing Ies4-Myc were analyzed to detect changes in the electrophoretic mobility of Ies4- Myc.

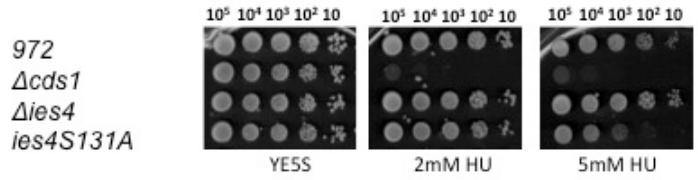


Fig. 33 I Ies4S131A is sensitive in HU plates. Cells were grown in YE5S and were spotted from 10 to 10^5 in YE5S plates containing HU concentrations and incubated at 30°C for 3 to 4 days

Still, we checked this mutant for HU sensitivity and what we saw was that has opposite phenotype of deletion *ies4* (Fig. 33). This only could means that Ies4 has different roles, and that phosphorylation is just one way or regulation.

3.7 Ies4 bind to MBF promoters opposite to Yox1

We demonstrated that Ies4 interacts with Cdc10 and that Ies4 is bound to MBF promoters, but we were wondering the nature of this binding. We decided to check the binding of Ies4 to MBF promoters in different MBF mutants and also if the nature of Ies4 binding is same throughout cell cycle.

We tagged Ies4 with Myc in different backgrounds, *Δrep2, Δyox1, Δres1, Δres2* and *cdc25-22*. We were wondering to see if Ies4 binding to MBF promoters was changing if any of MBF regulators is lost. While in *Δrep2, Δres1, Δres2* binding was more or less unchanged, in *Δyox1,* Ies4-Myc was more recruited (Fig. 34, A). That means that Ies4 was bound to MBF promoter when those genes were highly transcribed,

suggesting that ies4 could have an activating role in MBF transcription. Also, Ies4 was isolated with Cdc10 in *Δnrm1* background, where MBF transcription is at its maximum, and once more, when *Δyox1* is not present.

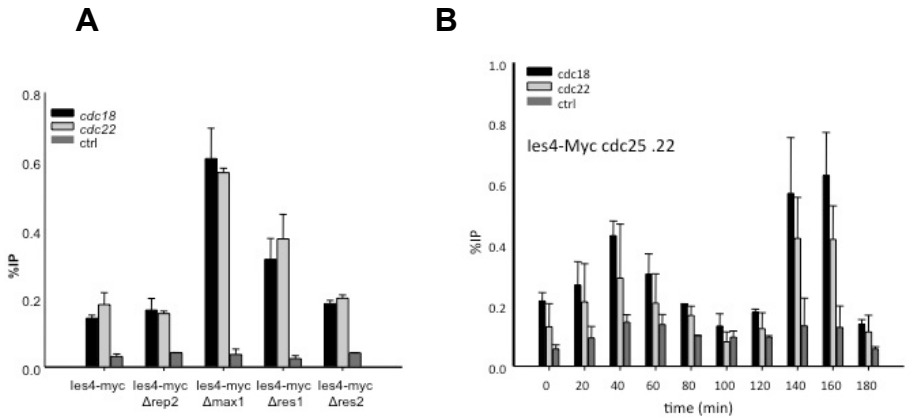


Fig. 34 I Representative ChIP data for Ies4 occupancy at MBF genes promoters in different MBF mutants (A) and throughout cell cycle (B). Occupancy at MBF promoters was measured using α-myc antibodies. Data was obtained from three independent experiments and are expressed as mean ± SD.

Then we decided to check if Ies4 binding was periodic as MBF transcription. In order to do that we tagged Ies4 with Myc in *cdc25-22* background, that allowed us to synchronize cells. We observed that Ies4 binding was periodic, with maximum binding when MBF transcription is fully on, and minimum when MBF is inactive (Fig 34, B). This goes opposite way than binding of Yox1. This also confirms that Ies4 could act as activator.

We also checked the protein level of Ies4 in *cdc25-22* background. Although Ies4 protein levels did not change, we saw that phosphorylation was cell cycle dependent (Fig. 35).

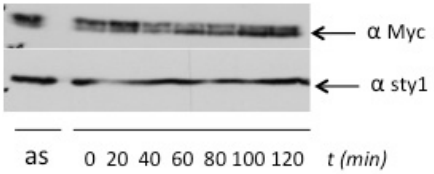


Fig. 35 I Phosphorylation of Ies4 is cell cycle dependent. TCA extract were prepared from Ies4-Myc *cdc25-22* asynchronous culture every 20 minutes after release from block at non permissive temperature.

We were never able to see one band of Ies4-Myc in acryl-amid band. Ies4 apart form Cds1 consensus site has conserved sites for other kinases. Probably it phosphorylated by more than one kinase, and that phosphorylation could form a part of a same path or not. One is sure, though we were not able to obtain Ies4S131A-Myc strain to confirm that is phosphotylated by Cds1, in *Δcds1* mutant main S phase checkpoint kinase, we were never observed lower band upon HU, that is normally observed in wild type strain, meaning that Cds1 is putative kinase for Ies4, that phosphorylates it upon replicative stress.

3.8 Ies4 is necessary for proper timing for MBF dependent transcription

We saw that in asynchronous culture of *Δies4* MBF dependent transcription was lower, but since we saw a periodic binding of Ies4 in MBF promoter we were wondering

if Ies4 influence cyclic nature of MBF genes. In order to do that, we deleted *ies4* in *cdc25-22*. We synchronized the culture by shifting it to non-permissive temperature for four hours, after what time cells are blocked in late G2. We released the culture to 25 °C and obtained total RNA every 20 minutes during next 2 hours.

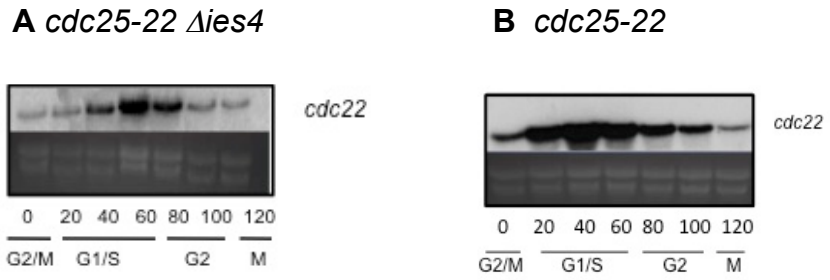


Fig. 36 I Ies4 is necessary for proper time inducing of MBF dependent genes A), Cyclic nature of *cdc22* in *cdc25-22*, for comparing B) Total RNA was prepared from synchronous cultures, and analyzed by hybridization to *cdc22* probe.

We could see that although *cdc22* was cycling upon release as cell cycle was progressing, in *Δies4* mutant full expression comes later, for about 20 minutes (Fig. 36). From this we could conclude that Ies4 is important for proper time induction of MBF dependent genes.

3.9 H2A.Z positioning is important for MBF transcription

Previously during checking for INO80 mutants, we saw that when deleting histone variant H2A.Z pht1 transcription of MBF genes was lower. Further, when we checked two mutants of Pht1, Pht1-4KR and Pht1-4KQ in this mutants

MBF transcription was also disturbed. Pht1-4KR has mutated four lysines to arginine, this mutations disables acetylation, leaving Pht1 constantly unacetylated, with positive charge. On the other hand Pht1-4KQ has four glutamines instead of lisynes, mimicking acetylation, and neutralizing positive charge.

We also saw that, as some other mutants, *Δpht1* was reverting *Δcds1* phenotype, had genomic instability and had misregulated exit from S phase block, after release from HU treatment. It was already reported that in *S. cerevisiae* genes that were cell cycle regulated and important for G1/S transcription CLN2 and CLB5 in *Δpht1* were downregulated (Dhillon et al., 2006)

From so far published literature we could read that INO80 is important for proper positioning of Pht1 at +1 nucleosome of regulated genes, but also for proper acetylation, where proper positioning of Pht1 is important of its acetylation/ deacetylation.

Knowing this, we were wondering to see how nucleosomes are positioned at MBF dependent promoters. As described for many other promoters of inactive genes that are regulated upon specific conditions or cell cycle phase, MBF promoters are characterized by a nucleosome-depleted region (NDR), a region that is relatively depleted of nucleosomes and

enriched with transcription factor binding sites (TFBSs), just upstream of the transcription start site (TSS).

In order to check nucleosome positioning we treated isolated chromatin with MNase, isolated mononucleosomes, After we obtained pure DNA, we performed RT-PCR using oligos for given promoters. Whole protocol is written in Materials and Methods.

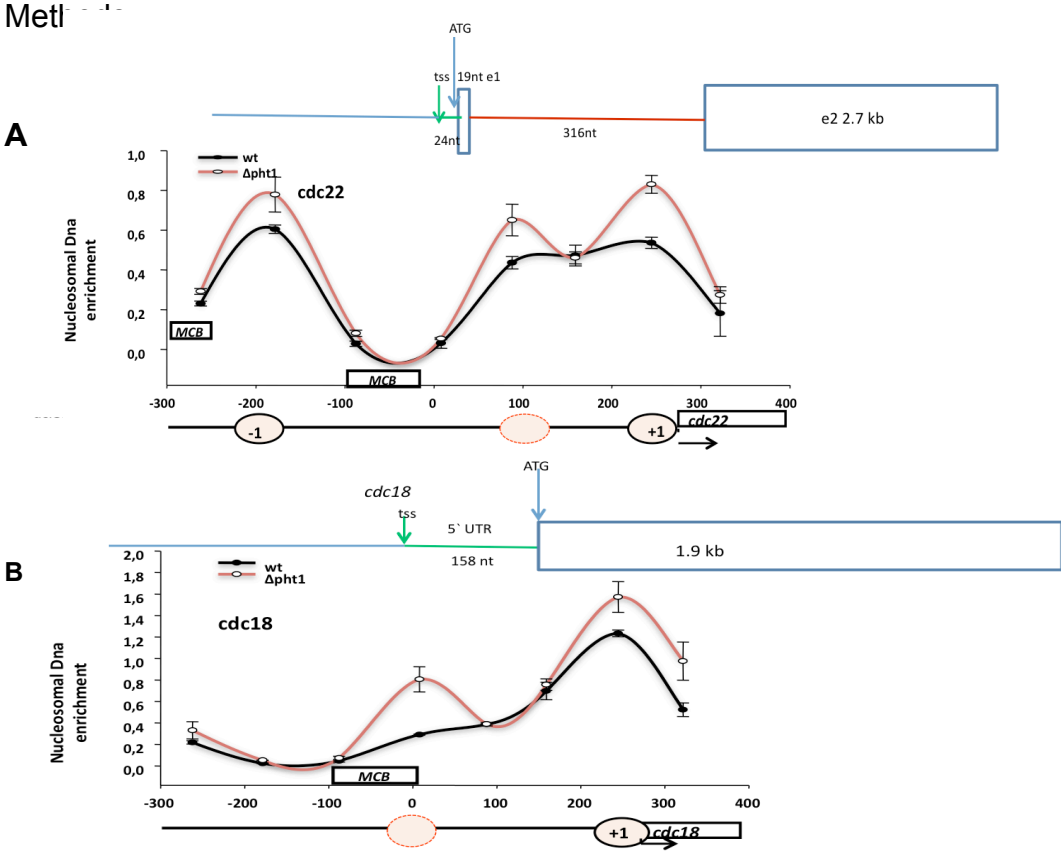


Fig. 37 I Nucleosome positioning in wild type and *Δpht1* strains, in *cdc22* (A) and *cdc18* (B) promoters. MCB box are given as sites of MBF binding. Cells were grown asynchronously, and chromatin was isolated. We treated isolated chromatin with MNase, isolated mononucleosomes, after what we obtained pure DNA, and performed RT-PCR using oligos for given promoters. Data was obtained from three independent experiments and are expressed as mean ± SD.

From the figure 37 we could see that nucleosome positioning are different in *Δpht1* compared to wild type cells in both MBF promoters. While in *cdc22* promoter in *Δpht1* we observed two prominent nucleosomes, +1 and +2 compared to more fuzzy ones in wild type, in *cdc18* promoter we saw that in *Δpht1* appeared stable prominent nucleosome before +1, at NDR, and just where MBF complex is bound.

Pht1 histone is making nucleosome more unstable than H2A, and that type of nucleosomes are more flexible and easily evicted. When Pht1 is not present some other histone (probably H2A) is replacing it, making this nucleosomes again, more stable. In this case nucleosome dynamics would be impaired. The result is that instead of probably fuzzy and flexible nucleosomes in wild type upon *pht1* deletion we get two more prominent and stable nucleosomes. In this case, transcription would be hindered.

In order to ensure that this was specific for MBF genes (or cell cycle regulated) but not for other inducible genes, we have performed nucleosome scanning for two other promoters *ctt1* and *gpd1*, genes that get induced upon oxidative stress. For these two promoters in *Δpht1* nucleosomes are positioned just as in wild type. This confirmed us that upon deletion of *pht1* repositioning of nucleosomes are not genome wide, and that there are some

genes that are affected while others remained unchanged (Fig. 38).

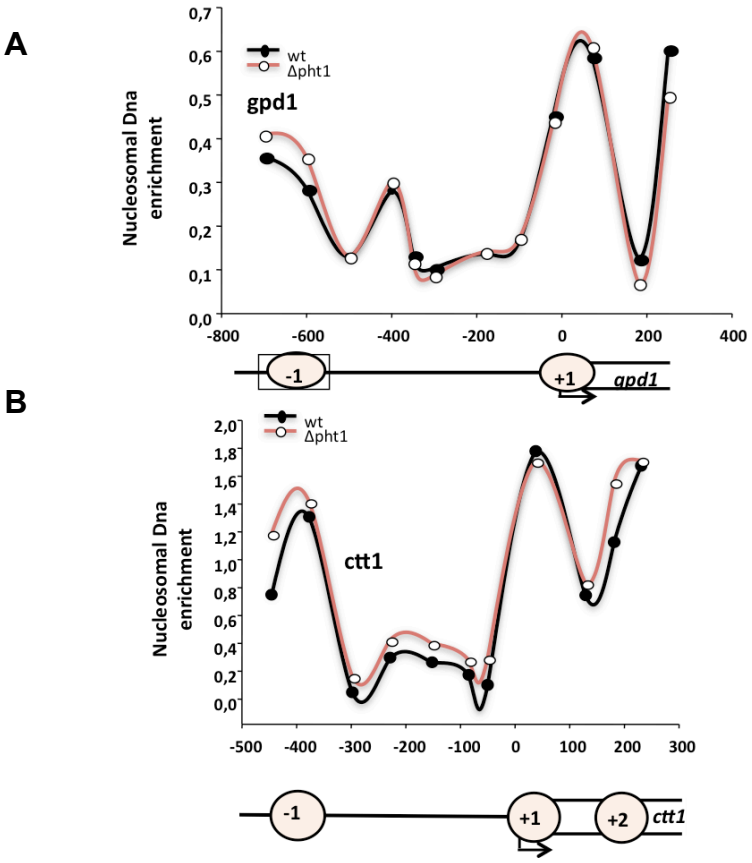


Fig. 38 Nucleosome scanning in wild type and *Δpht1* mutant in *gpd1* (A) and *ctt1* gene promoter. Nucleosome positioning of *ctt1* and *gpd1* gene promoters are wild type in *Δpht1* strain. Cells were grown asynchronously, and chromatin was isolated. We treated isolated chromatin with MNase, isolated mononucleosomes, after what we obtained pure DNA, and performed RT-PCR using oligos for for given promoters.

We were wondering also to see how nucleosomes are positioned in two Pht1 mutants, Pht1-4KQ and Pht1-4KR. In this case the profile is totally different (Fig. 39).

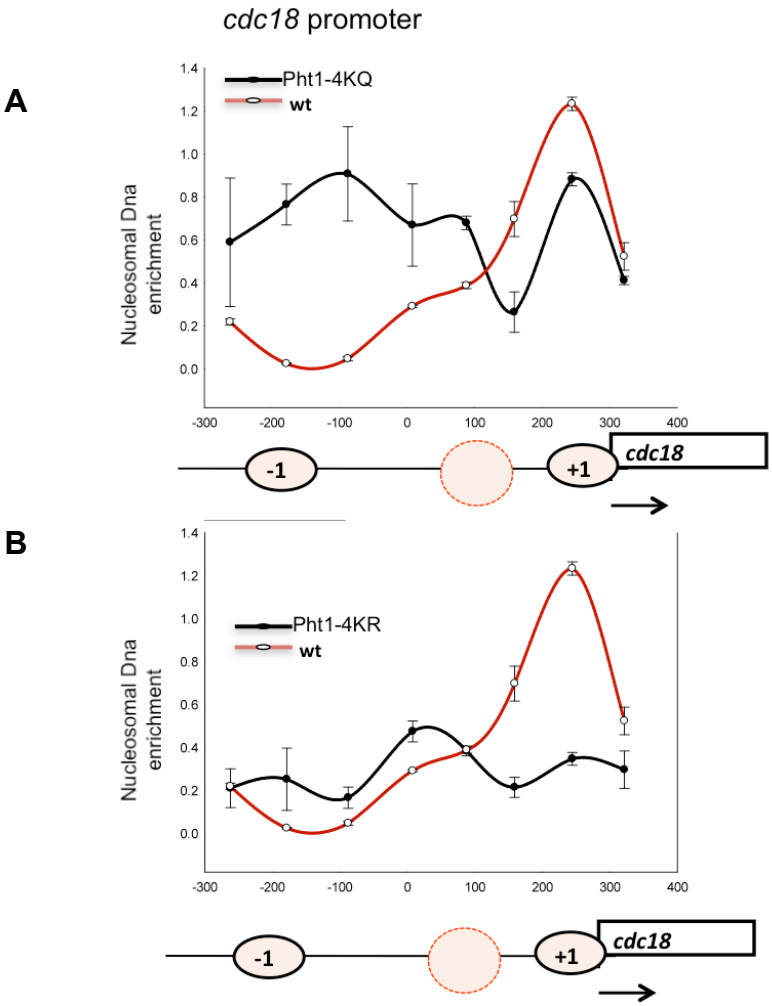


Fig. 39 I Nucleosome positioning in wild type and Pht1-4KQ (A) and Pht1-4KR (B) strains, *cdc18* promoters. Cells were grown asynchronously, and chromatin was isolated. We treated isolated chromatin with MNase, isolated mononucleosomes, After what we obtained pure DNA, and performed RT-PCR using oligos for given promoters. Data was obtained from three independent experiments and are expressed as mean ± SD.

Although we could not explain why there is so many difference in NDR region in Pht1-4KQ or why +1 nucleosome almost disappears in Pht1-4KR, it is obvious that acetylated and unacetylated forms of Pht1 have completely different profiles compared to each other, but also compared to wild type.

Also, we cannot be sure that in these two mutants we have histone with H2A.Z variant, or is replaced with H2A. Maybe in case of Pht1-4KQ where lysines are constantly acethylated that leads to unstable form of H2A.Z, this histone is evicted and replaced with H2A that is tightly bound to DNA.

In case of Pht1-4KR, lysines can not be acethylated and histone stays positively charged, that promotes binding to chromatin. It is possible that for that reason we see nucleosome enrichment before +1 nucleosome, still it does not explain why Pht1-4KR loses +1 nucleosome.

In both mutants nucleosome enrichment is observed before +1 nucleosome in the promoter sequence where MBF is bound. If this were the case, MBF would have troubles to regulate transcription of their target genes. From this we could also conclude that acetylation is also important for spatial distribution of nucleosomes.

We have also checked another MBF promoter, *cdc22* in Ph1-4KQ and Pht1-4KR (Fig. 40).

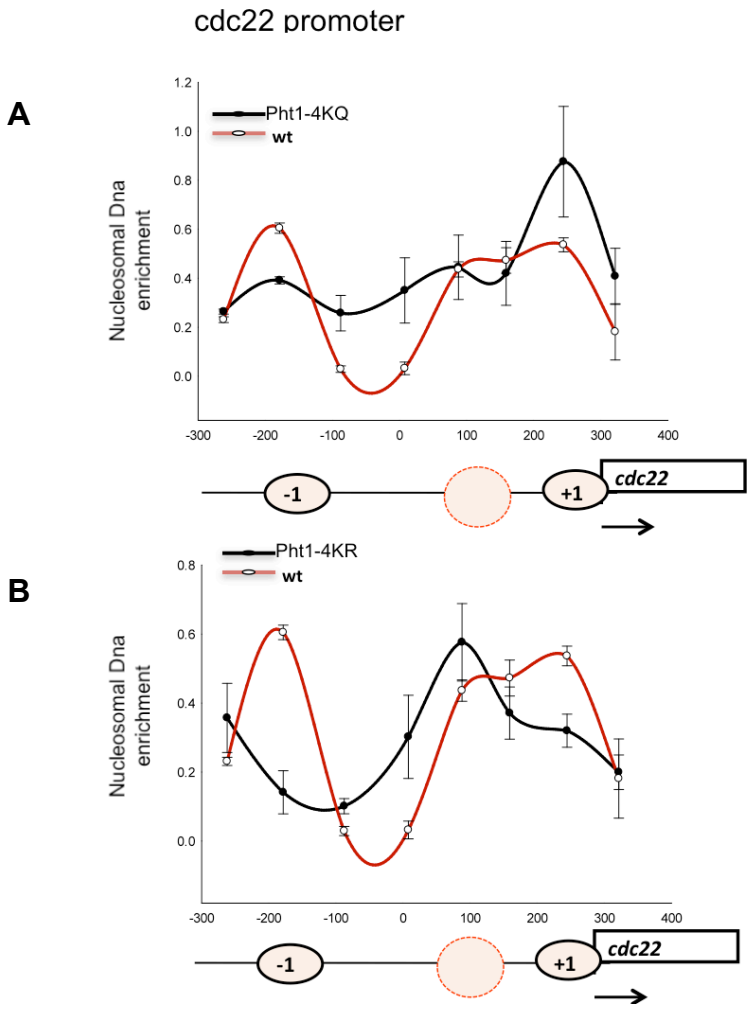


Fig. 40 I Nucleosome positioning in wild type and Pht1-4KQ (A) and Pht1-4KR (B) strains, *cdc22* promoters. Cells were grown asynchronously, and chromatin was isolated. We treated isolated chromatin with MNase, isolated mononucleosomes, After what we obtained pure DNA, and performed RT-PCR using oligos for given promoters. Data was obtained from three independent experiments and are expressed as mean ± SD.

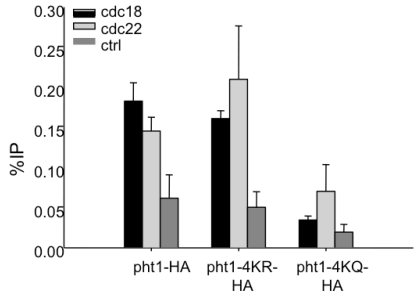
In *cdc22* promoter we observed almost same pattern. In Pht1-4KQ mutant we observed nucleosomal enrichment where in wild type we found NDR, while in Pht1-4KR we observed an clear additional nucleosome before annotated +1 nucleosome. In both cases once more, we saw more nucleosomal enrichment where MBF would be bound and where in wild type we did not observed nucleosomal enrichment.

After analyzing these two promoters of MBF regulated genes, we could conclude that nucleosome profiles are different in every of three mutants, *Δpht1,* Pht1-4KR and Pht1-4KQ, but share one thing: in all three mutants we observed that nucleosomes where distributed across the sequence of MCB boxes, or just after it. This could impede regulation of MBF transcription and even obstruct further progress of RNA polymerase.

3.10 Binding of Pht1 mutants to MBF prompters differs among each other

We also checked occupancy of Pht1 mutants at MBF promoters, *cdc18* and *cdc22.* We could observe from our data that Pht1 is bound to the promoters of MBF genes. When we checked for Pht1-4KR and Pht1-4KR binding to this

promoters we saw difference. While Pht1-4KR mutant that is mimicking unacetylated form, hence retains positive charge, was binding to the promoters as wilt type Pht1, Pht1-4KQ drastically lowers the percentage of IP. This mutant is constantly acethylated, neutralizing histone charge, and binding of Pht1-4KQ to the MBF promoters is down to negative control levels (Fig. 41).



Fig, 41 I Representative ChIP data for Pht1, Pht1-4KR and Pht1-4KQ occupancy at MBF genes promoters. Occupancy at MBF promoters was measured using α-HA antibodies. Data was obtained from three independent experiments and are expressed as mean ± SD.

Having in mind that acethylated form of Pht1 is unstable and is evicted from nucleosomes, our results could be natural consequence of acetylation of histone. Comparing wild type histone to Pht1-4KR binding to MBF promoter, we saw that the percentage does not change a lot. Once Pht1 gets acetylated nucleosomes becomes unstable, and acetylated Pht1 could be rapidly evicted when transcription is on. Since this experiment is done in asynchronous culture, where majority of cells are in G2, we probably see only unacetylated

histone bound to MBF promoters. This would explain the same IP percentage of Pht1 and Pht1-4KR.

This does not mean that nucleosome are absent in Pht1-4KQ mutant, just because Pht1-4KQ is not present in MBF promoters. As we saw from nucleosome scanning in this mutant nucleosome distribution is impaired, and H2A.Z histone is probably replaced with other histone.

3.11 Binding of H2A histone to MBF promoters differs in Pht1 mutants

We were interested to see if acetylation or deletion of Pht1 histone would lead to different behaviour of H2A histone. We wanted to know if the binding of H2A to MBF promoters changes in different Pht1 mutants.

We observed that in Pht1-4KQ and Pht1-4KR strains nucleosome profile of MBF promoters changes. We also know form literature that affinity of un/acetylated histones to chromatin changes. But, what we wanted to know if this influences binding of other histone H2A.

In order to do that we decided to perform ChiP experiment of H2A in wild type, Pht1-4KQ, Pht1-4KR and *Δpht1*. We decided to use oligos that amplify DNA sequence that is

normally wrapt around histone octamer, i.e. we were amplifying -1 and +1 nucleosome sequence established previously from our experiments.

We could see that H2A histone was present in -1 and +1 nucleosomes in both *cdc18* and *cdc22* promoter in wild type cells (Fig. 42. A, B).

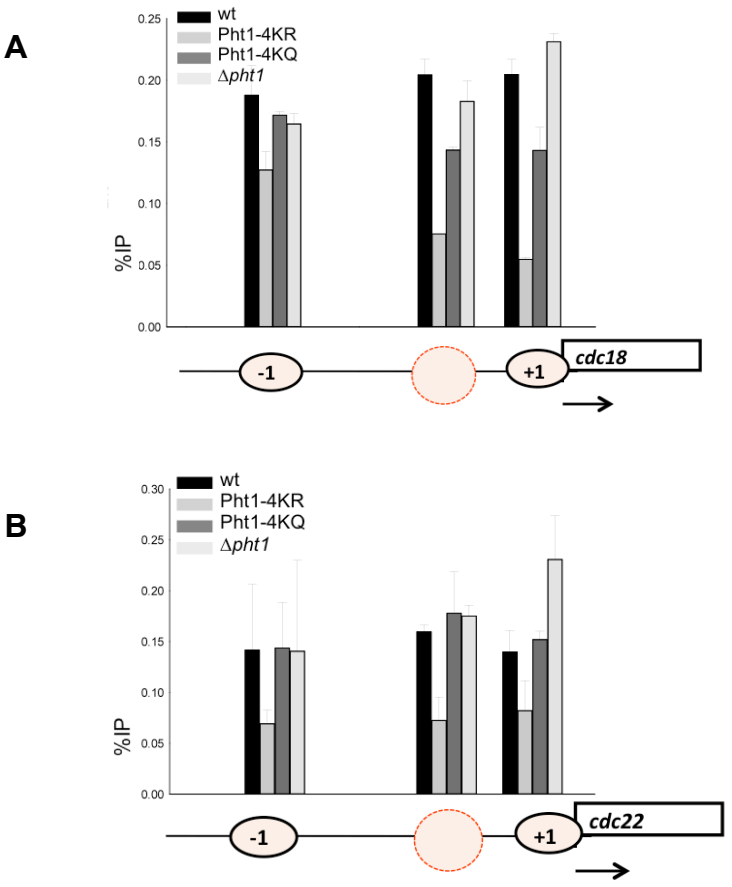


Fig 42 I Binding of H2A histone to MBF A) *cdc18* and B) *cdc22* promoters in wild type, Pht1-4KQ, Pht1-4KR and *Δpht1* strains. Occupancy at MBF promoters was measured using α-HA antibodies.

Further what was very interesting was that in Pht1-4KR in both promoters and both nucleosomes H2A binding was diminished. As already stressed before this mutate cannot be acethylated, and maintains positive charge resulting in stronger binding to DNA.

Normally, acetylated and unacetylated forms of Pht1 are exchanged in dynamic way in regulated promoters. When acetylation is disabled this histone variant is even more stable that H2A, and stays strongly bound to DNA.

SWI-RC and INO80 are regulating replacement of H2A to H2A.Z in histone octamer, but before H2A.Z is evicted it is acethylated. When we perform ChIP experiment we see binding of both histones, as average of occupancy. Yet, when H2A.Z is mutated and cannot be acethylated it is not evicted and as a result H2A cannot bind. As a result in Pht1-4KR mutant we see less binding of H2A to MBF promoters.

3.12 Induction of MBF dependent genes in a cell cycle dependent manner is impaired in Pht1 mutants

After we saw that in asynchronous conditions expression of MBF genes was impaired in Pht1 mutants, as a probable consequence of mislocalization of nucleosomes in the promoters of those genes, we were also interested to see MBF expression in a synchronous cultures.

For that reasons we constructed strains of three Pht1 mutants, deletion, 4KR and 4KQ in *cdc25-22* background that allowed us to synchronize cultures. After arresting cells for four hours at restrictive temperature at 36°C, cultures were released at 25°C and total mRNA was obtained every 20 minutes.

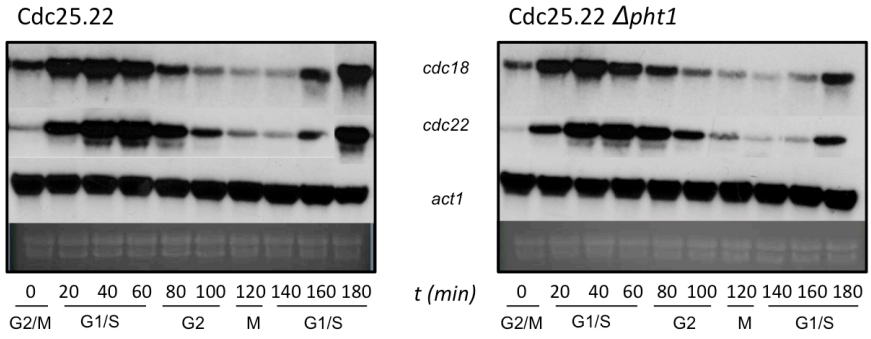


Fig. 43 l Cdc15.22 Δ*pht1* mutant has not impaired cell cycle regulation of MBF genes, but does have less level of transcription. Total RNA from *cdc25-22* and *cdc25-22* Δ*pht1* synchronized culture was obtained every 20 minutes and analyzed by Northern blot, and hybridized with *cdc18* and *cdc22* and *act1* probe.

Although in *cdc25-22* Δ*pht1* mutant MBF genes are cycling just as in wild type, it shows less transcription at every point of cell cycle (Fig. 43).

We have also checked cell cycle transcription for two other mutants, Pht1-4KQ (Fig. 44) and Pht1-4KR (Fig. 45). In case of Pht1-4KQ transcription almost left to be cell cycle dependent, especially starting second round of the cell cycle. Although cycling nature of MBF genes could be observed it is reduced to half. This phenotype was observed only in one

other mutant *Δrep2*, the gene that encodes for the only one established activator of MBF complex, Rep2. I will point out that Pht1-4KQ *cdc25-22* had no cell cycle defects, progresses through cell cycle as wild type, with same time of reaching G1 and S phase.

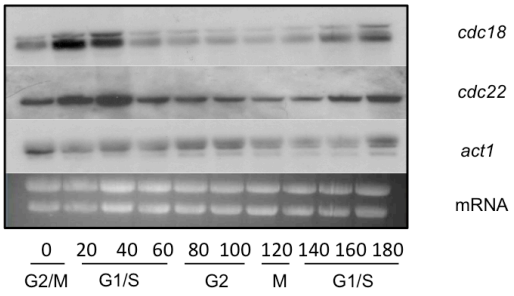


Fig. 44 I Induction of cell cycle transcription of MBF dependent genes is impaired in Pht1-4KQ Cdc25.22 strain. Total RNA from *cdc25-22* and *cdc25-22* Pht1-4KQ synchronized culture was obtained every 20 minutes and analyzed by Northern blot, and hybridized with *cdc18* and *cdc22* and *act1* probe.

We also performed this experiment in a Pht1-4KR *cdc25-22* strain. In this mutant cell cycle regulation of MBF genes is lost.

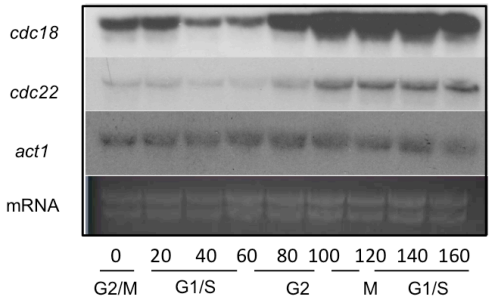


Fig. 45 I Induction of cell cycle transcription of MBF dependent genes is misregulated in Pht1-4KR Cdc25.22 strain. Total RNA from *cdc25-22* and *cdc25-22* Pht1-4KR synchronized culture was obtained

every 20 minutes and analyzed by Northern blot, and hybridized with *cdc18* and *cdc22* and *act1* probe.

In this mutant MBF genes were unable to induce at the proper way at the right time. Although we saw finally accumulation of MBF dependent transcripts, cell cycle nature of those genes was lost. Out of many mutants that were tested throughout years, this was the first time that we saw that cycling nature of MBF genes expression is completely misregulated. This mutant of histone cannot be acethylated; hence probably stays bound to DNA and obstruct transcription. This mutant also progresses slower though cell cycle.

Although neither deletion of the two mutants is wild type in MBF dependent transcription, the most severe affect we saw in Pht1-4KR *cdc25-22*, meaning that acetylation is absolutely necessary for proper MBF transcription.

3.13 Binding of acethylated Pht1 to MBF promoters is cell cycle regulated

After we saw how transcription was changed in Pht1 mutants were wondering to see what was happening to Pht1 histone binding through out cell cycle. In order to do that we performed ChIP experiments in *cdc25-22* strain that allows us synchronicity, and using panAC-Pht1 antibody.

We saw that the binding of acethylated Pht1 was in a cell cycle manner, with the peak of binding when MBF of transcription was at maximum (Fig. 46). This could mean that this is timing when Pht1 gets acetylated, then being evicted and allowing transcription to occur.

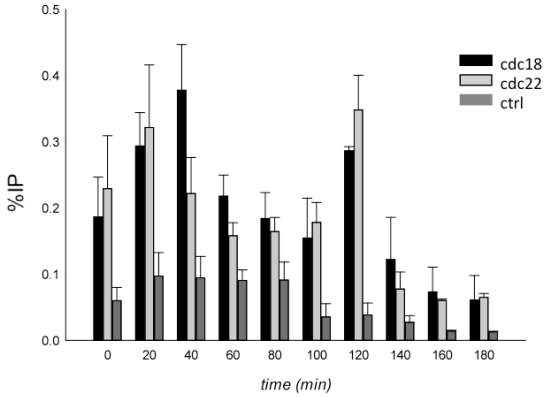


Fig. 46 I Representative ChIP data for Pht1-panAC occupancy at MBF genes promoters throughout cell cycle. Occupancy at MBF promoters was measured using α-panAC-Pht1 antibodies. Data was obtained from three independent experiments and are expressed as mean ± SD.

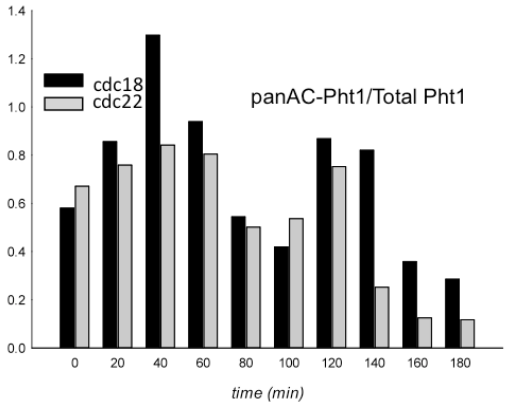


Fig. 47 I Representative ratios of ChIP data for Pht1-panAC and total Pht1 occupancy at MBF genes promoters throughout cell cycle. Occupancy at MBF promoters was measured using α-panAC-Pht1 and α-HA antibodies.

In order to be sure that this cyclic binding is due to acetylation and not total amount of Pht1 we also performed ChIP against total Pht1. When we divide percentage of IPs we obtain ratio that gives us information of binding of Pht1-panAc compared to total Pht1. From that experiment we could conclude that cycling nature of Pht1-panAc binding was not due to total amount of Pht1 (Fig. 47).

3.14 Nucleosome profile changes as transcription does

Since we saw changes in binding of Pht1-panAC throughout cell cycle, we were also interested to see if nucleosome profile around promoters also changes as transcription goes on. For that reason we decided to perform nucleosome scanning experiment in *cdc25-22* strain. We purified nucleosomes from four samples, as culture was release we obtained samples at the beginning of transcription, at the middle, at the maximum of transcription, and at the and of the G1/S wave.

As we expected, nucleosome profile at the promoter was changing as the transcription was progressing (Fig. 48). At the start of transcription nucleosomes are well established. As transcription goes on, nucleosomes are less prominent. At the maximum of transcription, nucleosomal enrichment is down to half, and the peaks are less prominent. As transcription finishes we could observe reconstitution of nucleosomes.

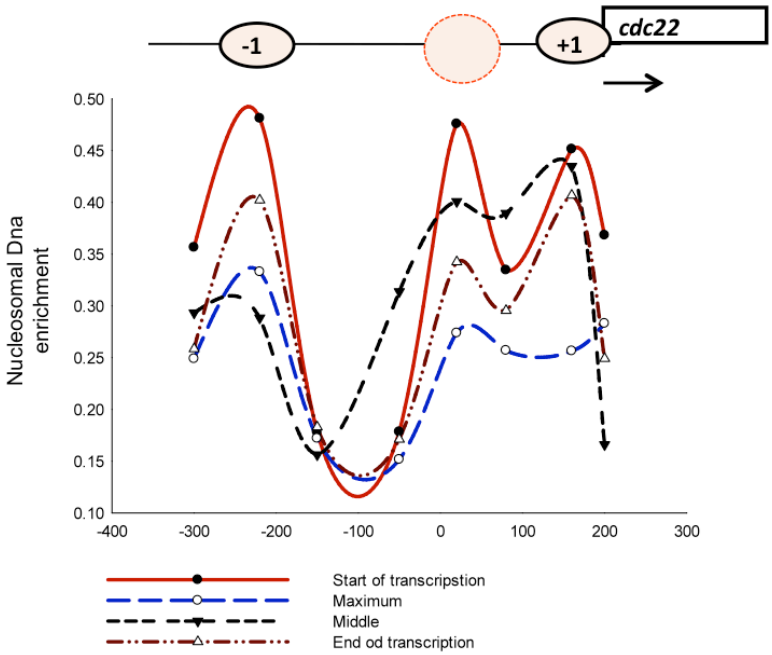


Fig. 48 I Nucleosome positioning in *cdc25-22* strain, of *cdc22* promoter. Cells were blocked for four hours at non-permissive temperature and the realised, samples were collected and chromatin was isolated. We treated isolated chromatin with MNase. After we obtained pure DNA, we performed RT-PCR using oligos for given promoters.

We wanted to know if at the maximum of transcription in *Δpht1* strain nucleosome profile is different then in the wild type. We synchronized *cdc25-22 Δpht1* strain and obtained samples at the maximum and at the end of transcription (Fig. 49).

We could conclude from this experiment that compared to the wild type in *Δpht1* mutant at the maximum of transcription, nucleosomes were still well defined.

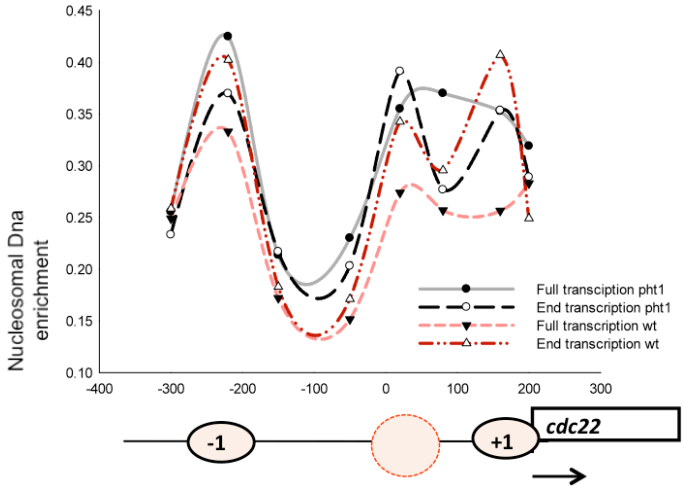


Fig. 49 I Nucleosome positioning in *cdc25-22* strain and *cdc25-22* Δpht1, of *cdc22* promoter. Cells were blocked for four hours at non-permissive temperature and the realised, samples were collected and chromatin was isolated. We treated isolated chromatin with MNase. After we obtained pure DNA, we performed RT-PCR using oligos for given promoters.

We also speculate that some of the phenotypes of INO80 strains could be also observed in mutants missing acetylase for H2A.Z. One possible acetylase is Mst1, a protein that was already annotated to form a complex with Arp4.

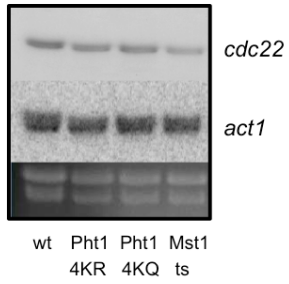


Fig. 50 I Mst1 mutant shows decrease in MBF dependent transcription. Total RNA was prepared from asynchronous cultures, and analyzed by hybridization to *cdc22* and *act1* probe.

We observed lower expression of *cdc22* in Mst1 mutant (Fig. 50). Still further experiments are needed to confirm an implication of Mst1 acetylase in possible regulation of MBF dependent transcription.

DISCUSSION

Identification of MBF interactors

Main objective of this Project was to further understand the regulation of *S. pombe* transcription factor MBF. In the beginning we focused on characterization of putative interactors from previous purification, while also performing new purification in a specific condition where MBF complex is fully active.

We started characterizing several proteins as possible regulators of MBF, HMG proteins and Mbf1, proteins that have been isolated already through affinity purification. Also we were able to repeat immunoprecipitation of Cdc10, *Δnrm1* background where MBF genes are constantly induced, since we failed to obtain healthy strain *cdc25-22* Cdc10-HA that would allows us to synchronize the culture and obtain the sample at the onset of MBF activation, as originally was the idea.

Since carboxy tagging of Cdc10 produced a non wild type phenotype in *cdc25-22* background, we also keep in mind to tag Cdc10 with HA on amino side, hoping that Cdc10 function would be less affected then, since all major regulation of Cdc10 is happening on carboxi terminus. Is this is the case, synchronization of cells and isolating MBF from different cell cycle phases would be possible and reliable.

From this purification we focused on Ies4 and Ino80 protein, but also knowing function of INO80 complex, we tried to understand the nature of H2A.Z histone in organization of nucleosomes in promoters of MBF regulated genes.

Also, there is a deeper analysis to be done. Other possible MBF regulators might be among the proteins that we purified with high ratios, as components of APC or splicing factors, as several uncharacterized proteins. We are also interested in the functional characterization of MBF during meiosis, since the composition of the nuclear core of MBF also changes when *S. pombe* cells enter into meiosis (Ayte et al., 1997).

Characterization of HMG proteins

Two proteins containing a High Mobility Group domain were isolated in affinity purification, SPBC28F2.11 and SPAC57A10.09c. HMG proteins are most abundant non-histone chromatin proteins. Together with another HMG protein SPBC19G7.04, those mutants were tested for survival in HU plates, MBF transcription with or with out HU treatment, and we did not observe any clear phenotype.

Anyhow, when we checked SPBC28F2.11 mRNA and protein level, upon HU we observed downregulation of both mRNA and protein level. We wanted to know if this downregulation was due to activation of S phase checkpoints, so we tested it in *Δcds1*, and in its upstream kinase *Δrad3* background. Yet,

downregulation stayed the same, meaning that this invent is independent of at least the two major checkpoint kinases.

Furthermore, we checked the protein level throughout cell cycle, using *cdc25-22* background, and demonstrate that expression of SPBC28F2.11 is cell cycle dependent with the peak in S phase. Protein level also peaks at S phase. This cell cycle regulation could mean that SPBC28F2.11 could be important for progression throughout cell cycle, and having in mind that is chromatin protein, it could be important for organization of chromatin after DNA synthesis, that would explain why is S phase protein. Also, being downregulated upon HU when cells are arrested at the onset of S phase, could contribute to the hypothesis that serves in reorganization of chromatin after DNA synthesis.

Although we also confirmed SPBC28F2.11 occupancy at the MBF promoters by ChIP experiments, SPBC28F2.11 deletion did not affect MBF transcription in asynchronously growing culture or in *cdc25-22* background.

From everything tested and observed, we could conclude, that SPBC28F2.11 although important for S phase and found in MBF promoters does not influence MBF transcription. Since this protein is architectural, it is expected to interact with various transcription factors, but from all the evidence that we have, we could not state that it is MBF specific.

Characterization of Mbf1 protein

Another protein that has been brought to our attention was Multiprotein Bridging Factor 1, Mbf1.

We checked the HU sensitivity, and observed that had no phenotype upon these conditions. We were able to Co-immuno precipitate it with Cdc10 and Yox1, confirming the interaction of MBF complex and Mbf1 protein.

When we checked for localization tagging it with GPF we observed that it was cytoplasmatic as well as nuclear. When we synchronized the cells using *cdc25-22* background we observed that one-hour after release Mbf1 started to acummulate in nucleus, that corresponds to peak of S phase.

We also demonstrate that Mbf1 protein level was cell cycle regulated, and that in G2 phase was downregulated, and up regulated in S. The accumulation that we observed at the very beginning of the release at G2/M is probably the accumulation from S phase that cannot be regulated due to the block.

Once again, we had protein that accumulates in S phase, suggesting importance of it in this very phase. Still, when we delete Mbf1, MBF transcription was not delayed, not in asynchronous, upon HU or in *cdc25-22* background. Anyhow, deleting *mbf1* in *cdc25-22* strain led to speeding up of cell cycle for about 20 minutes.

We were also surprised to see that *mbf1* gene was up regulated upon HU, that could mean that is important for S phase check point, and this up regulation was independent of MBF since in ∆*yox1* profile was unchanged.

From all experiments done we could not unambiguously confirm that Mbf1 has any role in MBF regulation.

Immunopurification of MBF complex

We wanted to purify MBF complex using same technique that was already done and established previously (Gomez-Escoda et al., 2010). We were interested to purify MBF under conditions where it would be fully active, in order to find its putative activators.

In order to do that we decided to put Cdc10-HA strain in *cdc25-22* mutant. *cdc25-22* has punctual mutation of Cdc25 phosphatase that makes it temperature sensitive. After four hours at non-permissive temperature at 36°C degrees cells are blocked at G2/M phase, and once they are released at 25 C degrees they synchronously progress through cell cycle. After about 30 minutes cells are in G1/S phase where MBF transcription is in its maximum.

Yet when we checked Cdc10-HA *cdc25-22* strain it was not wild type. For whatever reason HA tag on Cdc10 made this mutant slower, and MBF genes were misregulated. The probable explanation is that tagging Cdc10 on its carboxy

terminal, where Cdc10 is being regulated, is impairing its full functionality. For that reason we have had to leave the idea to work under this condition.

We have also tried to work with anti Cdc10 antibodies, using only *cdc25-22* background but we have also left this approach since anti Cdc10 antibody was not good enough for Immunoprecipitations.

Since we still wanted to obtain samples when MBF was active, we decided to use *Δnrm1* background where MBF transcription is constantly upregulated. We used the same HA tag on Cdc10.

We performed affinity immunopurification, and sample was sent to MS together with control, *Δnrm1.* Total of 389 protein was found. Cdc10-HA once precipitated only in main purificataion. We were also able to catch Res2.

Four different subunits of same complex INO80 were co-immunoprecipitating with Cdc10; Ies5, Alp5, Ies4, and Rvb1. This confirmed previous purification where main subunit Ino80 helicase, together with Rvb1 was precipitating with Cdc10.

INO80 characterization

First we have focused on confirming physcal interaction of INO80 and MBF complex. We have demostrated that Ino80

core subunit of the complex and Ies4 subunits interact with Cdc10 using CoImmunoprecipitaion technique. We also showed that these two subunits occupy MBF dependent promoters *cdc18* and *cdc22*.

Individual deletion of various INO80 subunits led to imparied MBF transcription in asynchronous culture. The major effect we saw in the mutants that had many growth problems and polyploidy, *Δarp5* and *Δies6.* On the other hand, mutants as *Δies2, Δies4, Δpht1 and Δnht1* had no apparent growth problems and still had downregulated MBF transcription. Also, unacetylated mutant of Pht1, Pht1-4KR had also downregulated transcription.

We also show that Cdc10 binding to its promoters depends on INO80 complex, and that the level of expression could be explained by the percentage of occupancy of Cdc10 in its promoters in INO80 mutants.

INO80 mutants did not show phenotype in HU plates. But once we put them in *Δcds1* background we observed that INO80 mutants were rescuing *Δcds1* phenotype. Since that was something we did not expect, considering what was so far described on INO80 role, we were wondering why deletion of INO80 would led to resistance to HU. We have checked for genomic instability, and found out that the mutants that confer HU resistance of *Δcds1* had severe chromosome instability. Many different situations can lead cells to chromosome

instability, like defects in chromosome segregation, DNA replication, spindle assembly and dynamics, cell-cycle regulation and mitotic checkpoint control, and mutations in more than 100 genes involved in all these processes have been reported to cause chromosomal instability in yeasts (Jallepalli and Lengauer, 2001).

To further understand the nature of INO80 mutants upon HU treatment we followed the exit from S phase upon HU arrest of *Δies4, Δpht1, Δnht1* individually and in *Δcds1* background. We saw that *Δnht1* and *Δpht1* progress faster through cell cycle after release from HU, and that in double mutants *Δcds1* also progress faster.

From this three individual experiments we could conclude that INO80 mutants have aberrant S phase regulation, but more importantly since those cells exit faster from S phase block, we suggest that INO80 is important for the very S phase checkpoint when cell cycle is compromised. When cells are treated with HU, soon checkpoint is activated and cells are blocked in G1/S not able to pass through “Start”. Checkpoint activation does not allow cells to progress until the DNA stress in unsolved. If cells progress through cell cycle under this condition, they would enter S phase and experience aberrant DNA replication that would cause genomic instability.

It has been reported that upon DNA damage INO80 is recruited to DNA by H2A phosphorylation, through Nht1 subunit. Phosphorylated H2AX (γH2A) signalling is the initial step of the checkpoint response. The phopshorylation acts as a scaffold for the recruitment of other proteins of the checkpoint cascade in the surroundings of the damaged sites. H2A phosphorylation is critical in both situations: replication-associated DNA damage (when replication fork progression is paused or arrested at particular sites at the genome during replication) and external replication stress (like in responses to hydroxyurea, which stalls replication forks).

The sensor kinase ATR (Rad3) is activated phosphorylates H2A, and Cds1 that eventually phosphorylates Yox1. INO80 facilitates, through its chromatin remodelling activity DNA repair. In the absence of INO80 chromosome instability would be expected. Still that does not explain why INO80 mutants revert *Δcds1* phenotype, a kinase that is downstream of Rad3. Genetic interaction between Cds1 and INO80 suggests that once Rad3 is activated it is regulating the recruitment and activation of INO80 by several mechanisms. One is by phosphorylation of H2A and another by its effectors kinase that could regulate directly or indirectly some subunits of INO80.

We have also demonstrated that upon HU Ies4 is phosphorylated. This phosphorylation is characteristic for

replicative stress since it was not observed under double strand brake conditions (MMS). Phosphorylation was lost in *Δcds1* background, strongly suggesting that this main effector kinase of replicative stress checkpoint is phosphorylating Ies4, and supporting previous statement that INO80 is involved in S phase regulation. Still deletion of *ies4* did not lead to impaired induction of MBF genes upon HU. One explanation would be that Ies4 is redundant with another protein.

At the same time as this discussion was written, a paper from Kapoor P., was published on a direct link between INO80 complex and checkpoint kinase RAD53 in *S. cerevisiae.* They claim that upon DNA damage phosphorylation of IES4 enhances RAD53 activity. Although in *S. pombe* we saw phosphorylation upon replication stress (HU), but not under DDR (MMS), hence regulation by INO80 upon different stresses could differ among organism, this strongly confirmed our hypothesis of S phase regulation by INO80. Further, I could dare to speculate that INO80 is regulating S phase by influencing cell cycle arrest, by the mechanism still undiscovered.

Phosphorylation of Ies4 is cell cycle dependent, and shift occurred at 60 minutes that coincides with S phase, once more suggesting that Ies4 is involved in S phase regulation by being phosphorylated. When we checked cell cycle

transcription in *Δies4* background we observed that this mutant had problem to induce MBF transcription on time.

When we checked for Ies4 binding to MBF promoter throughout cell cycle, we observed that its binding is periodic, and that the maximum of binding coincides with the maximum of MBF transcription. Also, binding of Ies4 was promoted in *Δyox1* strain. From all this experiments we conclude that Ies4 is involved in regulation of MBF transcription and for proper time induction of the MBF genes.

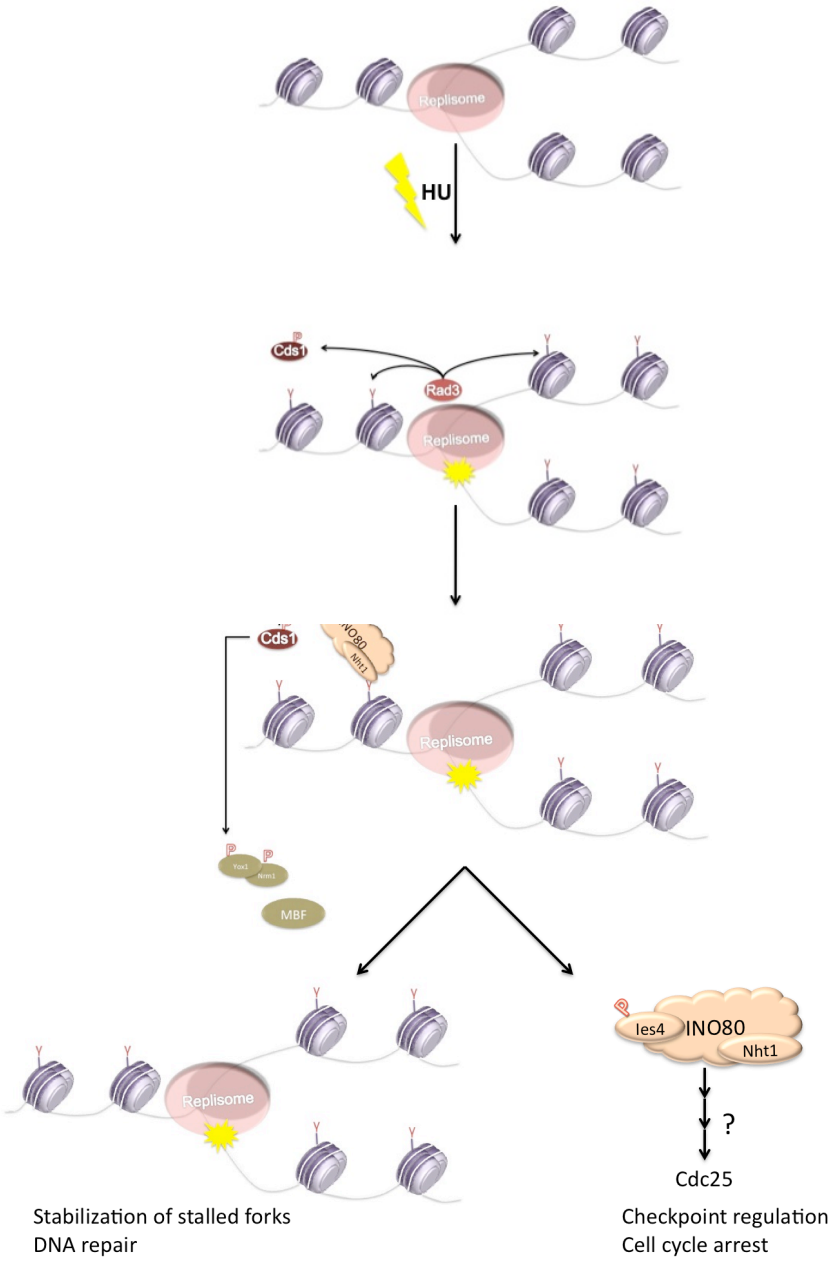


Fig. 51 I Possible role of Ies4 and INO80 complex upon replicative stress. Upon replicative stress Rad3 phosphorylates H2A histone but also Cds1. INO80 gets recruited to γH2A through Nht1 subunit, and Ies4 gets phosphorylated by Cds1. Apart form stabilizations of stalled forks (as already reported) INO80 could be involved in checkpoint regulation and cell cycle arrest.

H2A.Z positioning is important for MBF transcription

The main mechanism by witch INO80 is regulating replication and transcription is by exchanging the histone variants H2A and H2A.Z. H2A.Z is known to reside at almost all promoters of regulated genes. We have confirmed that in *Δpht1* as in Pht1-4KR MBF genes are downregulated. We were interested to investigate the architecture of MBF regulated promoters. We were wondering if the phenotypes that we observed in INO80 mutants including *Δpht1*, are reflection of changed architecture of the promoters. For that reason we purified mononulceosomes DNA and performed RT-PCR using oligos designed to amplify the promoters.

We confirmed that deletion of *pht1* led to more stable and defined nucleosomes around promoters. Nucleosomes at promoters are very dynamic, and susceptible to constant remodelling, sliding and histone exchange. What we observed with MNase assay is an average of nucleosome enrichment across the promoter. The more narrow the peaks the less dynamic is the region. In *Δpht1* mutant we observed more prominent peaks of nucleosomal enrichment that was telling us that the architecture in this mutant was more rigid. Nucleosomes containing H2A.Z instead of H2A are unstable once it is acetylated. Acetylation of H2A.Z is linked to activation of transcription since it would lead to opening of chromatin and facilitate progression of transcription machinery.

In wild type condition exchange of these two histones is non-stop process. If we delete H2A.Z, H2A, which is forming more stable nucleosome, would provoke more rigid architecture. This could mean an obstacle for transcription, and as a consequence less transcription of MBF genes.

In order to be sure that this architecture was specific for MBF promoters, we have checked two other promoters (regulated under stress). We did not see any change in *Δpht1* background. This confirmed us that upon deletion of *pht1* repositioning of nucleosomes are not genome wide, and that there are some genes that are affected while others stayed unchanged.

Pht1 mutants had, on the other hand, completely mislocalized nucleosomes. Both mutants had nucleosomes where in wild type these regions are depleted. This completely different profile compared to the *Δpht1* where the nucleosomes are only more prominent, tells us the importance of acetylation for the proper organization of chromatin. In both mutants nucleosome enrichment is observed before +1 nucleosome in the promoter sequence where MBF is bound. If this were the case, MBF would have troubles to regulate transcription of its genes.

To further understand the nature of MBF promoters in H2A.Z mutants we performed ChIP experiment. While Pht1-4KR

mutant that is mimicking unacetylated form, hence retains positive charge, was binding to the promoters as wild type Pht1, Pht1-4KQ drastically lowers the percentage of IP. This mutant is constantly acetylated, neutralizing histone charge, and binding of Pht1-4KQ to the MBF promoters is down to negative control levels. Since Pht1 binds as Pht1-4KR, and Pht1-4KQ not at all, this result tells us that when immunoprecipitating total Pht1 we were actually seeing the Pht1-4KR fraction only. As a conclusion we could state that unacetylated Pht1 is bound to DNA, but once it is acetylated it is being evicted, by INO80.

Since we also very curious to know if acetylation or deletion of Pht1 histone would lead to different behaviour of H2A histone, so we performed ChIP using anti H2A antibody and amplifying -1 and +1 nucleosome. What we saw is that in Pht1-4KR mutant binding of H2A drops to half. Since this mutant cannot be acetylated, has more affinity to DNA, and exchange to H2A is not occurring. As a result in Pht1-4KR mutant we see less binding of H2A to MBF promoters.

We have already saw that the MBF transcription in asynchronous culture was impaired, so we wondered to know how does the induction occurred in *cdc25-22* background.

We have come to conclusion that acetylation of H2A.Z is necessary for proper MBF genes induction. Induction of MBF

genes is worst in both Pht1-4KR and Pht1-4KQ mutants then in *Δpht1* background.

In Pht1-4KQ mutant induction as well as cycling nature of MBF genes was down to half. Although it was reported that cycling of those genes is not absolutely necessary for cell viability, this phenotype was only observed in *Δrep2* mutant. On the other hand in Pht1-4KR background cyclic nature of MBF is lost. This was the first time that we saw that cycling nature of MBF genes expression is completely misregulated. This mutant of histone cannot be acethylated; hence probably stays bound to DNA and obstruct transcription. With this we confirmed that that acetylation of H2A.Z is crucial for proper MBF gene transcription.

Further, when we tested binding of pan acetylated H2A.Z through cell cycle we observed that the binding coincides with MBF transcription.

Nucleosome profile changes as transcription does

We supposed that chromatin architecture at the promoters would change when genes are induced. For that reason we checked nucleosome positioning in *cdc25-22* background. As we suspected as transcription was reaching its maximum, nucleosomes were less prominent and enrichment was lower. The importance of H2A.Z is shown when we compare MBF

promoter at maximum of transcription in *cdc25-22* strain and in *Δpht1* background. At the maximum of transcription in *Δpht1* mutant nucleosomes are not evicted that results in high nucleosomal enrichment, and obstructs transcription.

We also think that the acetylase that acetylates H2A.Z would lead to same phenotype as INO80 mutants. A mutant of putative acetylase Mst1 exhibits decreased MBF transcription.

For proper activation of MBF dependent genes MBF complex needs to be bound to its promoters. Since MCB boxes are found at the NDR just before +1 nucleosome, any physical disruption of architecture of promoters impairs proper binding of transcription factor to its binding sequence. Also, acetylation of Pht1 is important as it recruits general transcription factors and machinery. Furthermore, as transcription progresses nucleosomes need to be evicted to allow free DNA to transcribe.

We suggest a model in which acetylation and eviction of Pht1 is important for proper MBF transcription. H2A and H2A.Z are exchanged at the onset of transcription, after which H2A.Z is acetylated. Acetylation of histones is usually a signal for transcription factors and machinery assembly. At the same time H2A.Z is evicted by INO80. As DNA gets free from histone, progression of transcription is possible.

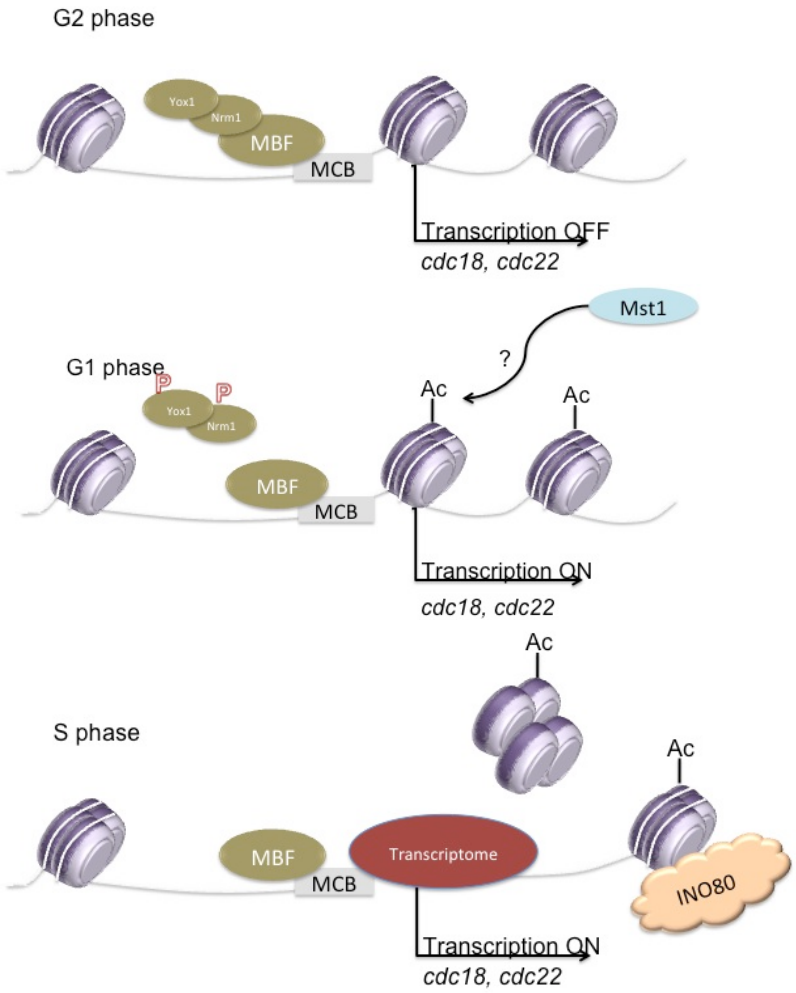


Fig. 52 I Model of MBF promoters regulation. At G2 phase MBF genes are inactive, repressors are bound to the core of MBF, and nucleosomes are well positioned. As MBF gets activated in late G1 by derepression, transcription can occur. To loose the chromatin and attract general transcription factors, histone acetylase (probably Mst1) acetylates Pht1. Than INO80 can evict dimmer H2A.Z-H2B (or entire nucleosome) that allows transcription machinery to progress.

CONCLUSIONS

1. Ino80 and Ies4 subunits of INO80 complex interact with MBF transcription factor.

2. Deletion of *Δies2, Δies4, Δarp5, Δarp8, Δies6, Δnht1*, *Δpht1* and Pht1-4KR exhibit lower expression of MBF dependent genes. *Δies4, Δapr5, Δnht1, Δpht1* are partially resistant to HU and rescue *Δcds1* phenotype, and overcome HU arrest.

3. Ies4 is phosphorylated upon replicative stress; phosphorylation is lost in Δcds1 background. Phosphorylation is also cell cycle dependent. Ies4 is putative substrate of Cds1 kinase.

4. Binding of Ies4 to MBF promoters is promoted in *Δnrm1* background. Binding of Ies4 is cell cycle regulated. Ies4 is necessary for proper timing for MBF dependent transcription

5. Nucleosome architecture of MBF promoters is changed in *Δpht1*, Pht1-4KR and Pht1-4KQ mutants. Acetylation of Pht1 is of absolute importance for induction of MBF dependent genes.

6. Induction of MBF dependent genes in a cell cycle dependent manner is impaired in Pht1 mutants.

MATERIALS AND METHODS

Strains. 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), 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. T emperature-sensitive strains *cdc25-22* were cultured at the permissive temperature (25°C) in a water shaker (INFORS HT) until mid log phase (3-4 x 106 cells ml -1) before shifting to non-permisive temperature (36°C) for 4 h as described. Synchronicity was messured by septation index using 4',6'-diamidino-2-phenylindole (DAPI) staining

Protein extraction and immunoprecipitation. Extracts were prepared in NET-N buffer (20mM Tris HCl pH 8.0, 100mM NaCl, 1mM EDTA, 0,5% NP40, 1mM dithiothreitol (DTT), 1mM phenylmethyl sulphonyl fluoride (PMSF), 5 µgml-1 aprotinin, protease inhibitor cocktail (Sigma, used as described by manufacturer), 2mM sodium fluoride (NaF), 0,2mM sodium orthovanadate (Na3VO4), 2mM β-glycerophosphate). Cells were broken in Spex 6770 Freezer Mill.. Immunoprecipitations (1 to 3 mg of whole-cell lysate) were performed with 10 µl of prot. G separose and 100 µl of tissue culture supernatant from the monoclonal hybridoma (HA or Myc). For HA immunoprecipitations, antibody

was previously crosslinked to protein G separose. Immunoprecipitates were washed after 1 hour of incubation three times with the same buffer and resolved in SDS-PAGE, transferred to nitrocellulose membranes and blotted with the indicated antibody.

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.

Affinity purification and iTRAQ analysis. Total protein extracts of two different strains (*Δnrm1* and Cdc10-HA *Δnrm1*) were prepared from 2 litres of asynchronous midlog grown cultures. Cells were frozen under liquid nitrogen and then broken in a Spex 6770 Freezer Mill. Cell lysates were resuspended in 10 ml of Lysis buffer and centrifuged 5 minutes at 4,000xg. Supernatant was collected and centrifuged in a Beckman centrifuge 40 minutes at 30,000xg. Total protein of each strain was precleared by incubation 1 hour at 4ºC with protein G-sepharose. Precleared supernatants were incubated 4 hours at 4ºC with protein G-sepharose crosslinked to α-HA antibody. Immunoprecipitates were washed 4 times in Bio-Rad Poly-prep Chromatography Columns with 5 ml of lysis buffer, and eluted from columns with 5 washes of

1 ml of glycine pH 2. The presence of Cdc10 in the eluates was checked by Western Blot and 1/5 of the selected eluate was loaded on a 12% SDS-PAGE followed by silver staining to compare the specificity of purification in both strains. The rest of the sample was dialyzed overnight against NH_4HCO_3 20mM using Spectra/Por dialysis membranes (Spectrum laboratories), and then lyophilized. Samples were analyzed by M/S and an iTRAQ labeling was performed (as described by manufacturer).

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

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

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

Chromatin Immunoprecipitation. ChIP experiments were performed as described (Moldon et al., 2008). All the experiments

were plotted as the average of at least three different biological replicates ± SD

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

Nucleosomal scanning. Cells were crosslinked with formaldehyde (final conc. 0.5% V/V) for 20 minutes, and quenched with glycine, for 10 minutes. To obtain spheroplasts we used zymolyase 20T (ICN Biochemicals). We obtained monolucleosomes using MNase (8Units/ml) for 20 minutes. From mononucleosomes DNA was purified by phenol-chlorophorm extraction. Purified DNA was used for qPCR with a set of overlapping primer pairs rendering amplicons of approximately 100 bp. For each primer pairs, numbers of Y-axis correspond to the relative value to the input, which was obtained using as template DNA from cells not treated with MNase.

Strains used in this work.

Strains

J972 h-

A1369 nrm1::kan cdc10-HA-Nat+

JA960 nrm1::kan h-

JA570 cdc10-HA Kan+ leu1-32 ade6-704 h+

JA1558 ino80-FLAG-NatR+ h-

JA1559 ies4-13XMyc-NatR+ h-

JA1651 ino80-FLAG-NatR+ cdc10-HA-KanR+ h?

JA1652 ies4-myc-NatR+ cdc10-HA-KanR h?

JA1700 cdc25-22 ies4-13xMyc:NatR+ h?

JA256 cdc25-22 leu1-32 h+

JA1536 ies2::KanR+ h+

JA1537 ies4::NatR+ h-

JA1545 arp5::KanR+ h?

JA1550 nht1::KanR+ h-

JA1551 iec3::KanR+ h-

JA1552 pht1::KanR+ h-

JA1553 iec6::KanR+ h?

JA1554 arp8::KanR+ h?

JA1648 pht1-3xHA-KanMX6 h-

JA1649 pht1-4KR-3xHA-KanMX6 h+

JA1650 pht1-4KQ-3xHA-KanMX6 h-

JA1821 cdc25-22 pht1-4KR-3xHA-KanMX6 h+

JA1822 cdc25-22 pht1-4KQ-3xHA-KanMX6 h+

JA1728 ies4-13XMyc-NatR+ ura- h+

JA1990 ies4-13XMyc-NatR+ rep2::KanR+ ura4-D18

JA1991 ies4-13XMyc-NatR+ max1::KanR+

JA1992 ies4-13XMyc-NatR+ res1::ura4 ura4-D18

JA1993 ies4-13XMyc-NatR+ res1::ura4 ura4-D18

JA1044 cdc25-22 cdc10-3xHA-KanMX6

JA1123 mbf1-GFP-KanMX6

JA1124 mbf1-GFP-KanMX6 cdc25-22

JA1046 mbf1::KanR

JA1324 mbf1-13myc-KanR h-

JA1325 SPBC28F2.11-3HA-KanR h-

JA1371 SPBC28F2.11-3HA-KanR rad3::kan

JA1327 SPBC28F2.11-3HA-KanR cdc25-22

JA1374 SPBC28F2.11::kan cdc25-22

JA1540 cdc25-22 ies4::NatR+

JA1555 cds1::phleo ies2::KanR+

JA1556 cds1::phleo ies4::NatR+

JA1653 cdc25-22 nht1::KanR+

JA1658 cds1::NatR+ pht1::KanR+

JA1659 cds1::NatR+ nht1::KanR+

JA1698 ies4-13xMyc:NatR+ cds1::KanR+

JA1700 cdc25-22 ies4-13xMyc:NatR+

JA1721 iec3::KanR+ Chr16

JA1722 nht1::KanR+ Chr16

JA1723 pht1::KanR+ Chr16

JA1724 arp8::KanR+ Chr16

JA1725 ies2::KanR+ Chr16

JA1726 mst1::ura4+ leu1::nmt-mst1L344S leu1+ ura4-D18 ade6-M216 h+

BIBLIOGRAPHY

Abraham, R. T. (2001). "Cell cycle checkpoint signaling through the ATM and ATR kinases." Genes Dev 15(17): 2177-96.

Adam M., et al. “H2A.Z is required for global chromatin integrity and for recruitment of RNA polymerase II under specific conditions.” (2001) Mol Cell Biol. 21(18):6270-9.

Affar el B.” Essential dosage-dependent functions of the transcription factor yin yang 1 in late embryonic development and cell cycle progression.” (2006) Mol Cell Biol. 26(9):3565-81

Aligianni, S., D. H. Lackner, et al. (2009). "The fission yeast homeodomain protein Yox1p binds to MBF and confines MBF-dependent cell-cycle transcription to G1-S via negative feedback." PLoS Genet 5(8): e1000626

Antequera, F. (2004). "Genomic specification and epigenetic regulation of eukaryotic DNA replication origins." Embo J 23(22): 4365-70.

Arents G., et al, “The nucleosomal core histone octamer at 3.1 A resolution: a tripartite protein assembly and a left-handed superhelix.” (1991) Proc Natl Acad Sci U S A 88(22):10148-52.

Asano M., et al, (1996) “Ectopic E2F expression induces S phase and apoptosis in Drosophila imaginal discs.” Genes Dev. 10(11):1422-32

Attwooll, C., E. Lazzerini Denchi, et al. (2004). "The E2F family: specific functions and overlapping interests." Embo J 23(24): 4709-16.

Ayte, J., C. Schweitzer, et al. (2001). "Feedback regulation of the MBF transcription factor by cyclin Cig2." Nat Cell Biol 3(12): 1043-50.

Ayte, J., J. F. Leis, et al. (1995). "The Schizosaccharomyces pombe MBF complex requires heterodimerization for entry into S phase." Mol Cell Biol 15(5): 2589-99.

Ayte, J., J. F. Leis, et al. (1997). "The fission yeast protein p73res2 is an essential component of the mitotic MBF complex and a master regulator of meiosis." Mol Cell Biol 17(11): 6246-54.

Azvolinsky, A., P. G. Giresi, et al. (2009). "Highly transcribed RNA polymerase II genes are impediments to replication fork progression in Saccharomyces cerevisiae." Mol Cell 34(6): 722-34.

Baum, B., J. Wuarin, et al. (1997). "Control of S-phase periodic transcription in the fission yeast mitotic cycle." Embo J 16(15): 4676-88.

Boddy, M. N., B. Furnari, et al. (1998). "Replication checkpoint enforced by kinases Cds1 and Chk1." Science 280(5365): 909-12.

Booher, R. N., C. E. Alfa, et al. (1989). "The fission yeast cdc2/cdc13/suc1 protein kinase: regulation of catalytic activity and nuclear localization." Cell 58(3): 485-97.

Branzei D, Foiani M. “Template switching: from replication fork repair to genome rearrangements.” (2007) Cell 131(7):1228-30

Broek, D., R. Bartlett, et al. (1991). "Involvement of p34cdc2 in establishing the dependency of S phase on mitosis." Nature 349(6308): 388-93.

Buck, V., et al. (2004). "Fkh2p and Sep1p regulate mitotic gene transcription in fission yeast." J Cell Sci 117(Pt 23): 5623-32.

Bueno, A. and P. Russell (1993). "Two fission yeast B-type cyclins, cig2 and Cdc13, have different functions in mitosis." Mol Cell Biol 13(4): 2286-97.

Bueno, A., H. Richardson, et al. (1991). "A fission yeast B-type cyclin functioning early in the cell cycle." Cell 66(1): 149-59.

Burkhart, D. L. and J. Sage (2008). "Cellular mechanisms of tumour suppression by the retinoblastoma gene." Nat Rev Cancer 8(9): 671-82.

Chambers AL, et al. “The INO80 chromatin remodeling complex prevents polyploidy and maintains normal chromatin structure at centromeres.” (2012) Genes Dev, 26(23):2590-603.

Chen L, et al.. “Multiple modes of regulation of the human Ino80 SNF2 ATPase by subunits of the INO80 chromatin-remodeling complex.” (2013) Proc Natl Acad Sci U S A 110(51):20497-502

Ciccia, A. and S. J. Elledge (2010). "The DNA damage response: making it safe to play with knives." Mol Cell 40(2): 179-204.

Claudio PP., et al. “The retinoblastoma family: twins or distant cousins?” (2002) Genome Biol. 3(9):reviews3012

Connolly, T. and D. Beach (1994). "Interaction between the Cig1 and Cig2 B-type cyclins in the fission yeast cell cycle." Mol Cell Biol 14(1): 768-76.

Cortez, D., Y. Wang, et al. (1999). "Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks." Science 286(5442): 1162-6.

Costanzo, M., J. L. Nishikawa, et al. (2004). "CDK activity antagonizes Whi5, an inhibitor of G1/S transcription in yeast." Cell 117(7): 899-913.

Coudreuse D., P. Nurse (2010). “Driving the cell cycle with a minimal CDK control network.” Nature 468, 1074–79.

Creanor, J. and J. M. Mitchison (1996). "The kinetics of the B cyclin p56cdc13 and the phosphatase p80cdc25 during the cell cycle of the fission yeast Schizosaccharomyces pombe." J Cell Sci 109 (Pt 6): 1647-53.

de Bruin, R. A., T. I. Kalashnikova, et al. (2006). "Constraining G1-specific transcription to late G1 phase: the MBF-associated corepressor Nrm1 acts via negative feedback." Mol Cell 23(4): 483-96.

de Bruin, R. A., T. I. Kalashnikova, et al. (2008). "DNA replication checkpoint promotes G1-S transcription by inactivating the MBF repressor Nrm1." Proc Natl Acad Sci U S A 105(32): 11230-5.

de Bruin, R. A., W. H. McDonald, et al. (2004). "Cln3 activates G1-specific transcription via phosphorylation of the SBF bound repressor Whi5." Cell 117(7): 887-98.

DeGregori, J. and D. G. Johnson (2006). "Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis." Curr Mol Med 6(7): 739-48.

DeGregori, J., G. Leone, et al. (1997). "Distinct roles for E2F proteins in cell growth control and apoptosis." Proc Natl Acad Sci U S A 94(14): 7245-50.

Dhillon N., et al. "H2A.Z functions to regulate progression through the cell cycle." (2006) Mol Cell Biol. 26(2):489-501

Diffley, J. F. (1996). "Once and only once upon a time: specifying and regulating origins of DNA replication in eukaryotic cells." Genes Dev 10(22): 2819-30.

Diffley, J. F. (2004). "Regulation of early events in chromosome replication." Curr Biol 14(18): R778-86.

Dirick, L., T. Bohm, et al. (1995). "Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of Saccharomyces cerevisiae." Embo J 14(19): 4803-13.

Dirick, L., T. Moll, et al. (1992). "A central role for SWI6 in modulating cell cycle Start-specific transcription in yeast." Nature 357(6378): 508-13.

Downs JA., Allard S., et al. "Binding of chromatin-modifying activities to phosphorylated histone H2A at DNA damage sites." (2004) Mol Cell. 16(6):979-90.

Dunn J. M., et al. "Identification of germline and somatic mutations affecting the retinoblastoma gene." (1988) Science 241(4874):1797-800

Dutta, C, P. K. Patel, et al. (2008). "The DNA replication checkpoint directly regulates MBF-dependent G1/S transcription." Mol Cell Biol 28(19): 5977-85

Ebbert R., et al. “The product of the SNF2/SWI2 paralogue INO80 of Saccharomyces cerevisiae required for efficient expression of various yeast structural genes is part of a high-molecular-weight protein complex.” (1999) Mol Microbiol. 32(4):741-51.

Epstein C., et al. 1992). "CLB5: a novel B cyclin from budding yeast with a role in S phase." Genes Dev 6(9): 1695-706.

Ewaskow S., et al. (1998). "Mutation and modeling analysis of the Saccharomyces cerevisiae Swi6 ankyrin repeats." Biochemistry 37(13): 4437-50.

Fan J., et al. (2002) “The essential histone variant H2A.Z regulates the equilibrium between different chromatin conformational states.” Nat Struct Biol. 9(3):172-6.

Field S., et al. (1996) “E2F-1 functions in mice to promote apoptosis and suppress proliferation.” (1996) Cell 85(4):549-61.

Fisher, D. L. and P. Nurse (1996). "A single fission yeast mitotic cyclin B p34cdc2 kinase promotes both S-phase and mitosis in the absence of G1 cyclins." Embo J 15(4): 850-60.

Forsburg S. and P. Nurse (1994). "Analysis of the Schizosaccharomyces pombe cyclin puc1: evidence for a role in cell cycle exit." J Cell Sci 107 (Pt 3): 601-13.

Furnari B., et al. (1997) Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase.” Science 277(5331):1495-7.

Gately D., et al. (1998). "Characterization of ATM expression, localization, and associated DNA-dependent protein kinase activity." Mol Biol Cell 9(9): 2361-74.

Gerhold C., et al. (2012) “Structure of Actin-related protein 8 and its contribution to nucleosome binding.” Nucleic Acids Res. 40(21):11036-46.

Gomez-Escoda, B., et al. (2011). "Yox1 links MBF-dependent transcription to completion of DNA synthesis." EMBO Rep 12(1): 84-9.

Gould K. and P. Nurse (1989). "Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis." Nature 342(6245): 39-45.

Guillemette B., et al. (2005) "Variant histone H2A.Z is globally localized to the promoters of inactive yeast genes and regulates nucleosome positioning." Plos Biol. 3(12):e384.

Harata M., et al. (1999) "The nuclear actin-related protein of Saccharomyces cerevisiae, Act3p/Arp4, interacts with core histones." Mol Biol Cell. 10(8):2595-605

Harper J., et al. (2007). "The DNA damage response: ten years after." Mol Cell 28(5): 739-45.

Harrison J., et al. (2006). "Surviving the breakup: the DNA damage checkpoint." Annu Rev Genet 40: 209-35.

Hayles, J., et al. (1994). Temporal order of S phase and mitosis in fission yeast is determined by the state of the p34cdc2-mitotic B cyclin complex. Cell. 78: 813-22.

Hendriks, G., et al. "Transcription and replication: far relatives make uneasy bedfellows." Cell Cycle 9(12): 2300-4.

Hofmann, J. and D. Beach (1994). "cdt1 is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis." Embo J 13(2): 425-34.

Hogan C., et al. (2010) "Fission yeast Iec1-ino80-mediated nucleosome eviction regulates nucleotide and phosphate metabolism." Mol Cell Bio,. 30(3):657-74.

Horak C., et al. (2002). "Complex transcriptional circuitry at the G1/S transition in Saccharomyces cerevisiae." Genes Dev 16(23): 3017-33.

Hou H., et al. (2010) "Histone variant H2A.Z regulates centromere silencing and chromosome segregation in fission yeast." (2010) J Biol Chem. 285(3):1909-18

Ishida S., et al. (2001) “Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis.” Mol Cell Biol. 21(14):4684-99.

Ivanova T., et al. (2013) “The DNA damage and the DNA replication checkpoints converge at the MBF transcription factor.” Mol Biol Cell. (21):3350-7.

Iyer V., et al. (2001). "Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF." Nature 409(6819): 533-8.

Jackson S. and J. Bartek (2009). "The DNA-damage response in human biology and disease." Nature 461(7267): 1071-8.

Jha S, Dutta A. (2009) “RVB1/RVB2: running rings around molecular biology” Mol Cell. 34(5):521-33.

Jin J., et al. (2005) “A mammalian chromatin remodeling complex with similarities to the yeast INO80 complex.” J Biol Chem. 280(50):41207-12

Jónsson Z., et al. (2004) “Rvb1p/Rvb2p recruit Arp5p and assemble a functional Ino80 chromatin remodeling complex.” Mol Cell 16(3):465-77.

Kaelin W., et al. (1997). "Alterations in G1/S cell-cycle control contributing to carcinogenesis." Ann N Y Acad Sci 833: 29-33.

Kalocsay M., et al. (2009) “Chromosome-wide Rad51 spreading and SUMO-H2A.Z-dependent chromosome fixation in response to a persistent DNA double-strand break” Mol Cell 33: 335–43

Kapoor P., et al. (2014) “Mechanisms of nuclear actin in chromatin-remodeling complexes.” Trends Cell Biol. 24(4):238-46

Kapoor P., et al. (2015) “Phosphorylation-Dependent Enhancement of Rad53 Kinase Activity through the INO80 Chromatin Remodeling Complex” Mol. Cell.

Keyomarsi K., et al. (1995) “Inhibition of cyclin-dependent kinases by p21.” Mol Biol Cell. 6(4):387-400.

Kleine, H. and B. Luscher (2009). "Learning how to read ADP-ribosylation." Cell 139(1): 17-9.

Kabor M., et al. (2004) “A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin.” (2004) Plos Biol. 2(5):E131.

Koch C., et al. (1993). "A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase." Science 261(5128): 1551-7.

Kovesdi I., et al. (1986) “Identification of a cellular transcription factor involved in E1A trans-activation.” Cell 45(2):219-28.

Krogan N., et al. (2004) “Regulation of chromosome stability by the histone H2A variant Htz1, the Swr1 chromatin remodeling complex, and the histone acetyltransferase NuA4.” Proc Natl Acad Sci U S A 101(37):13513-8

Kuntz K., et al. (2009). "The G(2) DNA damage checkpoint: could this ancient regulator be the Achilles heel of cancer?" Cancer Biol Ther 8(15): 1433-9.

Lindahl T., et al. (2000). "Repair of endogenous DNA damage." Cold Spring Harb Symp Quant Biol 65: 127-33.

Lindsay H., et al. (1998) “S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in Schizosaccharomyces pombe.” Genes Dev. 12(3):382-95.

Lopez-Girona A., et al. (1998). "Negative regulation of Cdc18 DNA replication protein by Cdc2." Mol Biol Cell 9(1): 63-73.

Lowndes N., et al. (1992). "Control of DNA synthesis genes in fission yeast by the cell-cycle gene cdc10+." Nature 355(6359): 449-53.

Maqbool Z., et al. (2003). "MCB-mediated regulation of cell cycle-specific cdc22+ transcription in fission yeast." Mol Genet Genomics 269(6): 765-75.

Martin-Castellanos C., et al. (2000). "The puc1 cyclin regulates the G1 phase of the fission yeast cell cycle in response to cell size." Mol Biol Cell 11(2): 543-54.

Matsumoto S. and M. Yanagida (1985). "Histone gene organization of fission yeast: a common upstream sequence." Embo J 4(13A): 3531-8.

McInerny C., et al. (1995). "Positive and negative roles for cdc10 in cell cycle gene expression." Nucleic Acids Res 23(23): 4761-8.

Meneghini M., et al. (2003) “Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin.” Cell. 112(5):725-36.

Millar C., et al. (2011) Acetylation of H2AZ Lys 14 is associated with genome-wide gene activity in yeast.” (2011) Genes Dev. 20(6):711-22.

Millar J., et al. (1991). "cdc25 M-phase inducer." Cold Spring Harb Symp Quant Biol 56: 577-84.

Misteli T., et al. (2009). "The emerging role of nuclear architecture in DNA repair and genome maintenance." Nat Rev Mol Cell Biol 10(4): 243-54.

Miyamoto M., et al. (1994). "res2+, a new member of the cdc10+/SWI4 family, controls the 'start' of mitotic and meiotic cycles in fission yeast." Embo J 13(8): 1873-80.

Mizuguchi G., et al. (2004) “ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex.” (2004) Science 303(5656):343-8.

Mondesert O., et al. (1996). "Cig2, a B-type cyclin, promotes the onset of S in Schizosaccharomyces pombe." Mol Cell Biol 16(4): 1527-33.

Mondesert O., et al. (1996). "Cig2, a B-type cyclin, promotes the onset of S in Schizosaccharomyces pombe." Mol Cell Biol 16(4): 1527-33.

Morrison A., et al. (2007) “Mec1/Tel1 phosphorylation of the INO80 chromatin remodeling complex influences DNA damage checkpoint responses.” Cell 130(3):499-511.

Murakami H., et al. (1995). "A kinase from fission yeast responsible for blocking mitosis in S phase." Nature 374(6525): 817-9.

Nakashima N., et al. (1995). "Fission yeast Rep2 is a putative transcriptional activator subunit for the cell cycle 'start' function of Res2-Cdc10." Embo J 14(19): 4794-802.

Nishitani, H. and P. Nurse (1997). "The cdc18 protein initiates DNA replication in fission yeast." Prog Cell Cycle Res 3: 135-42.

Nitiss, J. L. (2009). "DNA topoisomerase II and its growing repertoire of biological functions." Nat Rev Cancer 9(5): 327-37.

Ostapenko D., et al. (2011) “Anaphase promoting complex-dependent degradation of transcriptional repressors Nrm1 and Yhp1 in Saccharomyces cerevisiae.” Mol Biol Cell. 22(13):2175-84.

Papamichos-Chronakis M., et al. (2011) Global regulation of H2A.Z localization by the INO80 chromatin-remodeling enzyme is essential for genome integrity.” Cell 144(2):200-13

Pardee A. (1989). "G1 events and regulation of cell proliferation." Science 246(4930): 603-8.

Partridge J., et al. (1997). "Cell cycle-dependent transcription of CLN1 involves swi4 binding to MCB-like elements." J Biol Chem 272(14): 9071-7.

Purtill F., et al. "A homeodomain transcription factor regulates the DNA replication checkpoint in yeast." Cell Cycle 10(4): 664-70.

Ray S, Grove A. (2009) “The yeast high mobility group protein HMO2, a subunit of the chromatin-remodeling complex INO80, binds DNA ends.” Nucleic Acids Res. 37(19):6389-99.

Reymond, A. and V. Simanis (1993). "Domains of p85cdc10 required for function of the fission yeast DSC-1 factor." Nucleic Acids Res 21(16): 3615-21.

Rhind, N. and P. Russell (2000). "Chk1 and Cds1: linchpins of the DNA damage and replication checkpoint pathways." J Cell Sci 113 (Pt 22): 3889-96.

Rotman, G. and Y. Shiloh (1999). "ATM: a mediator of multiple responses to genotoxic stress." Oncogene 18(45): 6135-44.

Russell, P. and P. Nurse (1986). "cdc25+ functions as an inducer in the mitotic control of fission yeast." Cell 45(1): 145-53.

Rustici G., et al. (2004). "Periodic gene expression program of the fission yeast cell cycle." Nat Genet 36(8): 809-17.

Sabourin M. and V. A. Zakian (2008). "ATM-like kinases and regulation of telomerase: lessons from yeast and mammals." Trends Cell Biol 18(7): 337-46.

Shen X., et al. (2000) “A chromatin remodelling complex involved in transcription and DNA processing.” Nature 406(6795):541-4.

Shiloh Y. (2003). "ATM and related protein kinases: safeguarding genome integrity." Nat Rev Cancer 3(3): 155-68.

Shimada K., et al. (2008) “Ino80 chromatin remodeling complex promotes recovery of stalled replication forks.” Curr Biol. 18(8):566-75

Shrivastav M., et al. (2008). "Regulation of DNA double-strand break repair pathway choice." Cell Res 18(1): 134-47.

Sidorova J. and L. Breeden (1993). "Analysis of the SWI4/SWI6 protein complex, which directs G1/S-specific transcription in Saccharomyces cerevisiae." Mol Cell Biol 13(2): 1069-77.

Siegmund, R. and K. Nasmyth (1996). "The Saccharomyces cerevisiae Start-specific transcription factor Swi4 interacts through the ankyrin repeats with the mitotic Clb2/Cdc28 kinase and through its conserved carboxy terminus with Swi6." Mol Cell Biol 16(6): 2647-55.

Siliciano J., et al. (1997). "DNA damage induces phosphorylation of the amino terminus of p53." Genes Dev 11(24): 3471-81

Simanis V. and P. Nurse (1989). "Characterization of the fission yeast cdc10+ protein that is required for commitment to the cell cycle." J Cell Sci 92 (Pt 1): 51-6.

Stillman B. (1993). "DNA replication. Replicator renaissance." Nature 366(6455): 506-7.

Stucki M. and S Jackson (2006). "gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes." DNA Repair (Amst) 5(5): 534-43.

Tahara S., et al. (1998). "Functional domains of rep2, a transcriptional activator subunit for Res2-Cdc10, controlling the cell cycle "start"." Mol Biol Cell 9(6): 1577-88.

Takeda D., et al. (2005). "DNA replication and progression through S phase." Oncogene 24(17): 2827-43.

Tanaka K., et al. (1992). "A new cdc gene required for S phase entry of Schizosaccharomyces pombe encodes a protein similar to the cdc 10+ and SWI4 gene products." Embo J 11(13): 4923-32.

Tapia-Alveal C., et al. (2014) “H2A.Z-dependent regulation of cohesin dynamics on chromosome arms.” (2014) Mol Cell Biol. 34(11):2092-104

Tercero, J., et al. (2003). "A central role for DNA replication forks in checkpoint activation and response." Mol Cell 11(5): 1323-36.

Tibbetts R., et al. (2000). "Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress." Genes Dev 14(23): 2989-3002.

Tibbetts R., et al. (1999). "A role for ATR in the DNA damage-induced phosphorylation of p53." Genes Dev 13(2): 152-7.

Tosi A., et al. (2013) “Structure and subunit topology of the INO80 chromatin remodeler and its nucleosome complex.” Cell 154(6):1207-19.

Trimarchi J., et al. (2002). "Sibling rivalry in the E2F family." Nat Rev Mol Cell Biol 3(1): 11-20.

Vincent J., et al. (2008) “ATP-dependent chromatin remodeling shapes the DNA replication landscape.” Nat Struct Mol Biol 15(5):477-84.

Walworth N., et al. (1993) “Fission yeast chk1 protein kinase links the rad checkpoint pathway to cdc2.” Nature 363(6427):368-71

Walworth N., et al. (1996) “Rad-dependent response of the chk1-encoded protein kinase at the DNA damage checkpoint.” Scieince 271(5247):353-6.

Weber C., et al. (2014) “Nucleosomes are context-specific, H2A.Z-modulated barriers to RNA polymerase.” Moll Cell. 53(5):819-30.

Weinberg R. (1995) “The retinoblastoma protein and cell cycle control.” Cell 81(3):323-30

White S., et al. (2001). "The role of DSC1 components cdc10+, rep1+ and rep2+ in MCB gene transcription at the mitotic G1-S boundary in fission yeast." Curr Genet 40(4): 251-9.

Whitehall S., et al. (1999). "Cell cycle-regulated transcription in fission yeast: Cdc10-Res protein interactions during the cell cycle and domains required for regulated transcription." Mol Biol Cell 10(11): 3705-15.

Williams J., et al. (2010). "gammaH2A binds Brc1 to maintain genome integrity during S-phase." Embo J 29(6): 1136-48.

Wu W., et al. (2005) “Swc2 is a widely conserved H2AZ-binding module essential for ATP-dependent histone exchange.” Nat Struct Mol Biol. 12(12):1064-71.

Wuarin, J., et al. (2002). "Stable association of mitotic cyclin B/Cdc2 to replication origins prevents endoreduplication." Cell 111(3): 419-31.

Yahya G., et al. (2014) “A Whi7-anchored loop controls the G1 Cdk-cyclin complex at start.” Mol Cell. 53(1):115-26

Yen K., et al. (2012) “Genome-wide nucleosome specificity and directionality of chromatin remodelers.” Cell 149(7):1461-73.

Zeng, Y., et al. (1998). "Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1." Nature 395(6701): 507-10.

Zilahi E., et al. (2000). "The S. pombe sep1 gene encodes a nuclear protein that is required for periodic expression of the cdc15 gene." FEBS Lett 481(2): 105-8.