universität wien

DISSERTATION

ACTIVATION OF THE G2/M SPECIFIC GENE *CLB2* REQUIRES MULTIPLE CELL CYCLE SIGNALS

Verfasser

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angestrebter akademischer Grad

Doktor der Naturwissenschaften (Dr. rer.nat.)

Wien, August 2009

Studienkennzahl	A 091 419
Dissertationsgebiet	Chemie
Betreuerin / Betreuer:	ao. Univ.-Prof. Dr. Gustav Ammerer

DANKSAGUNG

Ich möchte mich bei allen meinen bestehenden und ehemaligen Kollegen herzlichst dafür bedanken dass ich die letzeten Jahre in ihrer Gegenwart arbeiten durfte, dazu gehören Aurora, Christina, Ilse, Alexandra, Eva, Andreas, Stephan, Christoph und viele Andere, die in den letzten Jahren die Belegschaft der Labors 5 und 6 am Institut für Biochemie im 5 Stock der Dr.Bohrgasse 9 (oder wie man heutzutage so schön sagt am *MFPL - a pioneering structure for visionary research*) bildeten.

Nur einige wenige möchte ich gesondert hervorheben, aufgrund Ihrer besonderen Verdienste um mein wissenschaftliches und seelisches Heil. Da wären vor allem Manfred und Helene an deren Arbeit ich anknüpfte und in deren Anwesenheit ich die wohl produktivsten Jahre meiner bisherigen Laufbahn hatte. Ein besonderer Dank gilt auch Veerle, die nicht nur immer ein offenes Ohr für Disskusionen jeder Art hatte sondern die sich auch meiner englischen Texte annahm, eine Aufgabe die wohl nur jene in ihrer ganzen Tragik erfassen können, denen diese Aufgabe nach Veerles Abgang aufgehalst wurde. Hiermit komme ich direkt zum Wolfi. Sein milder und lebensfreudiger Charakter hatte mir nicht nur wessentlich das Erstellen dieser Dissertation erleichtert sondern auch das wissenschaftliche Arbeiten freudiger gestalltet. Das gilt auch für Syam von dem wir alle hoffen, dass sein unbändiger Optimismus nicht nur seiner Jugend geschuldet wird.

Vor allem gilt mein Dank meinem Doktorvater Gustav, welcher immer ein leuchtendes Beispiel eines Wissenschaftlers war, welcher aus purer Freude an der Erkenntnis Forschung betreibt, abseits jeglicher eitler materieller Güter und Würden.

Diese Arbeit ist meiner Frau Zsuzsanna und meinen Töchtern Esther, Judith, Martha und Rebekka gewidmet, die mir in all der langen Zeit fortlaufend und zunehmend ein glückliches Zuhause boten, und mir somit die zwangsläufigen und notwendigen Gedanken über Sinn und Unsinn der Wissenschaftlichen Arbeit erträglich gestaltet haben .

1 ABSTRACT

In the yeast *Saccharomyces cerevisiae*, G2/M specific genes such as the mitotic cyclin gene *CLB2*, are transcribed from S phase to the exit of mitosis. The transcription of these genes is initiated after START, the main restriction point of the budding yeast cell cycle, and is maintained by a positive feedback loop consisting of the DNA binding protein Fkh2, the transcriptional activator Ndd1 and the Cdc28/Clb2 kinase. However, this positive feedback loop can neither account for the initiation of transcription nor for the repression of G2/M specific genes in G1. This thesis focuses on factors responsible for promoter repression in G1, and the regulation of Ndd1 dependent activation

Genetic evidence has so far indicated that Fkh2 functions not only as an activator but also as a repressor of G2/M specific transcription. I could show that promoter repression is not an intrinsic function of the Fkh2 protein but is due to its ability to mediate cell cycle specific binding of the Sin3/Rpd3 histone de-acetylase. Regulated recruitment of Sin3 is likely mediated by the interaction between Sin3 and a 306 amino acids residue N-terminal region of Fkh2.

I present evidence that the Sin3/Rpd3 release from G2/M promoter requires the activity of G1 cyclins, which indicates that this process is one of the many START related activities of this cell cycle kinase. The release of Sin3/Rpd3 is reflected at the promoter region by the transient acetylation and eviction of one nucleosome. Sin3/Rpd3 release, and nucleosome acetylation are independent on the transcriptional activator Ndd1. However, nucleosome eviction requires Ndd1 as its presence at the promoter is a prerequisite for the recruitment of the chromatin remodeler Swi/Snf.

Finally I tried to characterise the mechanism that is responsible for the initial recruitment of Ndd1 to G2/M promoters, a process that has been commonly associated with B-type cyclin kinase activity. I provide evidence that cells can prevent Ndd1 from chromatin recruitment when either DNA damage occurs or cells fail to initiate replication origin firing. Both processes are independent on the previously described feedback loop involving Clb2.

In our model, transcriptional onset of G2/M promoters in early S phase is best characterized by a multi-step process of de-repression and activation, that is triggered by the switch from G1 cyclin associated to B-type cyclin associated kinase activity.

1 ZUSAMMENFASSUNG

In der Sprosshefe *Saccharomyces cerevisiae* werden G2/M spezifische Gene wie jenes des mitotischen Zyklins Clb2 von Beginn der S Phase an bis in den mitotischem Exit hinein transkripiert. Die Transkription dieser Gene wird nach dem sogenannten START, dem wichtigsten Restriktionspunkt im Zellzykluss der Sprosshefe, initiert und fortlaufend mittels eines positiven Feedbacks aufrecht erhalten, welcher aus dem DNA Bindungsprotein Fkh2, dem transkriptionellen Aktivator Ndd1 und Clb2 besteht. Dieser positive Feedback ist jedoch weder für die Initiation der Transkription noch für die Gen-Repression verantwortlich. In dieser Arbeit widme ich mich jenen Faktoren die für die Repression der G2/M spezifischen Gene in der G1 Phase verantwortlich sind, und der Frage nach der Initierung der mit Ndd1 verbundenen Aktivierung.

Die Genetik hat darauf hingewiesen, dass Fkh2 nicht nur in der Aktivierung sondern auch in der Repression der G2/M Gene involviert ist. Ich konnte zeigen dass die Repression des Promoters nicht Fkh2 selbst zugeschrieben werden kann, sondern vielmehr von seiner Fähigkeit, die zellzyklussabhängige Bindung der Sin3/Rpd3 Histon Deacetylase an den Genpromoter zu vermitteln, abhängt. Die regulierte Rekrutierung von Sin3/Rpd3 basiert höchstwahrscheinlich auf seiner Interaktion mit einer 306 Aminosäuren grossen Domäne von Fkh2.

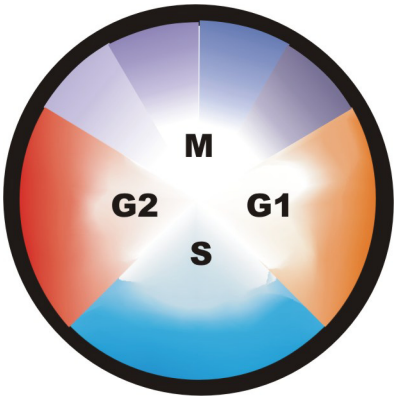
Ich zeige auch, dass das Loslösen von Sin3/Rpd3 von den entsprechenden Promotoren von der Aktivität der G1 Zykline in der Zelle abhängig ist, was darauf hinweist, dass dieser Mechanismus einer der vielen START abhängigen Prozesse ist. Das Ablösen von Sin3 hat zur Folge, dass ein einzelnes Nukleosom acetyliert und vom Promotor entfernt wird. Während die Abdissoziierung von Sin3 und die Acetylierung des einzelnen Nukleosoms unabhängig von Ndd1 vor sich gehen, verlangt die Abdissoziierung des Nukleosomes das Vorhandensein von Ndd1, da dieses für die Rekrutierung des Chromatin-Remodelers Swi/Snf ist notwendig ist.

Letztendlich versuche ich jenen Mechanismus zu charakterisieren welcher für die erstmalige Bindung von Ndd1 an G2/M Promotoren verantwortlich ist, und welcher zumeist mit B-Typ Zyklinen in Verbindung gebracht wird. Ich präsentiere Ergebnisse, welche darauf hindeuten, dass Zellen die Rekrutierung von Ndd1 verhindern können wenn die DNA geschädigt wird oder das Feuern der Replikationsorigins verhindert wird. Beide Vorgänge sind unabhängig von dem bereits bekannten positiven Feedback.

In unserem Model beschreiben wir die transkriptionelle Aktivierung von G2/M Promotoren als einen zweistufigen Prozess, bestehend aus De-repression und Aktivierung, welcher von dem Wechsel von G1-Zyklin assoziierter zu B-Typ-Zyklin assoziierter Kinaseativität angetrieben wird.

ACTIVATION OF THE G2/M SPECIFIC GENE CLB2 REQUIRES MULTIPLE CELL CYCLE SIGNALS

2 INTRODUCTION



2.1 The cell cycle and its checkpoints

To proliferate, living organisms need to duplicate all vital components of a cell and to divide them in two equal parts. The period of events from cell division to cell division is called the cell cycle and the molecular mechanisms driving it are highly conserved among species. In unicellular organisms such as bacteria and yeast every round of the cell cycle is creating a new individual.

The cell cycle has been traditionally divided into 4 stages; the Gap1 or G_1-phase, the Synthesis or S-phase, the Gap 2 or G_2-phase and mitosis. This subdivision was based on differences in amount of DNA, extent of chromatin condensation and segregation of chromosomes.

During S-phase the decondensed chromatin serves as a template for DNA replication whereas in mitosis the freshly duplicated DNA is equally distributed to both progenitor cells. Because of the great number of consecutive events, mitosis was further subdivided into four phases based on certain visual landmarks that can be easily observed in the microscope; prophase (chromosome condensation), metaphase (alignment of the chromosomes upon the spindle) anaphase (separation of sister-chromatids) and telophase (de-condensation of chromosomes).

Another basic requirement for most cells, besides DNA replication, is the duplication of cell mass. In most organism this is accomplished in G1 (Gap1), a cell cycle phase which is situated after mitosis and before S-phase. In analogy to Gap1, the period in cell cycle progression that follows S-phase and precedes mitosis is defined as Gap2 or G2-phase.

In G1 a check-point mechanism ensures that the cells accumulated sufficient nutrients for the next round of DNA replication. In the budding yeast *Saccharomyces cerevisiae* this control point has been designated START, whereas in most other organisms it is referred to as restriction point. Under optimal conditions, once START is passed the cells are committed to undergo DNA replication and cell division.

Several additional check-points from the onset of DNA replication into early mitoses ensure that DNA replication and distribution is correctly performed; during S-phase the Intra-S or DNA damage checkpoint senses events which deteriorate DNA replication and mitotic checkpoints prevent the cell from precocious separation of sisterchromatids or their aberrant distribution to daughter cells.

The molecular machinery which triggers cell cycle progression from one phase to the other is unidirectional and conserved in all eukaryotes from yeast to man. In principle it is controlled by a set of kinases, whose activity depends on their association with regulatory proteins called cyclins. Cyclins do not only exhibit a cell cycle dependent appearance but also provide the substrate specificity to the kinase. How, and through which mechanisms the uni-directionality as well as the surprising robustness of the cell cycle machinery is achieved is still issue of discussion, even in such a thoroughly studied model organism like the budding yeast. However, increasing evidence suggests that the cell cycle dependent regulon is controlled by multiple levels of regulation including protein modification, protein stability, transcriptional activation and repression, which results in a wave-like appearance of positive and negative feed-back loops adding up to an oscillating network named the cell cycle clock.

2.1.1 The kinase and its cyclins

Cyclin-dependent protein kinases or CDKs were first discovered in a genetic analysis of the cell cycle in yeast (Hartwell, 1974; Nasmyth and Reed, 1980) and in Xenopus as a component of the Maturation-promoting factor (MPF) (Lohka and Maller, 1988; Masui and Markert, 1971). MPF was known to trigger maturation of oocytes prior to the first meiotic division, but the fact that MPF could also trigger cell division in mitotic cells made clear that MPF is a general factor driving cell cycle activity (Dunphy et al., 1988). Even more interestingly it was found that MPF consists of two subunits, one of them showing a periodic appearance in the cell which correlated to MPF activity. Because of this periodic activity it was named mitotic cyclin (Evans et al., 1983; Gerhart et al., 1984). The second subunit was a protein kinase named cyclin dependent kinase (CDK) (Nurse, 1990). CDKs are proline-directed kinases that phosphorylate serine or threonine in the S/T-P consensus motifs (Shenoy et al., 1989) and in most organism they occur in excess without any major changes in protein levels during the cell cycle.

In *Saccharomyces cerevisiae* there are five different CDKs, namely Cdc28, Pho85, Kin28, Ssn3, and Ctk1, but only Cdc28 is required for cell-cycle progression. The importance of Cdc28 for cell cycle progression is reflected by the fact that the *cdc28* deletion mutant is lethal. However, several temperature sensitive *cdc28* alleles are known which either arrest at START (Mendenhall et al., 1987; Reed, 1980) or, like *cdc28-1N*, in G2 (Piggott et al., 1982; Surana et al., 1991). Full activation of Cdc28 as well as any other CDK generally requires two major events, namely a stimulatory phosphorylation and the binding of the appropriate cyclin, which provides the substrate specificity to the kinase. CDKs can also be inactivated by the binding of inhibitory proteins, the CKIs (CDK inhibitors), as well as by inhibitory phosphorylation. (Kitagawa et al., 1996; Songyang et al., 1994; Songyang et al., 1996).

Budding yeast has 22 cyclins, but only nine of them confer substrate specificity to Cdc28. These Cdc28 associated cyclins have been classified into two groups: three G_1 cyclins; (Cln1,Cln2 and Cln3) and six B-type cyclins (Clb1 to Clb6). G1 cyclins primarily regulate events during the G_1 phase of the cell cycle. B-type cyclins are expressed in three successive waves from START to Mitoses. With the exception of Cln3, most cyclins are functionally redundant (Cln1/2, Clb5/6, Clb3/4, Clb1/2), with both members of each pair sharing a common overall amino-acid sequence and a similar pattern of transcription. G1 cyclins help the cell to complete G1 and to enter S phase, whereas Clb5/6, which are also called S-phase

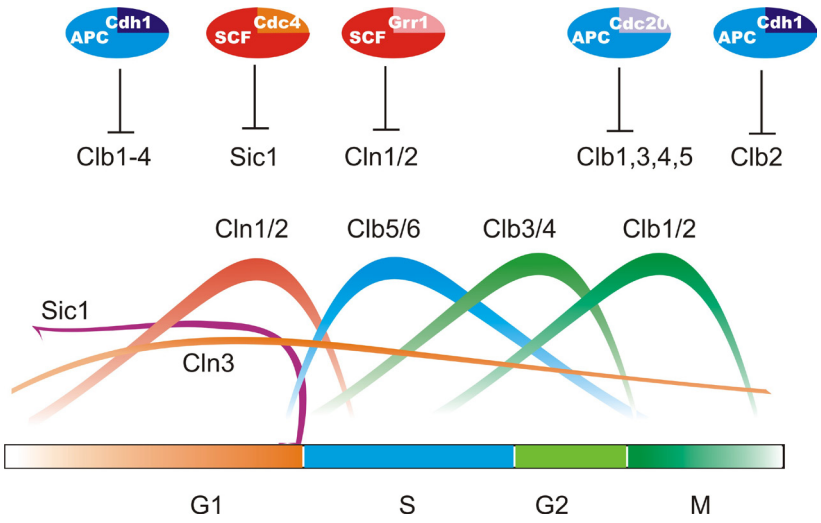
cyclins, are required for the initiation of DNA replication. The mitotic cyclins Clb1, Clb2 Clb3 and Clb4 are partly redundant although they might have distinct roles in the formation of the mitotic spindle. Clb2 seems to be the most important mitotic cyclin, as deletion of *CLB2* alone is sufficient to slow down mitosis and is lethal in combination with its closest homolog Clb1. Clb1 and Clb4 seem to have more distinct roles in meioses, whereas Clb2 is dispensable for meioses (Breeden, 2000; Breeden, 2003; Dahmann and Futcher, 1995; Koch et al., 1996; Lee et al., 2002). Significantly, each of the cyclins confers a limited and partially overlapping range of functions to Cdc28. As CDK function is primarily controlled by changes in cyclin levels, the question arises how cyclin expression is regulated.

2.1.2 Regulation of Cyclins – transcription, inhibition and degradation

In general, cyclin activity in the cell is regulated at three different levels; cyclin transcription, inhibition of cyclin associated CDK activity and targeted proteolysis of cyclins. Transcriptional regulation of cyclins constitutes a closed circuitry of positive and negative feedback loops, where cyclin associated kinase activity of the actual cell cycle phase drives the expression of cyclins of the next phase. Once the new cyclin activity is established it represses the transcription of all cyclins associated to the past cell cycle stage, thus making the process irreversible. Apart from transcriptional control and thus cyclin abundance, Far1 and Sic1, two predominant inhibitors of cyclin associated Cdc28 activity in yeast (CKIs), can inhibit CDK activity via substrate exclusion from the active sites of Cdc28. Far1 inhibits Cln/Cdc28 activity in response to pheromone (Peter and Herskowitz, 1994), thereby ensuring that cells synchronise their cell cycle in G_1. In contrast to Far1, Sic1 is an inhibitor of B-type cyclin associated Cdc28 activity. Two major cellular functions have been assigned to Sic1. Its appearance in late anaphase leads to down-regulation of Clb/Cdc28 kinase activity and allows the cell to enter telophase and subsequently G1. Later in the cell cycle, Sic1 degradation is required for a switch like transition from G_1 to S phase. (Schwob et al., 1994).

Interestingly, cyclins as well as their inhibitors are under a further level of regulation. Both can be substrate for cell cycle specific targeted proteolysis by the 26S proteosome. This regulatory function is displayed by two E3-ubiquitin ligase complexes, the SCF (Skip1-cullin-F-box protein) complex and the APC (anaphase promoting complex) (Zachariae and Nasmyth, 1999). The SCF complex is active throughout the cell cycle but is especially

required at the G1/S transition. It is composed of four core subunits and two regulatory subunits that determine substrate specificity; Cdc4 and Grr1 (Tyers and Jorgensen, 2000). Cdc4 targets the CDK inhibitors Sic1 and Far1, whereas Grr1 targets the cyclins Cln1 and Cln2 for degradation. In contrast to the SCF complex the APC is active from late S phase to late G1. It consists of at least 11 core subunits and two regulatory subunits, Cdc20 and Cdh1 (Tyers and Jorgensen, 2000) and APC^{Cdc20} is required for destruction mitotic cyclins Clb3 and Clb5. The association of APC with Cdh1 requires low CDK activity and alters the specificity of the complex towards Clb1 and Clb2 (Zachariae and Nasmyth, 1999), thereby enabling mitotic exit.



Model 1. Cell cycle dependent cyclin abundance in the cell, and its regulation by ubiquitin dependent degradation

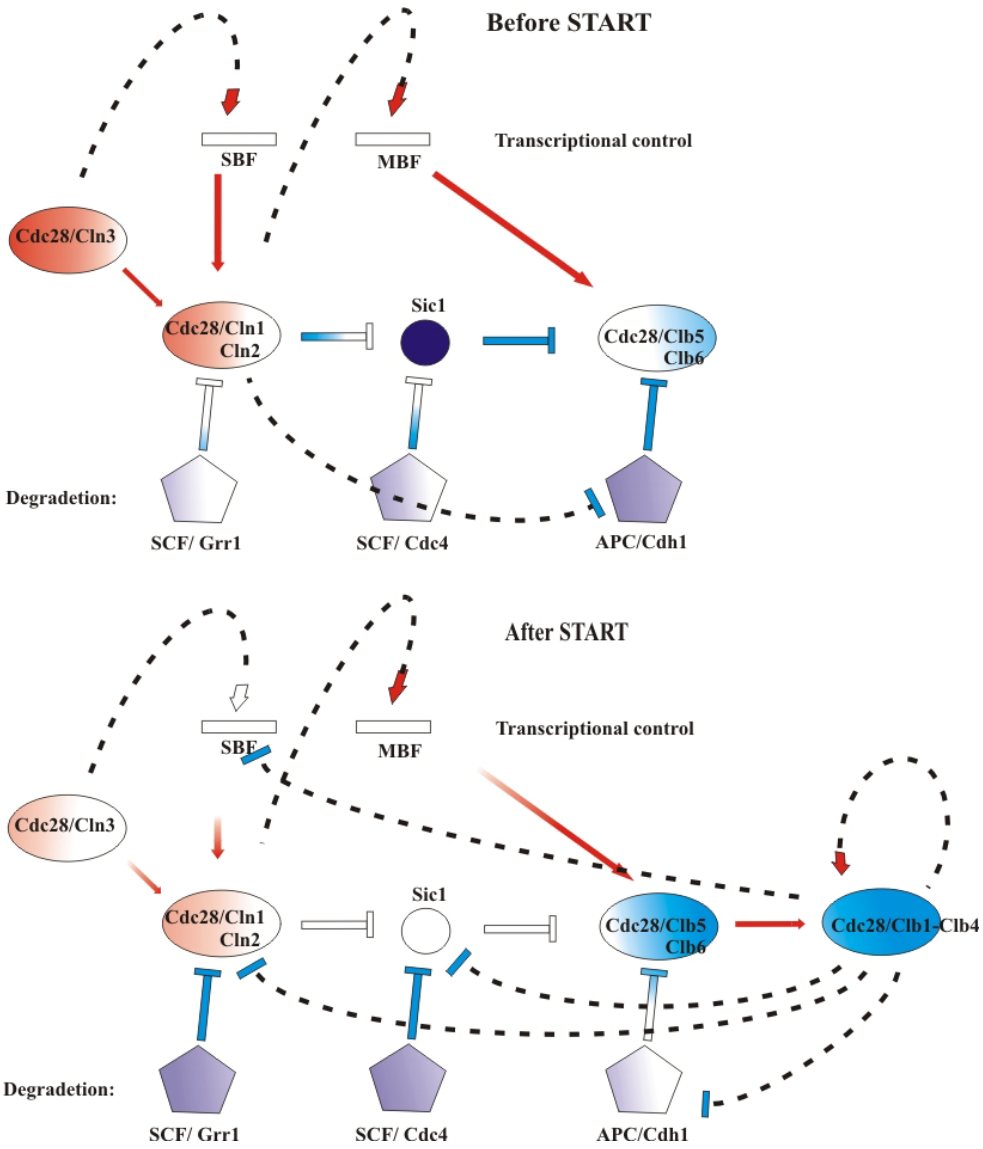
2.1.3 Throughout G1 to START

The GAP1 or G1 phase is defined as the period between cytokinesis and START. In budding yeast, this time point is irreversible and constitutes a sharp borderline between G1 and S phase. On the molecular level the G1 phase is best characterised by the complete absence of Cyclin B associated Cdc28 kinase activity. This is achieved by two mechanisms. First, B-type cyclins are actively targeted for ubiquitin mediated proteolysis by the APC^{Cdh1} complex. Second B-type cyclin associated Cdc28 activity is blocked by the stoichiometric inhibitor Sic1 (Knapp et al., 1996; Schwob et al., 1994). Apart of the active repression of B-type cyclins there are three different G1 cyclins, Cln1, Cln2 and Cln3 which accumulate throughout G1 and their activity peaks immediately prior to START. All three G1 cyclins mediate the transcription of the G1 regulon, which contains about 200 different genes. These genes are under the control of two different transcriptional complexes named SBF (SCB binding factor) and MBF (MluI binding factor). Both, SBF and MBF, contain Swi6 as a common regulatory subunit, which is recruited to the promoter by two different DNA-binding proteins, namely Mbp1 (MBF) and Swi4 (SBF) (Moll et al., 1992; Sidorova and Breeden, 1993). Mbp1 in complex with Swi6 is recruited to promoters containing the MCB element (Mlu1 cell cycle box, ACGCGTNA, recruiting MBF) whereas SBF binds the SCB sequence motif (Swi4/6 cell cycle box, CACGAAA, recruiting SBF) (Mendenhall and Hodge, 1998).

Cln1 and Cln2 are functional and structural homologs as they share 72% identity in their N-terminal region and the single deletion of either *CLN1* or *CLN2* has only weak effect on cell cycle progression. In contrast, Cln3 has only a 20 -25% identity to his namesakes and is constitutively transcribed throughout the cell cycle from a nutrient dependent promoter. Cln3 tends to accumulate in the nucleus as the cells gain in size (Mendenhall and Hodge, 1998). There, in complex with the cell cycle kinase Cdc28, it triggers the phosphorylation of several transcription complexes such as are SBF and MBF and the transcriptional inhibitor of SBF Whi5 (Costanzo et al., 2004; de Bruin et al., 2004). Phosphorylated Whi5 rapidly exits the nucleus which is the rate-limiting step for transcriptional activation. Because Cln1 and Cln2 are both transcribed from SBF bound promoters, Cln3 was suggested to be the only G1 cyclin required for appropriate timing of START. However, in *CLN3* deletion strains Cln1 and Cln2 can drive their own transcription (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991), which led to speculations that there must be a positive feedback loop involving Cln1/2 in *CLN3* deletion strains. Whether this positive feedback loop is of any relevance in wt-type cells has

been a matter of debate for almost 15 years. For long time this idea was rejected, because in a *CLN1,CLN2* double deletion cells SBF and MBF dependent promoters are induced at the same time like in wt-cells (Stuart and Wittenberg, 1995). Only recently, single cell analysis revealed that in *cln1, cln2* cells the time-coherence of SBF/MBF promoter activation is completely lost. The previously published observations were obviously based on experimental bias as a consequence of ensemble analysis methods. Thus, a positive feedback loop involving Cln1/2 seems to be essential for the coherence of the G1 regulon induction prior to START (Skotheim et al., 2008).

At START, the G1 cyclin associated Cdc28 phosphorylates the B-type cyclin inhibitors Cdh1 and Sic1. In the case of Sic1, this triggers its ubiquitination and degradation via the E2 enzyme Cdc34 and the E3 ubiquitin ligase SCF^{Cdc4} complex (Verma et al., 1997). Peaking G1 cyclin associated kinase activity also drives the expression of the S-phase cyclin genes *CLB5* and *CLB6* as these are under the control of MBF dependent promoters. Thus, once the B-type cyclin specific inhibitor Sic1 is inactivated, Clb5 and Clb6 activity is rapidly stabilized in the cell. The S-phase cyclins Clb5/6 are not only involved in S-phase associated processes like bud formation and DNA synthesis but are also suggested to trigger the expression of mitotic cyclins Clb1 and Clb2 (Pic-Taylor et al., 2004). In a turnaround process the mitotic cyclin associated Cdc28 kinase actively represses SBF, and is thus involved in the transcriptional shutdown of G1 cyclin genes (Koch et al., 1996). The switch-like transition from G1 cyclin associated to B-type cyclin associated cell cycle kinase activity is favoured by the fact that targeted proteolysis of Sic1 requires the phosphorylation of at least 6 of its 9 consensus sites. This mechanism allows Sic1 to ignore low levels of Cln-CDK activity in early G1 phase, and then respond decisively once Cln-CDK activity has exceeded a threshold level (Nash et al., 2001). Although Sic1 contributes to the accuracy of the transition, new data suggest that it is not essential for the process *per se*. When non-phosphorylatable Sic1 is expressed in the cell at endogenous levels, cells are still able to enter S-phase. However, the process is significantly delayed which is most probably due to the fact that Clb5 and Clb6 have to out-compete Sic1. Cells can tolerate endogenous levels of non-degradable versions of Sic1, however these cells require appropriate B-type cyclin levels, which indicates that in wt cells there is a well established balance between Sic1 and B-type Cyclin abundance (Cross et al., 2007).



Model 2. Positive and negative feedback loops stabilizing G1 and B-type cyclin activity at START

2.1.4 The S phase

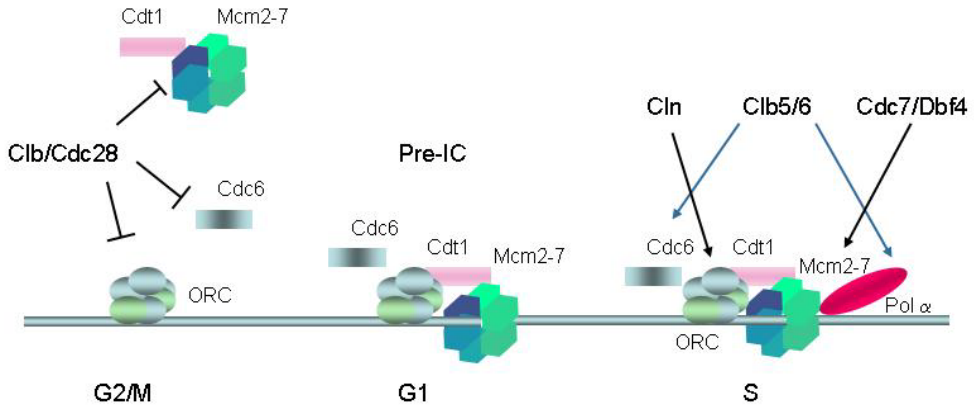
2.1.4.1 Formation of the pre-replicative complex and its regulation by CDK

During the S-phase, cells have to duplicate their DNA in a regulated and well orchestrated manner. In all eukaryotes, DNA replication is started at numerous well defined positions in the genome in order to accomplish a complete duplication of the template in a considerable timescale. The loci at the DNA where the Replication Forks are allowed to form are thus called origins of replication. The situation in yeast is different from other eukaryotes as origins of replications are defined by a specific DNA motif which is called autonomously replication sequence (ARS) (Bell and Dutta, 2002). The ARS is bound throughout the cell cycle by a hetero-hexameric complex which is called origin-recognition complex (ORC) (Robinson and Bell, 2005). Prior to the initiation of replication the ORC complex recruits the DNA replication licensing factors Cdc6 and Cdt1. Cdt1 is required for the loading of another hetero-hexameric complex called Mcm2-7 on the ARS (Tanaka and Diffley, 2002). The Mcm2-7 complex posses DNA-helicase activity and is required for unwinding of DNA during the process of replication (Labib and Diffley, 2001; Labib et al., 2001). However, in G1 this complex consisting of ORC, Mcm2-7, Cdc6 and Cdt1 is still inactive and thus named Pre-initiation complex (Pre-IC). Once START is passed the DNA polymerase Polα becomes recruited and the Cdc7/Dbf4 kinase phosphorylates Mcm2-7 (Bell and Dutta, 2002) which initiates the firing of the respective origin. From the time of origin firing one can distinguish between early and late origins.

The process of Pre-initiation complex assembly and activation is tightly regulated by different cell cycle specific kinase activities in order to prevent either premature DNA replication or DNA re-replication outside of the S-phase. As prevention of re-replication is especially important in the G2/M phase, mitotic cyclins inhibit the formation of a new Pre-IC by three different mechanism; they trigger the export of the Cdt1-Mcm2-7 complex outside of the nucleus (Nguyen et al., 2000), the phosphorylation induced degradation of Cdc6 (Drury et al., 2000) and the inhibition of the ORC itself (Nguyen et al., 2001). In contrast to the inhibitory role of mitotic cyclins in the G2/M phase, prevention of premature DNA replication in G1 is associated to low levels of CDK activity at this stage of the cell cycle. Once mitotic

cyclin levels break down after mitosis the Pre-IC is formed but remains inactive until throughout G1 until START. There, G1 cyclin associated kinase activity phosphorylates the ORC, whereas S-phase cyclins are necessary for the phosphorylation of Cdc6 and Polα, and thus for origin firing.

Apart from DNA bound factors, there is evidence that the state of chromatin might influence the timing of late origin firing. Especially the correct histone acetylation pattern of DNA seems to be required for the appropriate timing, as hyperacetylated DNA causes premature firing of the adjacent late origins (Vogelauer et al., 2002).

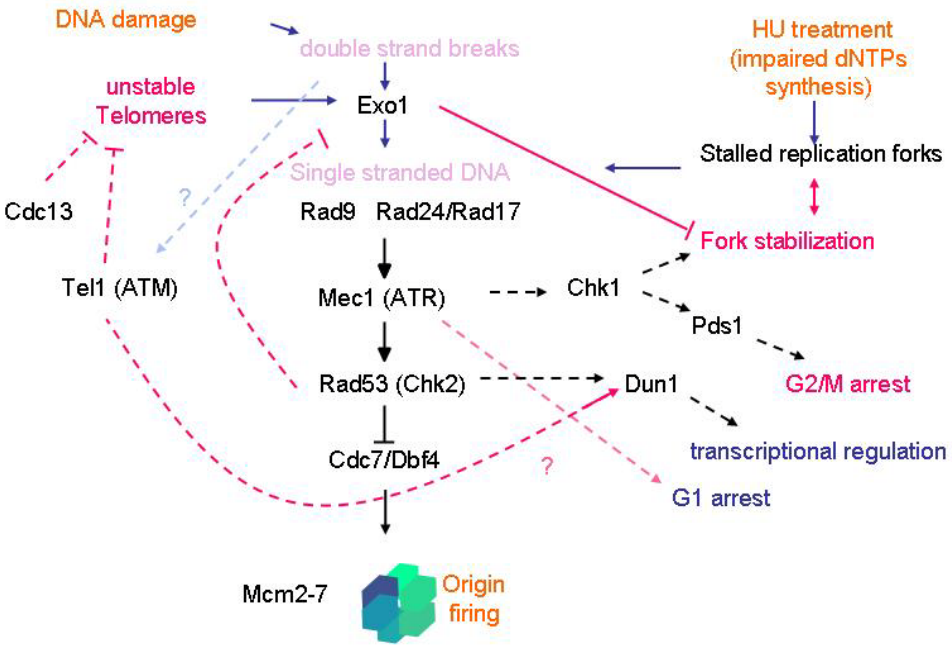


Model 3. Regulation of origin formation and firing throughout the cell cycle

2.1.4.2 The DNA damage and the intra S-checkpoint

Once the initiation complex is assembled, cells are committed to replicate their DNA. Firing of individual origins can be halted by the so-called intra-S checkpoint. This checkpoint can be induced by DNA damage, caused by such agents like MMS, or by stalled replication forks, which can experimentally be induced by treating the cells with hydroxyurea (HU). HU inhibits the ribonucleotide reductase (RNR) and thereby impairs dNTP levels in cells. In yeast, the DNA damage checkpoint involves a kinase cascade which is mainly regulated by the ATR homolog Mec1 and its downstream effector Rad53, which is a homologue to human Chk2 kinase (Zhou and Elledge, 2000). Upon induction of the checkpoint, this cascade is involved in many processes such as DNA repair, transcriptional activation of DNA repair genes, regulation of origin firing and stabilisation of the replication fork. Stalled replication forks as well as DNA damage or uncapped telomeres create double strand brakes which recruit the exonuclease Exo1 in order to generate single stranded (ss)DNA (Lewis et al.,

2002). The ssDNA is coated with replication protein A, which in turn recruits the Mec1 kinase to a site close to the double strand brake (DSB) (Dubrana et al., 2007). Mec1, in collaboration with other proteins such as Rad9, Rad17 and Rad24 activates the downstream kinases Rad53 and Chk1. Phosphorylated Rad53 is recognised by the FHA domain (see chapter 2.3.1.1.) of the Dun1 kinase (Lee et al., 2008), which in turn becomes phosphorylated by Rad53 and thus activated. Dun1 itself triggers the transcription of genes involved in DNA repair (Chen et al 2007). Rad53 also stabilises the replication fork as it phosphorylates Exo1 (Smolka et al., 2007) and prevents further creation of single stranded DNA (Segurado and Diffley, 2008). However, Rad53 cannot account for the replication fork stabilisation alone as Mec1 and Chk1 also seem to be involved (Segurado and Diffley, 2008). Rad53 also upregulates dNTP synthesis as it inactivates the ribonucleotide reductase (RNR) inhibitor Sml1 (Nyberg et al., 2002). Another downstream target of the Rad53 kinase is the Cdc7/Dbf4 kinase, which is required for origin firing. The Rad53 dependent phosphorylation of Dbf4 thereby prevents firing of other origins (Ogi et al., 2008; Weinreich and Stillman, 1999). Outside of the S-phase, activated Chk1 also blocks mitosis as it is thought to stabilise the mitotic securin Pds1, which degradation is required for sister-chromatid separation (Tyers and Jorgensen, 2000). In mammals the ATM kinase is mainly involved in DSB detection, In yeast the ATM homologue in Tel1 is less important. However, recent data suggest that Tel1 can bypass Mec1 activity, although its signalling becomes only apparent when multiple DSBs are generated (Mantiero et al., 2007).



Model 4. A simplified view of the DNA damage network in yeast

2.1.5 G2/M phase

The period in the cell cycle from early S phase until late anaphase can be described as a period of high B-type cyclin associated kinase activity between two switch-like transitions, which are START and mitotic exit. High levels of B-type cyclins are maintained through positive feedback loops and by the phosphorylation-dependent degradation of the B-type cyclin inhibitor Sic1. As mentioned above there are four mitotic B-type cyclins, Clb1, 2,3 and 4 which become expressed from S-phase to early M-phase. They have widely redundant roles in regulation of cell cycle progression. Clb3/4 seem to have additional roles in the assembly of the early mitotic spindle. Cells deleted for *CLB1* and *CLB2* arrest in G_2 with a fully formed bipolar mitotic spindle, whereas cells deleted for all 4 mitotic cyclins arrest without spindle formation.

Clb1/2 cyclins are expressed after Clb3/4 and seem to be required for completion of mitosis. Furthermore Clb1/Clb2 are responsible for switching from apical to isotropic growth as disruption of both genes prevents this switch (Lew and Reed, 1995). Disruption of *CLB2* alone delays the switch to uniform growth, giving a possible explanation for the pseudohyphal like growth of strains displaying low Clb2 levels (see this study).

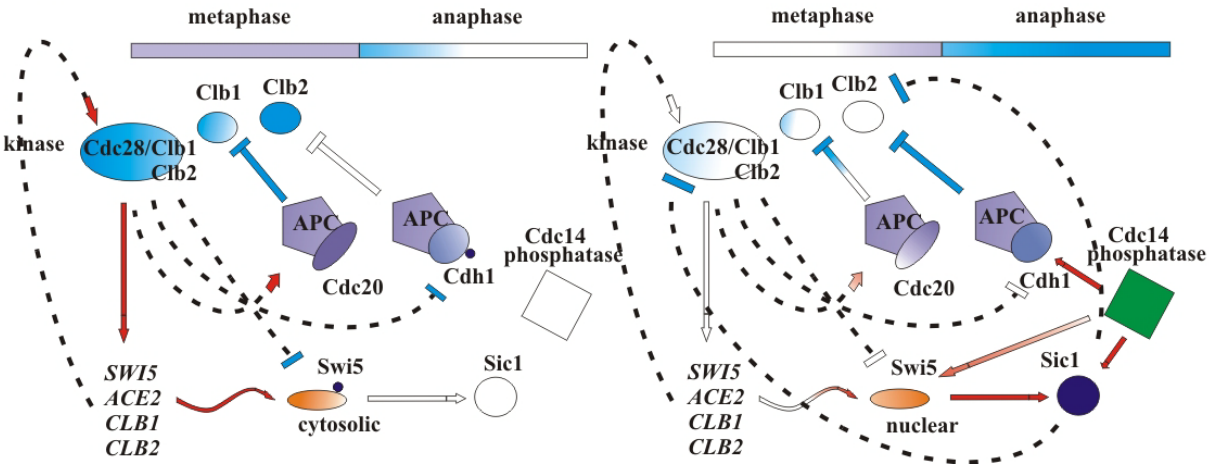
Mitotic cyclins levels peak at the metaphase/anaphase transition. At this stage of the cell cycle, the already segregated chromosomes arrange in the so-called metaphase plate, where sisterchromatids are held together by the cohesin complex while being attached to a bi-polar spindle via their kinetochores. The mitotic spindles are microtubuli emanating from two spindle pole bodies which are located at the opposite site of the cell (Jaspersen and Winey, 2004). Thus, correct chromosome distribution requires the attachment off all kinetochores as well as the subsequent cleavage of the cohesin complex. When cells cannot ensure the correct attachment of all chromosomes or when mitotic cyclin levels are impaired, the metaphase/anaphase checkpoint is activated and the cells arrest in metaphase.

Once the metaphase-anaphase checkpoint is passed, the process of chromosome segregation is triggered by the APC^{Cdc20} dependent degradation of the mitotic securin Pds1. After the degradation of the securin, the Esp1 protease is activated and cleaves Scc1, the essential subunit of the cohesin complex (Ciosk et al., 1998; Nasmyth, 1999; Uhlmann, 2001).

At the level of cyclin activity, multiple negative feedback loops lead to the subsequent down regulation of B-type cyclins, which ultimately results in mitotic exit. First, peaking CDK activity activates APC^{Cdc20} and thereby triggers the degradation of mitotic cyclins except for

Clb2. Because of this, APC^{Cdc20} cannot completely abolish CDK activity. This task is accomplished in late anaphase when the substrate specific subunit of APC, Cdc20 is replaced by Cdh1, which is specific for Clb2. Cdh1 itself remains active throughout the G1 phase until START, where it becomes phosphorylated and thus inactivated by the peaking Cln/Cdc28 activity and loses its ability to interact with the APC (Zachariae et al., 1998). However, de-phosphorylated Cdh1 triggers Clb2 degradation via the APC^{Cdh1} complex, which leads to the complete shut off of CDK activity in late anaphase. De-phosphorylation of Cdh1 is mediated by the phosphatase Cdc14, which is one of the key player in late anaphase (Jaspersen et al., 1999; Visintin et al., 1998). Cdc14 is bound in the nucleolus prior to anaphase, and is released by protein kinase cascade called mitotic exit network (MEN) which involves Cdc5 and Esp1 to name a few (McCollum and Gould, 2001).

A second mechanism leading to the break down of CDK activity involves the B-type cyclin inhibitor Sic1. The transcription of the *SIC1* gene is triggered by the transcriptional activator Swi5. The *SWI5* gene is already activated during S-phase as it is expressed from a G2/M specific promoter (see chapter 2.3) but the Swi5 protein remains cytosolic during G_2 and early M phase. This is due to the phosphorylation of its nuclear localization sequence by the Clb1,2/Cdc28 kinase. Once CDK activity decreases in anaphase, Swi5 is de-phosphorylated, enters the nucleus and triggers transcription of Sic1 (Knapp et al., 1996; Moll et al., 1991; Toyn et al., 1997). Here again, Swi5 as well as Sic1 are de-phosphorylated and thus stabilized by the phosphatase Cdc14. Sic1 remains stable until late G_1, thereby suppressing B-type cyclin associated kinase activity until START.



Model 5. Switch from high to low CDK activity at the metaphase-anaphase transition

2.2 Transcription and chromatin

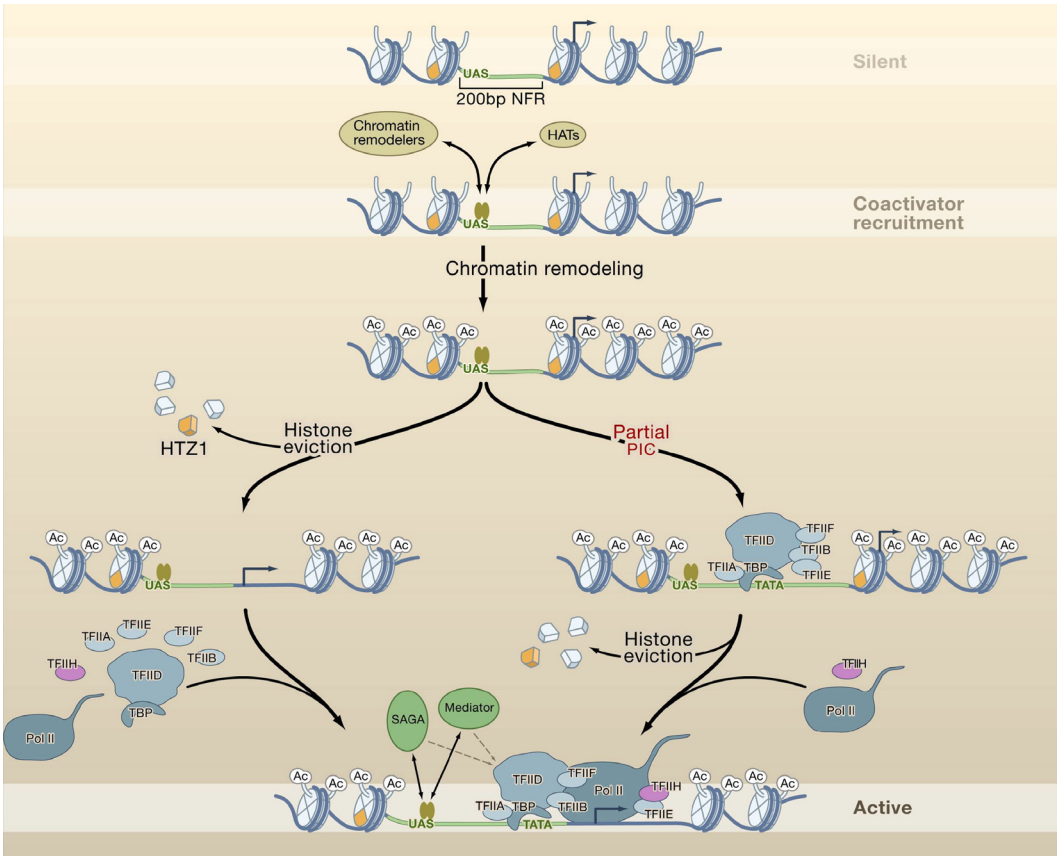
2.2.1 Initiation of transcription

In eukaryotes, chromatin consist of nucleosomes distributed quite evenly along the DNA. Each nucleosome is composed of 147bp of DNA wrapped around a protein octamer, consisting of two histones H2A/H2B dimers, and one H3/H4 tetramer (Luger et al., 1997). However the interaction between the histone octamer and DNA is very strong, the nucleosome itself is not a simple static unit, as transcriptional initiation as well as transcriptional elongation require the repositioning of nucleosomes or even nucleosome displacement (Li et al., 2007). Because probably presence of nucleosomes also impedes the recruitment of transcriptional activators most promoters in yeast exhibit a nucleosome free region (NFR) upstream of the core promoter (including the TATA box) which spans about 200bp, and frequently contains an upper activation sequence (UAS) which recruits transcriptional activators. Interestingly the nucleosomes flanking the NFR are enriched for the H2A.Z (Htz1) histone variant (Raisner et al., 2005). The recruitment of promoter specific transcription factors to the UAS triggers the recruitment of coactivators and chromatin remodelers such as are SAGA or SWI/SNF. These histone posttranslational modifications increase the binding of activators, particularly for those bound within nucleosomal regions. More importantly, histones are acetylated at promoter-proximal regions, and these nucleosomes become much more mobile.

Subsequently, Pol II is recruited to the promoter, either in association with or shortly after the recruitment of general transcription factors (GTF: e.g. TFIID, TFIIA and TFIIB), in order to assemble the pre-initiation complex (PIC). Another general transcription factor TFIIH then melts around 10bp of DNA in order to allow Pol II to encompass single stranded template DNA. During the first 30bp of transcription TFIIH phosphorylates the carboxy-terminal domain (CTD) of Pol II which in turn starts to recruit factors associated to transcriptional elongation (Buratowski, 2003).

The formation of the PIC is most probably facilitated by the fact that H2A.Z is acetylated and evicted from the DNA, most probably as a dimer with H2B, thereby extending the nucleosome free region surrounding the UAS. However the transcribed region is not

completely nucleosome free, as acetylated H3 and H4 continue to accumulated during gene activation (Pokholok et al., 2005), supporting the idea that the eviction of the H2A.Z H2B tetramer creates enables nucleosome sliding (Li et al., 2007).



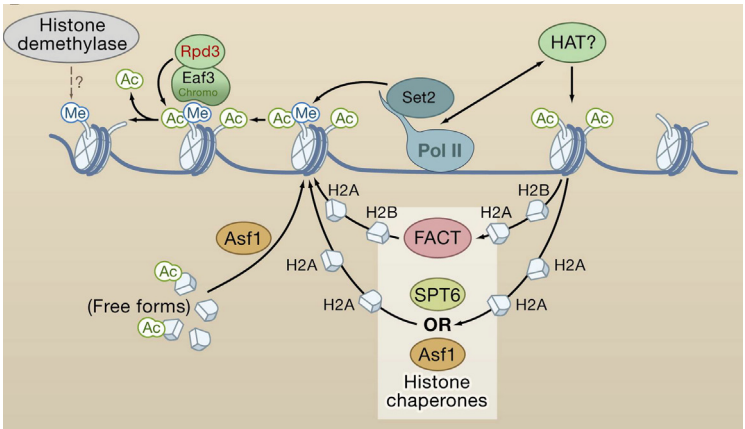
Li et al. 2007

Model 6. Two models how chromatin might be regulated upon transcriptional initiation.

Left: A combination of histone modifications and chromatin remodeling directly results in the loss of H2A.Z-containing nucleosome, thereby exposing the entire core promoter to the GTFs and Pol II. SAGA and mediator then facilitate PIC formation through direct interactions. **Right:** PIC is partially assembled at the core promoter prior to the loss of H2A.Z containing nucleosomes. It is the binding of Pol II and TFIIH that leads to the displacement of HH2A.Z-containing nucleosomes and the full assembly of PIC.

2.2.2 Transcriptional elongation and maintenance of chromatin stability

Cells do not only require efficient mRNA synthesis, but they also have to preserve chromatin integrity while Pol II proceeds along the DNA. The process of transcriptional elongation is tightly regulated in yeast, as Pol II and its ability to recruit cofactors is modified successively. First at the upstream region of an open reading frame TFIIH phosphorylates Ser5 inside of the CTD of Pol II . Later as transcription continues, Ser2 inside the CTD is phosphorylated by the Ctk kinase (Buratowski, 2003). In vitro data show that Pol II needs to disassemble at least one H2A/H2B dimer to allow passage along one nucleosome. Interestingly chromatin remodeling during elongation is determined by different factors associated with the respective phosphorylation forms of Pol II. At the 5' end of the gene Ser5 phosphorylated Pol II recruits factors which trigger H2AB ubiquitination and H3K4 tri-methylation as well as chromatin remodeling. When Pol II migrates into promoter distal regions it recruits histone acetyl-transferases (HATs) to acetylate the nucleosome in front of the elongation machinery. The passage of PolII cause histone displacement, which is accomplished by chromatin remodelers such is the FACT complex or histone chaperons like the Asf1. The freshly depositioned nucleosomes are methylated at H3K36 by the Set2 methyltransferase which is recruited depending on the Ser2 phosporylation of Pol II (Krogan et al., 2003). Subsequently H3K36 methylated nucleosomes are recognised by the small Sin3/Rpd3 histone deacetylase complex, de-acetylated and left in a stable state (Keogh et al., 2005).



Li et al. 2007

Model 7. Disassembly and re-assembly of nucleosomes during transcription

2.2.3 Histone modification and remodeling

As already mentioned above nucleosomes can be altered in two different ways. Either histones are replaced by histone variants or they can be target of posttranslational modifications. These modification include acetylation, methylation, ubiquitination, ADP- rybosylation and sumolation of lysines, methylation of arginines and phosphorylation of serines and threonines (Li et al., 2007). There are two mechanistically different explanations, why histones are a target of such a vast variety of regulation. First, all histone modifications, except for methylation, change the net charge of the protein, thereby altering their ability to interaction with DNA. This is especially the case for histone acetylation as acetylated histones exert an increased mobility at DNA in vitro (Chandy et al., 2006; Reinke and Horz, 2003). This explains why acetylated histones are often associated to transcriptionally active genes where they are target of chromatin remodeling processes. Second, histone modifications can serve as specific recognition marks for other chromatin binding factors, which might be also components of other histone modifying enzymes. To give a prominent example already mentioned in previous chapters; during transcriptional elongation methylated H3K36 is bound by the chromodomain of Eaf3 which in association with the Rpd3S HDAC de-acetylates the underlying chromatin.

Histone acetylation is carried out by numerous histone acetyltransferase complexes (HATs), like Gcn5 or Esa1, and typically occurs at multiple lysine residues (brown et al 2000). Whether the impact of histone acetylation on the process of transcriptional regulation is based upon a specific pattern of acetylated histones and their residues, or whether it is a consequence of a cumulative effect is still a mater of debate (Kurdistani et al., 2004). Histone acetylation is counteracted by histone deacetylases (HDACs) which are most often associated with transcriptional repression. One of the most prominent HDACs is the Sin3/Rpd3 complex which will be discussed in more detail in the context of G2/M specific transcription. Whereas all HATs are able to modify any histone tail residue accessible for acetylation, other histone modifications are often catalyzed by a particular enzyme at a specific residue inside of a specific histone, which often results in locally restricted function of chromatin.

Apart from histone modification a group of chromatin modifying complexes use ATP hydrolysis in order to replace histones or whole nucleosomes. Generally they can be subdivided in two groups; chromatin remodelers such as Swi/Snf or the RSC complex and histone chaperones like Asf1 and Nap1 (Bruno et al., 2003; Lorch et al., 2006). In most cases

their activity has been associated to transcriptionally active genes where they help PolII to surpass the nucleosome barrier. However, data suggest that also chromatin repression requires histone-remodeling activity. Especially the Isw2 histone remodeling complex was shown to suppress anti-sense transcription in a genome wide manner (Whitehouse et al., 2007).

2.2.4 Chromatin & cell cycle specific transcription

Quite little is known about chromatin modification and remodeling at cell cycle specific promoters as research focused mainly on the recruitment of site specific transcription factors and their modification by the various feedback loops which drive cell cycle progression. The best studied promoter related to the cell cycle was for long time the HO promoter. HO codes for a site specific endonuclease which is specifically expressed in mother cells upon cytokinesis and which triggers mating type switch, thus enabling mating type diversification of a previously homogenous population. The mechanism is driven by Swi5, a transcription factor that enters the nucleus in anaphase and binds to two DNA regions inside of the HO promoter; URS1 and URS2. Swi5 binding is transient, as the protein remains bound for only 5 minutes. However, this is sufficient to mediate the recruitment of the Swi/Snf complex, the Mediator and the Gcn5 HAT. These complexes remain bound to the HO promoter throughout G1, until peaking G1 cyclin activity leads to the recruitment of Pol II and initiation of transcription (Cosma et al., 2001). Recent data suggest that the Swi/Snf, the mediator and Gcn5 are firstly recruited to the URS1 promoter region and later transferred to the URS2 promoter region, which one is more adjacent to the transcriptional start. This transfer is dependent on the transient presence of the histone remodeling complex FACT and the histone chaperone Asf1 within URS2, thus suggesting that FACT and Asf1 have to alter the nucleosomal structure of URS2 prior to Swi/Snf, Mediator and Gcn5 binding (Takahata et al., 2009). This finding is unique as it shows requirement for chromatin remodeling within a highly structured promoter region. Interestingly, the Sin3/Rpd3 HDAC complex, in association with Ash1, seems to negatively regulate the HO promoter, as in Ash1 daughter cells HO transcription is similar to mother cells (Carrozza et al., 2005). Sin3/Rpd3 might even play a role in mother cells too, as it was shown that *ash1* mutation allows the recruitment of Swi/Snf to HO URS1 even in the absence of the normally required Gcn5 (Mitra et al., 2006).

Involvement of the Sin3/Rpd3 HDAC was described at two other sets of cell cycle specific promoters transcribed in G1. First, at promoters which contain binding sites for Ace2 and Swi5, Swi5 specific trans-activation is repressed by Sin3/Rpd3 presence, making these promoters Ace2 dependent (Voth et al., 2007). More interestingly Sin3/Rpd3 recruitment to these promoters is mediate by Fkh2, another DNA binding protein which will be discussed in more detail in the next chapter. Second, Sin3/Rpd3 is recruited throughout G1 to SBF dependent promoters by a yet unknown mechanism. Prior to START, the Sin3/Rpd3 HDAC is removed from SBF dependent promoters, thus showing a cell cycle dependent recruitment pattern (Stephan and Koch, 2009).

2.3 G2/M specific gene promoters and their regulators

In S phase the transcription of 33 genes is activated by a common transcriptional machinery, via a common upper activation sequence (UAS) inside of the promoter regions of these genes. Because this gene cluster, including the most essential mitotic cyclins *CLB2*, is active throughout the G2 phase until early anaphase, the genes inside of this cluster have been named G2/M specific genes or *CLB2* cluster genes. Many of the *CLB2* cluster genes, like *CDC20, CDC5, SWI5*, *CLB1*,*CLB2* or *ACE2* code for cell cycle relevant proteins, which are often involved either in CDK activation or repression. The transcriptional activation of the G2/M regulon thus determines the correct onset of several positive and negative feedback loops which drive the cell cycle through mitosis. One of these positive feedback loops involves Clb2 and was rewarded to stabilize the expression of the *CLB2* cluster itself (Amon et al., 1993). However, the mechanism by which the transcription of the *CLB2* cluster genes is initiated is still unclear. In general, it is assumed that a ternary complex consisting of the DNA binding proteins Mcm1 and Fkh2 co-operatively bind the G2/M specific UAS and remains bound throughout the cell cycle. In early S phase the transcriptional activator Ndd1 is recruited to the promoter via its interaction with Fkh2 and remains bound until late anaphase (Koranda et al., 2000). In order to identify the target sites of the cell cycle kinase which could explain the positive feed back loop as well as to decipher the mechanism by which transcription is initiated, the main players in G2/M specific transcription, Ndd1 and Fkh2 were subject of many investigations.

Although many phosphorylation sites inside of Ndd1 and Fkh2 were identified and have been assigned to different cell cycle specific kinases, deep controversies about the accuracy

and correct interpretation of the available dataset remain. Most contradictions are based on the fact that in vitro data do not match to in vivo experiments and, even worse, some in vivo results fail to be reproducible by other groups in the field. In the following chapters I would like to introduce the main players in more detail. I also want to discuss some of the controversy of the past.

2.3.1 A platform for additional modifications; Mcm1& Fkh2

As mentioned above *CLB2* cluster gene promoters are bound throughout the cell cycle by a protein complex consisting of Mcm1 and Fkh2 (Kumar et al., 2000), which bind to the MCE respectively to the SFRE elements inside of the UAS of their promoters.

Mcm1 was originally genetically identified by the inability of *mcm1*^{ts} mutants to stably maintain yeast minichromosomes (Mini Chromosome Maintenance 1) (Maine et al., 1984) Several studies have shown that Mcm1 is a nuclear localized transcription factor, involved in the activation and repression of multiple genes regulating diverse processes, such as are cell mating type control (Elble and Tye, 1991), osmotic-stress response and cell cycle progression (Althoefer et al., 1995; Lydall et al., 1991; Pramila et al., 2002). Mcm1 binds directly to the MCE element of the promoter region where it is suggested to form a homodimer in order to induce DNA bending (Lim et al., 2003). Mcm1 possesses 4 domains but only the first 98 residues including the first domain seem to be required for DNA binding as well for dimerization (Primig et al., 1991).

Regarding the regulation of G2 specific genes, Mcm1 is involved transcriptional activation as well as in transcriptional repression as its presence at the promoter is a prerequisite for Fkh2 recruitment. Promoters bearing mutations in the MCE element fail to recruit Fkh2 to the SFRE element in vitro, and DNA methylation at putative SFRE binding sites is not hindered in Mcm1 depleted cells in vivo (Althoefer et al., 1995; Lydall et al., 1991). Some data suggest that to promoters displaying both elements, MCE and SFRE, Mcm1/Fkh2 are already recruited as a complex (Hollenhorst et al., 2001).

Fkh2 is a forkhead transcription factor of 98kDa which was characterized as a major component of the Sff (SWI five factor), which was an at that time uncharacterized protein complex known to associate with Mcm1 at G2/M promoters (Kumar et al., 2000). In yeast Fkh2 has a close homolog named Fkh1. These two protein have an overall similarity of 47 %. Both proteins share two similar domains; the Dna Binding Domain (DBD) with an overall identity off 72%, and the Forkhead Associated (FHA) domain with an overall identity of 59%. Despite their similarity, Fkh2 and Fkh1 generally exert different influences on transcriptional regulation and bind to different sets of promoters (Hollenhorst et al., 2000; Hollenhorst et al., 2001). Fkh2 is preferentially bound to G_2/M specific promoters like *SWI5* or *CLB2*. However, in mutant cells depleted for Fkh2, Fkh1 presence at G2/M promoters is increased (Hollenhorst et al., 2000). The ability of Fkh1 to replace Fkh2 function in *fkh2* cells is reflected by the fact that only in strains deleted for both genes a phenotype, namely pseudohyphal growth, can be observed (Hollenhorst et al., 2000; Zhu et al., 2000). Pseudohyphal growth is generally associated to low Clb2 levels, as Clb2 associated kinase activity is required fro the switch from apical to isotrophic growth. Interestingly a similar phenotype can be observed in conditional *mcm1* and *ndd1* mutants (*mcm1* and *ndd1* full deletion are both lethal), indicating that Ndd1, Mcm1 and Fkh1/2 are all involved in transcriptional regulation of Clb2.

It is still not known, whether there is a genuine role for Fkh1 in G_2/M promoter regulation. Some data suggest that Fkh1 and Fkh2 have different effects on the steady state levels of *CLB2* mRNA and that they play contradicting roles in gene expression (Hollenhorst et al., 2000; Pic et al., 2000). Some authors even impose a regulatory role for Fkh1 and Fkh2 in the process of transcriptional elongation and termination of the G2/M regulon (Morillon et al., 2003). However, efforts undertaken in order to reproduced these data, failed by us and by other investigators (Voth et al., 2007). Furthermore it was claimed that Fkh1 can not interact with Mcm1 (Boros et al., 2003) although Fkh1 is able to bypass *FKH2* deletion.

As mentioned above the Mcm1/Fkh2 complex is bound throughout the cell cycle to G_2/M specific promoters. Chromatin immunoprecipitation (ChIP) studies revealed, that the transcriptional activator Ndd1 (Koranda et al., 2000), as well as the transcriptional co-repressor Sin3 (Manfred Koranda thesis) are recruited to *CLB2* cluster-gene promoters at different stages of the cell cycle.

Whereas Ndd1 binds the promoter from the onset of S-phase to early anaphase, the Sin3/Rpd3 histone de-acetylase complex seems to display opposite regulatory functions as its binding peaks in G1 and is completely abolished in S phase (Manfred Koranda thesis). Their antagonistic roles in regard to the G2/M regulon is probably best characterized by the finding

that the deletion of *SIN3* can bypass the lethality of *ndd1*. Furthermore, in cells deleted for both forkhead factors the binding of Ndd1 and Sin3 is lost. Interestingly, the presence of Fkh1 is sufficient to restore the recruitment of Ndd1 but not of Sin3, indicating that distinct interaction sites of FKH2 mediate the recruitment of Ndd1 and Sin3 (Jiri Veis diploma,).

2.3.1.1 The FHA domain of Fkh2

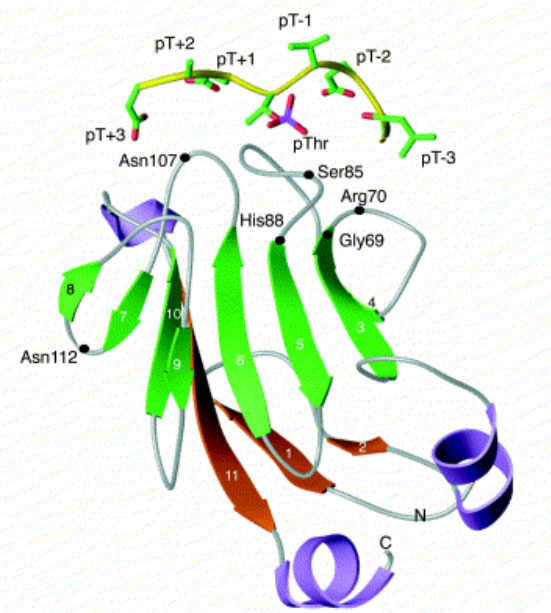
Both forkhead proteins Fkh1 and Fkh2 posses a phospho-recognition domain in their N-terminal region which is named forkhead associated domain or FHA domain. This highly conserved domain, binds to phosphorylated threonine residues of target proteins thereby mediating protein-protein interaction (Durocher and Jackson, 2002; Li et al., 2002). FHA domains are common in many proteins and organism and consist of 80-100 amino acids, formed into 11 β-sheets from which only 8 (β3-β11) define the core homology region (Durocher and Jackson, 2002). The α helical loops between the individual β sheets (mostly β3/4, β4/5 or β6/7) contain conserved phospho-threonine binding residues, similar to Arg70 and Ser85 in the FHA1 domain of Rad53, which was the first FHA domain structure solved in NMR (Durocher and Jackson, 2002). However, different FHA domains use different binding mechanism to confer their binding specificity beyond their common phospho-threonine requirement.

For example, there is strong evidence that the FHA domains of the DNA-damage checkpoint kinase Dun1 binds with 100x higher efficiency to the dual phosphorylated form of Rad53 than to the single phosphorylated protein (Lee et al., 2008). Furthermore, conserved residues like Gly 69 and His88 of the Rad53 FHA1 domain seem not to be directly involved in phospho-threonine recognition, but rather stabilize the overall architecture of the interaction (Durocher et al., 2000).

It was suggested that Fkh2 recruits Ndd1 to G2/M specific promoters via its FHA domain (Koranda et al., 2000). We tested this proposition in *fkh1, fkh2* cells which expressed *fkh2* mutants depleted either for their entire FHA domain or comprising alanine substitutions for the arginine 87 and histidine 123 residues (which correspond to Arg 70 and Histidine 88 in Rad53). In fact, Ndd1 binding to the *CLB2* promoter could not be re-constituted in both cases, although both Fkh2 mutants successfully bound to the promoter. Furthermore, these cells still displayed pseudohyphal growth, whereas cells transformed with the wt allele of *FKH2*

regained their normal cell shape (Manfred Koranda thesis, Jiri Veis diploma). In addition we could show that when the FHA domain of Fkh2 was fused to the DNA binding domain of Gal4, Ndd1 binding to the *GAL1-10* promoter could be observed. Even more this interaction was still cell cycle regulated, suggesting that it is the modification of Ndd1 which accounts for its regulation (Jiri Veis diploma).

It was published that the FHA domain of Fkh2 binds the single phospho-threonine T319 of Ndd1, and that this interaction is solely mediated by arginine 87 inside of the FHA domain of Fkh2 (Reynolds et al., 2003). However, both claims could not be reproduced by us. Thus, although the involvement of FHA domain in Ndd1 recognition is beyond any doubt, it remains unclear which concrete sites in Ndd1 are recognised.



Model 8. Ribbons representation of the Rad53^{FHA1} domain in complex with a phospho Threonine-containing peptide (shown in a ball-and-stick model). The core FHA homology (sheets β3–β10) is coloured in green. (Durocher et al. 2000)

2.3.2 Required for activation; Ndd1

Ndd1 is an essential protein consisting of 554 amino acids, with no known homology in any organism. This protein also does not contain any conserved domain. There is only one noteworthy motif; a polyglutamin stretch of 15 residues. However, this motif does not seem to

be essential, as Ndd1 deleted for this region displays normal regulatory activity and effectively suppresses the lethality of *ndd1* strains (Loy et al., 1999).

Ndd1 was first discovered as a multi-copy-suppressor of the *cdc28-1N* mutation, that arrests cells prior to mitosis (Loy et al., 1999), already indicating involvement in cell cycle regulation. Additional genetic evidence that Ndd1 is involved in the regulation of G2/M specific genes was given by David Lydall, who isolated Ndd1 as an up-regulator of a SWI5-UAS_{309}-ubiYlacZ construct. Manfred Koranda showed that Ndd1 is recruited to the promoters of *CLB2*-cluster genes in a cell cycle dependent manner in G2 (Koranda et al., 2000). As Ndd1 presence at G2/M specific gene promoters closely patterns the transcriptional activity expression of these genes, Ndd1 was recognised as a G2/M specific transcriptional activator.

The enzymatic function of Ndd1 is unknown, although it is clear that it activates transcription, as it was shown with reporter genes in one hybrid assays (Loy et al., 1999). The lethality of *ndd1* deletions is due to the lack of Clb2 protein, as cells arrest at the metaphase-anaphase plate and are not able to switch from polar to apical bud growth. Thus cells arrest with elongated bud formation and cannot undergo mitosis. However, *ndd1* cells are able to undergo a second round of DNA synthesis and appear thus to be quadruploid when observed by FACS analysis. Ndd1 recruitment to *CLB2* cluster gene promoters depends on an intact FHA domain of Fkh2, which is known to interact specifically with phosphorylated proteins (see previous chapter).

As Clb2/Cdc28 is known to be necessary for the transcription of *CLB2* cluster genes (Amon et al), Ndd1 is thought to be a possible target of CDK kinase activity. Indeed mutation of threonine 319 into alanine seems to completely abolish the interaction between Ndd1 and Fkh2.

However, Manfred Koranda replaced all four putative CDK sites (S/TPxxK/R) in Ndd1 (see Figure below) to alanine, among them also threonine 319. Unfortunately in our hands Ndd1 mutated in all four putative Cdc28 consensus sites is still phosphorylated, indicating that these four sites are not the only phosphorylation sites in Ndd1 (Helene Klug thesis). The quadruple Ndd1 mutant also continues to complement the *NDD1* deletion, and it is still recruited to the promoter at schedule (Helene Klug thesis). We made another attempt, in order to determine whether in the conditional Clb1-4 mutant Ndd1 is recruited to *CLB2* cluster genes promoters. Once again, we could not find any clear evidence for the involvement of mitotic cyclin associated CDK activity in Ndd1 recruitment (Helene Klug thesis).

Even when B-type Cyclin associated CDK activity is involved in the maintenance of a positive feed-back loop the question remains how Ndd1 recruitment to *CLB2* cluster gene

promoters is initiated. Recently involvement of the polo kinase Cdc5 was described and Ndd1 was shown to be phosphorylated at serine 85 by this kinase. The authors claimed that this phosphorylation is required for the appropriate timing of Ndd1 binding, as mutants where serine 85 was mutated to alanine displayed delayed recruitment to G2/M promoters (Darieva et al., 2006). However we repeated the experiment and could not observe any difference in recruitment kinetics between mutant and wild type Ndd1 (Syam Kumar pers. com.). However we realised that protein levels of the S85A Ndd1 mutant are decreased in the cell, which might explain the different experimental outcome, as threshold levels for signal detection might differ significantly in different ChIP experiments from group to group.

```
  1 MDRDISYQQN YTSTGATATS SRQPSTDNNA DTNFLKVMSE FKYNFNSPLP
 51 TTTQFPTPYS SNQYQQTQDH FANTDAHNSS SNESSLVENS ILPHHQQIQQ
101 QQQQQQQQQQ QQQALGSLVP PAVTRTDTSE TLDDINVQPS SVLQFGNSLP
151 SEFLVASPEQ FKEFLLDSPS TNFNFFHKTP AKTPLRFVTD SNGAQQSTTE
201 NPGQQQNVFS NVDLNNLLKS NGKTPSSSCT GAFSRTPLSK IDMNLMFNQP
251 LPTSPSKRFS SLSLTPYGRK ILNDVGTPYA KALISSNSAL VDFQKARKDI
301 TTNATSIGLE NANNILQRTP LRSNNKKLFI KTPQDTINST STLTKDNENK
351 QDIYGSSPTT IQLNSSITKS ISKLDNSRIP LLASRSDNIL DSNVDDQLFD
401 LGLTRLPLSP TPNCNSLHST TTGTSALQIP ELPKMGSFRS DTGINPISSS
451 NTVSFKSKSG NNNSKGRIKK NGKKPSKFQI IVANIDQFNQ DTSSSSLSSS
501 LNASSSAGNS NSNVTKKRAS KLKRSQSLLS DSGSKSQARK SCNSKSNGNL
551 FNSQ
```

Cdc28 kinase site
Manfred Koranda: 1
Raynolds et al: 2
Cdc5 kinase site: 3

Phosphorylation sites in Ndd1 mutated by Manfred Koranda, Reynolds et al, and Syam Kumar (Manfred Koranda thesis, Reynolds et al 2003, personal communication]

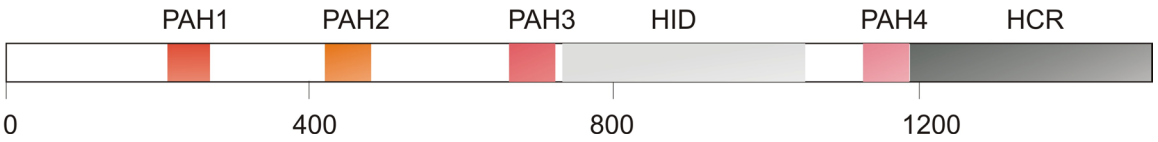
The *NDD1* gene is transcribed from a SBF related promoter (Loy et al., 1999) and mRNA levels peak prior to START, coincidentally with Cln kinase activity. Attempts to express Ndd1 from strong constitutive promoters, such as are the ADHpr or the GALpr during the G1 phase failed, indicating that Ndd1 is actively degraded in G1 (Helene Klug thesis). Helene Klug tested the E3-ubiquitin ligases SCF and APC as well as some of their substrate specific subunits for any possible involvement in Ndd1 degradation. Unfortunately any conclusive data could be obtained from these experiments (Helene Klug thesis).

2.3.3 Any need for active repression? a possible role for the Sin/Rpd3 HDAC

The Fkh2/Fkh1/Mcm1 complex does not only mediate transcriptional activation via Ndd1 recruitment but is also required for promoter repression in G1, as in *fkh1 fkh2* double deletion mutants G2/M cluster show a cell cycle independent basal transcriptional activity (Hollenhorst et al., 2000; Koranda et al., 2000; Zhu et al., 2000). Although this repressory activity was assigned to the C-terminal domain of Fkh2 (Reynolds et al., 2003), data from our lab strongly suggest that the C-terminal domain of Fkh2 is of minor importance for promoter repression (Jiri Veis diploma thesis). Manfred Koranda could show that the histone de-acetylase Sin3/Rpd3, which was known to trigger transcriptional repression (Kadosh and Struhl, 1998), is recruited to G2/M specific promoters. This interaction is most probably mediated through Fkh2 as cells depleted for Fkh2 fail to recruit Sin3 (Manfred Koranda pers. com.) and Fkh2 was shown to interact with Sin3 (Ho et al., 2002). Even more interesting was the finding that Sin3/Rpd3 function at G2/M promoters is independent on Ume6 (Manfred Koranda pers. com.), a DNA binding protein which targets the Sin3/Rpd3 complex to the URS1 sequence of other promoter regions (Kadosh and Struhl, 1997; Kurdistani et al., 2002).

In yeast Sin3/Rpd3 HDAC is thought to predominantly exist in two distinct complexes, a large one (Rpd3L) and a small one (Rpd3S) (Carozza et al 2005). Whereas Rpd3L is associated to histone de-acetylation which is carried out at promoter regions, Rpd3S is most often associated the open reading frame of a gene during elongation (lee et al 2007). The core component of both complexes consists of the scaffolding subunit Sin3, the yeast homologue of mammalian Sin3A and Sin3B, and the catalytic subunit Rpd3 which is the yeast homologue of HDAC1. Sin3 is a large acidic protein with a molecular mass of 174.9 kDa (Wang et al., 1990). The protein sequence contains four paired amphipathic α-helices (PAH domains), each domain consisting of two helices, separated by a spacer of approx. 20 amino acids in length, a histone interaction domain (HID) and a highly conserved region (HCR) (Wang et al., 1990; Wang and Stillman, 1993). The PAH domains are homologous to each other but each domain targets different kinds of proteins, allowing Sin3 to interact with a broad range of proteins. Sin3 itself has no enzymatic activity and is unable to bind DNA, thus it requires DNA binding factors which mediate its recruitment to chromatin. Interestingly much more is known about domain specific interaction partners in mammals than in yeast. In mammals it is thought that the PAH3 and the HID are required for the assembly of the core

complex including HDAC1 whereas PAH1 and PAH2 serve as the docking platform for promoter specific transcription factors (Grzenda et al., 2009).



Model 9. Sin3 and its interaction domains

2.4 Aim of work

This thesis focussed on several quite different questions, which all together might help to unravel the complex mechanism driving G2/M specific promoter repression and activation.

First, we wanted to determine at which cell cycle stages the Sin3/Rpd3 complex is targeted to G2/M promoters and which factors or domains are responsible for its cell cycle specific binding.

Second, we were interested whether distinct cell cycle kinase activities, that are present prior and after START, influence the recruitment of the Sin3/Rpd3 complex to G2/M promoters. We also purified the two main components of the Sin3/Rpd3 complex, in order to determine possible phosphorylation sites.

Third, we wanted to determine the state of chromatin inside of the gene locus of the G2/M specific gene *CLB2*, in order to understand how Sin3/Rpd3 and Ndd1 recruitment are reflected at the level of chromatin modification and remodeling. We hoped that this approach might help us to understand better the interdependency of these two processes.

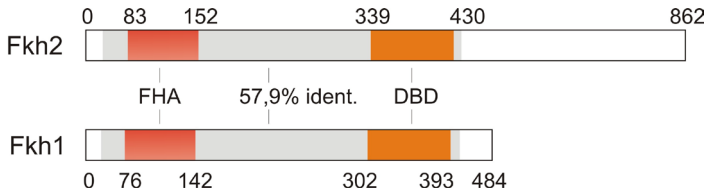
Furthermore, my thesis also targeted the question how Ndd1 binding is initiated and how transcriptional initiation of G2/M promoters is halted by the DNA damage checkpoint.

3 RESULTS I: A REPRESSED CELL CYCLE GENE CLUSTER LOOKS FOR ACTIVATION

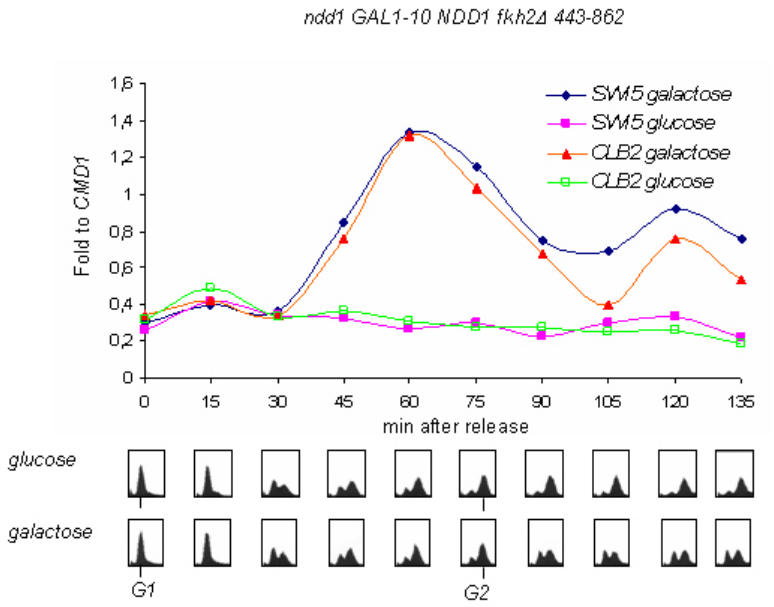
3.1 G2/M gene activation requires Ndd1 even in Fkh2 C-terminal mutants

The proposition for an active repression mechanism at G2/M specific promoters has its origin in the observation that *FKH2* deletion can suppress the viability defect of *ndd1* cells (Koranda et al., 2000). Moreover, genetic analysis as well as mRNA expression profiles suggested that the structural and functional analogue of Fkh2, Fkh1, is not involved in the repression of G2/M specific promoters (Hollenhorst et al., 2000; Hollenhorst et al., 2001; Zhu et al., 2000). As Fkh2 and Fkh1 share identity except for the C-terminal stretch of Fkh2, which is absent in Fkh1 (see Figure 1A), this C-terminal region has been suggested to mediate the repressory activity of Fkh2 towards G2/M specific promoters. In fact, in 2003 Raynolds et al presented data where cells expressing Fkh2 deleted for its C-terminal region were surpassing *ndd1* lethality. Even more surprisingly, the authors claimed that C-terminally truncated Fkh2 is able to re-establish proper cell cycle dependent activation of G2/M transcription even in absence of Ndd1. However, our findings do not support the model published by Reynolds et al. First, in a conditional *ndd1* strain carrying a C-terminally truncated *fkh2* allele transcriptional activation of the two G2/M cluster genes *CLB2* and *SWI5* could be only observed in the presence of Ndd1 (Figure 1B). However, the Fkh2 tested in our experiment was 13 amino acids shorter than the published one. In order to exclude the possibility that the contradicting results are based on slightly different *FKH2* alleles, we tested several C-terminally truncated versions of Fkh2 (Fkh2ΔC), tagged and untagged, for their ability to rescue the lethality of *ndd1* cells. To minimise the risk that secondary effects accumulated during homologous recombination, we generated all strains from diploids progenitors by sporulation. None of the tested *fkh2* alleles, among them one identical to Reynolds et al., could help the cells to bypass *ndd1* lethality. Only in a single case, in which a specific C-terminally HA tagged version of Fkh2 was used, we could observe viable *ndd1* cells (Figure1C). These results do not only demonstrate that Ndd1 is crucial for G2/M specific transcription, but also underlines the fact that experimental bias is created easily.

(A)



(B)



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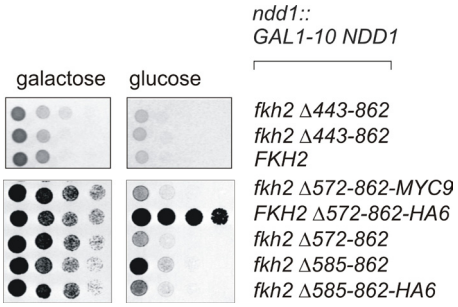


Figure 1 (A) Fkh1 is missing the C-terminal domain of Fkh2. The depicted domains are the forkhead associated domain (FHA)and the DNA binding domain (DBD) of Fkh2. **(B) Ndd1 is required for G2/M gene expression.** mRNA levels of the G2/M specific genes *CLB2 and SWI5* in cells released from α-factor block. A strain expressing an Fkh2ΔC mutant (JV333) was released in presence (galactose) and absence of ndd1 (glucose). Logarithmic growth and G1 -arrest were determined by FACS analysis. Blots were analyzed with STORM 840® phosphoimager (Molecular Dynamics) and signals were quantified with ImageQuant 5.0® software (Molecular Dynamics). **(C) Viability assay of various Fkh2 mutants in absence of Ndd1.** Diploid strains all heterozygous for *ndd1* and different *fkh2ΔC* alleles were sporulated, and homozygous haploids (JV 333, JV323, JV330, JV331, JV332, JV343, JV345) were tested for viability in the absence of Ndd1. Only one allele, associated to the abundance of an HA tag at AA position 572, was able to suppress the lethality of *ndd1*.

3.2 The Sin3/Rpd3 HDAC complex is involved in G2/M promoters repression

The failure to account the promoter repression to the C-terminal domain of Fkh2, shifted our interest into the identification of other proteins and/or complexes possibly involved in an Fkh2 mediated repression mechanism. Therefore, we tested loss of function mutants in several already known repressor systems for their ability to rescue the proliferation of *ndd1* cells. Whereas *ssn6* or *tup1* mutations did not give rise to viable *ndd1* progeny after sporulation of heterozygous ndd1 diploids, we found that deletion of *SIN3*, which is the supposed scaffolding subunit of the Sin3/Rpd3 histone de-acetylation complex (HDAC) allowed formation of *ndd1* colonies (Figure 2A). In order to prove whether the increased viability of ndd1 cells is based upon increased transcript levels of Ndd1 target genes, we performed a Northern blot analysis of *CLB2* cluster genes such as *SWI5* and *CLB2*. Indeed when cells were arrested in the G1 phase by *a*-factor, *sin3* cells exhibited elevated transcript levels when compared to wt cells (Figure 2B). To provide evidence that histone deacetylation is at the root of the repression mechanism in G1 we performed a similar set of experiments with an *RPD3* deletion strain, as Rpd3 is the de-acetylation subunit of the Sin3/Rpd3 HDAC. Once again, cells that combined deletions of *NDD1* and *RPD3* were viable (Figure 2A) and cells deleted for the *RPD3* subunit exhibited clearly de-repressed *CLB2* and *SWI5* mRNA levels when arrested by α-factor (Figure 2B).

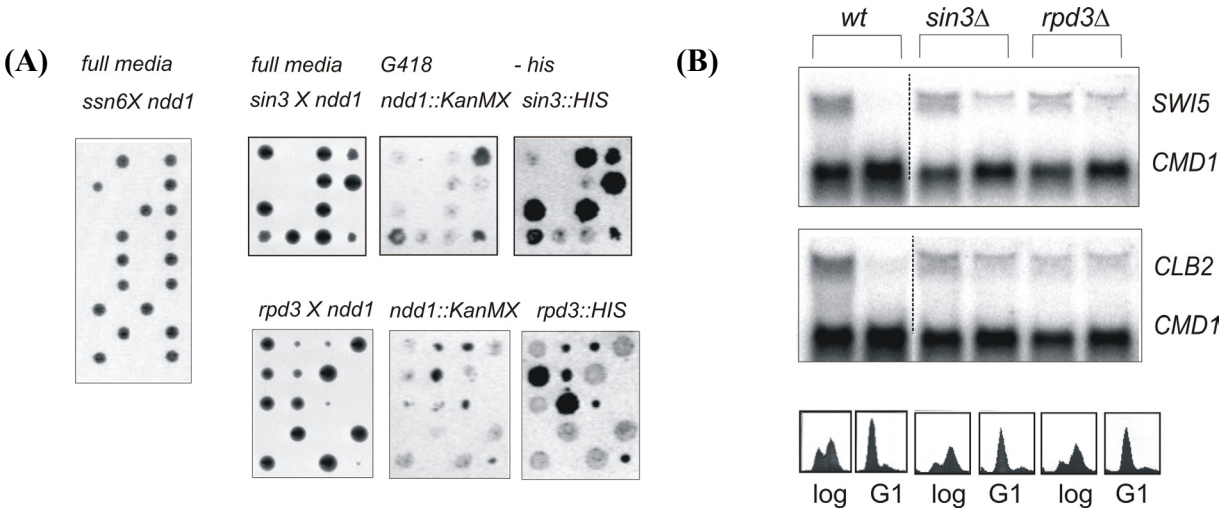


Figure 2 (A) Cells deleted for *SIN3* or *RPD3* do not require *NDD1* for viability. Diploid strains heterozygous for *ndd1* and either *ssn6* (MK259 X S1104), *sin3* (MK71 X K2957) or *rpd3* (JV375) were sporulated and spores were segregated and tested for growth. Each row represents one meiotic event. More than two colonies per row signify viable *ndd1* progeny. **(B) Deletion of *SIN3* or *RPD3* leads to promoter derepression in G1**. Wild type (JV1), *sin3* (JV361) and *rpd3* (JV363) strains were either exponentially grown (log) or arrested in G1 by α-factor. Expression levels of the G2/M specific genes *SWI5* and *CLB2* were determined by northern blot analysis. Logarithmic growth and G1 -arrest was determined by FACS analysis. Blots were analyzed with STORM 840® phosphoimager (Molecular Dynamics)

3.3 Sin3/Rpd3 recruitment at G2/M promoters is dependent on Fkh2

Our results clearly suggested the involvement of a Sin3/Rpd3 complex in G2/M gene regulation. Yet, we could not discount the possibility that these effects are of indirect nature as Sin3 complexes are part of many regulatory systems. We therefore measured promoter occupancy of *CLB2* cluster genes by HA-tagged Sin3 or Rpd3 using a classical chromatin-immunoprecipitation (ChIP) assay (see Material & Methods). PCR amplifications of *CLB2* and *SWI5* promoter regions indicated significant binding of Sin3-HA at these two G2/M promoters in comparison to either control promoters or the coding regions of *CLB2*, *CDC20* and *SWI5* (Figure 3A, see also Figure 4; thesis Manfred Koranda). Interestingly Sin3-HA failed to co-precipitate *CLB2* cluster promoter DNA in cells where *FKH2* was deleted. The fact that Sin3-HA continued to bind the *CLB2* promoter region in *fkh1* cells further strengthened the hypothesis that Fkh2 but not Fkh1 is involved in promoter repression (Figure 3A). We obtained similar results using cells expressing Rpd3-HA (Figure 3B). Consistent with the idea that Sin3 is the scaffolding subunit of the Sin3/Rpd3 complex we found that Rpd3 is not recruited in *sin3Δ* cells (Figure 3B), whereas Sin3 binding to G2/M promoters is independent from its catalytic subunit Rpd3 (Figure 3C). Not only did these findings implicate Sin3 complexes as effectors of Fkh2 function, they further emphasized the proposed functional differences between the two yeast forkhead proteins.

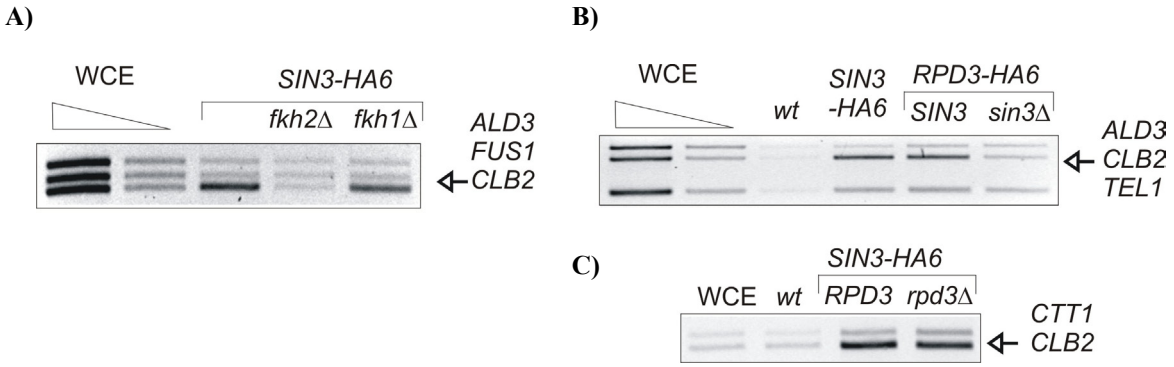


Figure 3 (A) Sin3-HA recruitment to the *CLB2* promoter requires Fkh2 and not Fkh1. Chromatin-immunoprecipitation (ChIP), directed against the HA-tag, was performed in *SIN3-HA* (MK257) and in tagged strains deleted either for *FKH2* (MK267) or *FKH1* (JV509). Co-precipitated DNA regions were visualized by multiplex PCR using the indicated primer pairs for the DNA regions of interest. *CLB2* cluster gene promoter regions are marked with an arrow. Unmarked DNA regions (e.g. *ALD3*, *FUS1)* serve as internal controls. Lane 1: whole cell extract (WCE) dilutions demonstrate equal amplification rates for all primer pairs comprised in the primer mix (Multiplex PCR). **(B) Rpd3 recruitment to G2/M promoters requires Sin3 presence.** Chromatin-immunoprecipitation, directed against the HA-tag, was performed in *SIN3-HA*, *RPD3-HA* and in *RPD3-HA sin3* (JV305, MK581, JV349) strains. Cultures were arrested in G1 by α-factor. **(C) Sin3 recruitment to G2/M promoters is independent on Rpd3.** Chromatin-immunoprecipitation, directed against the HA-tag, was performed in a *SIN3-HA* and in a *Sin3-HA rpd3* (JV305, JV350) strain. Logarithmic growing cultures are presented.

3.3.1 A region close to the FHA region of Fkh2 recruits Sin3 to the promoter.

To test whether the presence of Fkh2 at a promoter is sufficient for increased Sin3 recruitment we grafted the Gal4 DNA binding domain onto the amino-terminus of Fkh2. Expression of this hybrid protein led to a significant increase of Sin3-HA binding at the *GAL 1-10* promoter (Figure 4; 1), an observation that provided further evidence for the intimate connection between Sin3/Rpd3 and Fkh2. Since Fkh2 seems to function as a common platform for both the transcriptional activator Ndd1 and the Sin3 complex, we wondered whether Ndd1 and Sin3 would compete for similar binding sites. In order to map the Fkh2 regions involved in Sin3 recruitment, we generated several truncated and also mutant versions of Fkh2 fused to the Gal4 DNA binding domain ($Gal4_{DBD}$), and tested them for their ability to mediate Sin3-HA recruitment to the *GAL1-10* promoter. Sequences amino-terminal to the DNA binding domain of Fkh2 were able to recruit Sin3 to the *GAL1-10* locus (Figure 4A) suggesting that a region overlapping with the FHA domain was mediating this interaction. We also tested whether point mutations within the phospho-threonine recognition motif of Fkh2 that are known to prevent interaction between Ndd1 and Fkh2 (Reynolds et al., 2003), could also abolish recruitment of Sin3. An Fkh2 mutant containing alanine substitutions at positions R87 and H123 that completely abolishes Ndd1 recruitment (thesis Manfred Koranda) still allowed Sin3-HA recruitment to the *GAL1-10* promoter (Figure 4A). Any deletion located N-terminally to the FHA domain, abolished interaction with Sin3 (Figure 4; 2,4,5). In summary, structures competent for recruiting Sin3 seem to be located in a region covering the first 194 amino acids (aa) of Fkh2. Although this region includes the entire FHA domain, its function as a phospho-amino acid acceptor is dispensable. Finally, we cannot discount the possibility that more than one site in Fkh2 contributes to Sin3 recruitment as Fkh2 mutants missing the C-terminal domain seem to recruit Sin3 less efficiently to chromatin. We thus propose that Sin3 recruitment to the N-terminal region of Fkh2 is mechanistically distinct from the Fkh2 - Ndd1 interaction, which might even allow the simultaneous binding of Sin3 and Ndd1.

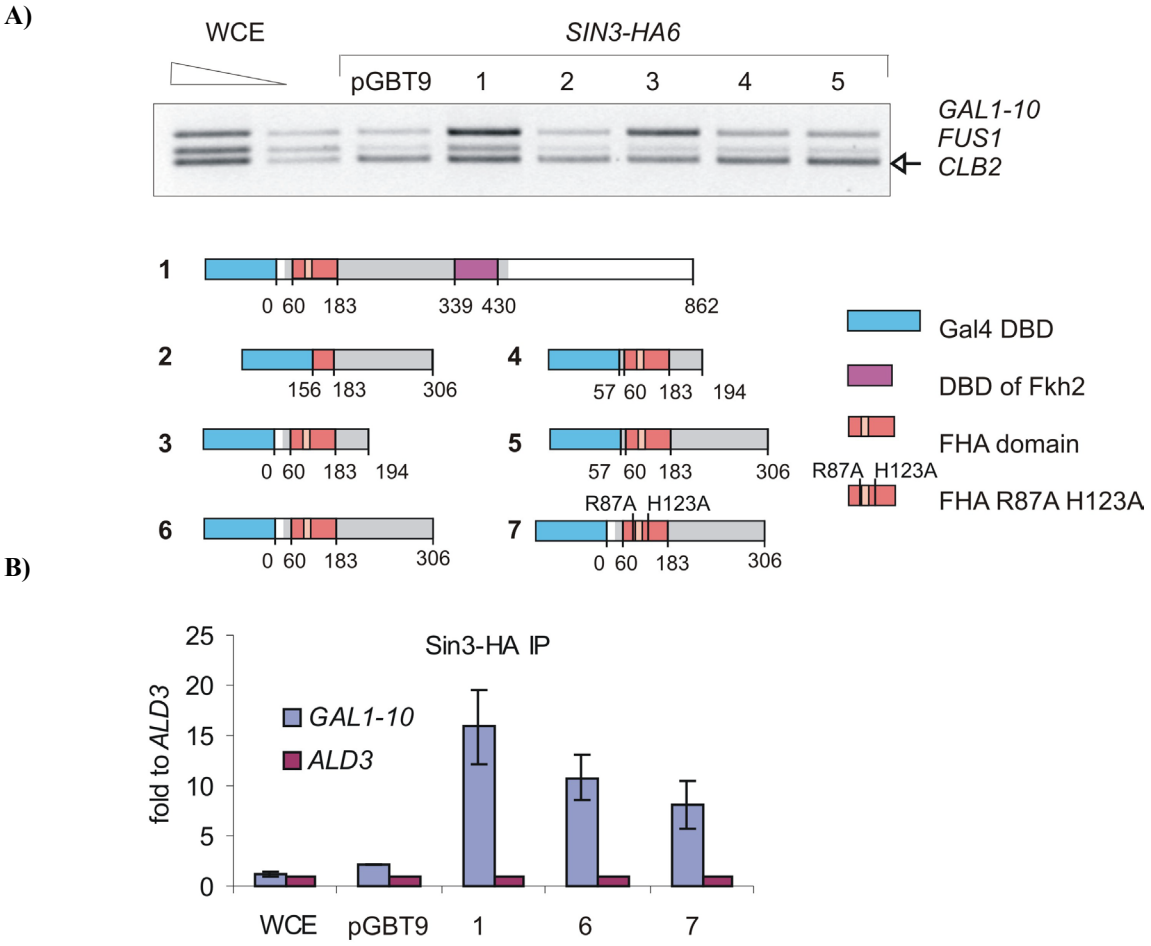


Figure 4 (A) Sin3 is recruited by the N-terminal region of Fkh2, including the FHA domain. Chromatin-immunoprecipitation, directed against the HA-tag, was performed in a *Sin3-HA* (JV413) strain, which was transformed with a series of vectors expressing different C and N terminally truncated alleles of Fkh2 fused to the binding domain of GAL4 (pJV251, pJV301, pJV403, pJV405, pJV408). **(B) The FHA site of Fkh2 interacts with Sin3 even in the absence of its phospho-interacting residues.** A *SIN3-HA* (JV305) strain was transformed with a series of vectors (pJV251, pJV287, pJV292) expressing truncated $Gal4_{DBD}$-Fkh2 fusion proteins as illustrated. Chromatin IP with α-HA antibodies was made in exponentially growing cultures and the co-precipitated DNA was amplified via multiple PCR as described in (B). Relative values for the *GAL1-10* DNA (*GAL1-10* fold to *ALD3*) were calculated using the ImageQuant® software as described in the Material and Methods section. Presented values are the mean of 3 independent experiments.

3.3.2 Sin3 and Rpd3 occupation rates oscillate during the cell cycle

To determine whether Sin3-HA binding at G2/M promoters is removed in a cell cycle dependent way, we looked at cells in different phases of the cell cycle. In cells arrested in G1 by pheromone exposure, the Sin3-HA protein efficiently co-precipitated with the *CLB2* promoter region. This result correlated well with the fact that the promoter is transcriptionally inactive at the G1 stage of the cell cycle (Figure 2B). When released from the pheromone block, Sin3-HA interaction with the *CLB2* promoter clearly diminished after twenty minutes, a time point when bud emergence and DNA synthesis become noticeable and the *CLB2* locus is activated (Figure 5). Interestingly, after the completion of S-phase, promoter occupancy by Sin3 was re-established. The timing of the Sin3 re-recruitment was validated by assaying cells treated with the microtubuli de-polymerizing agent nocodazole, which arrests cells in G2. This experiment showed that Sin3 is indeed associated with the promoters during the G2/M phase and that the initial observation is not an artefact emerging due to asynchrony of cell culture (compare Figure 2A and 2D). To confirm the cell cycle dependent binding of Sin3 to *CLB2* cluster genes, we also arrested cells in metaphase as we depleted cells for Cdc20, which is the essential cyclin specific subunit of the SCF complex required at the exit of mitosis (see introduction). At this time point of the cell cycle, the Sin3 signal at the *CLB2* promoter is already substantial. We released the cells from metaphase arrest by using a galactose inducible *GAL1-CDC20* construct. A time course showed that the Sin3-HA derived signal became only slightly stronger after release, to remain almost constant until it peaked during G1-phase. Sin3 occupation levels once again decreased as soon as the cells have reached S-phase (Figure 2C, upper panel, last lane). We also monitored cell cycle specific chromatin recruitment of a tagged version of Rpd3. These ChIP assays yielded a virtually identical pattern to that observed for Sin3. At the *CLB2* promoter, Rpd3 recruitment also peaked during early G1, followed by a rapid decline shortly before the onset of DNA synthesis. It also reappeared at the promoter during G2/M (Figure 2C lower panel). S-phase therefore seems to be the only cell cycle phase when the Sin3/Rpd3 complex is completely absent and perhaps actively removed from the promoter.

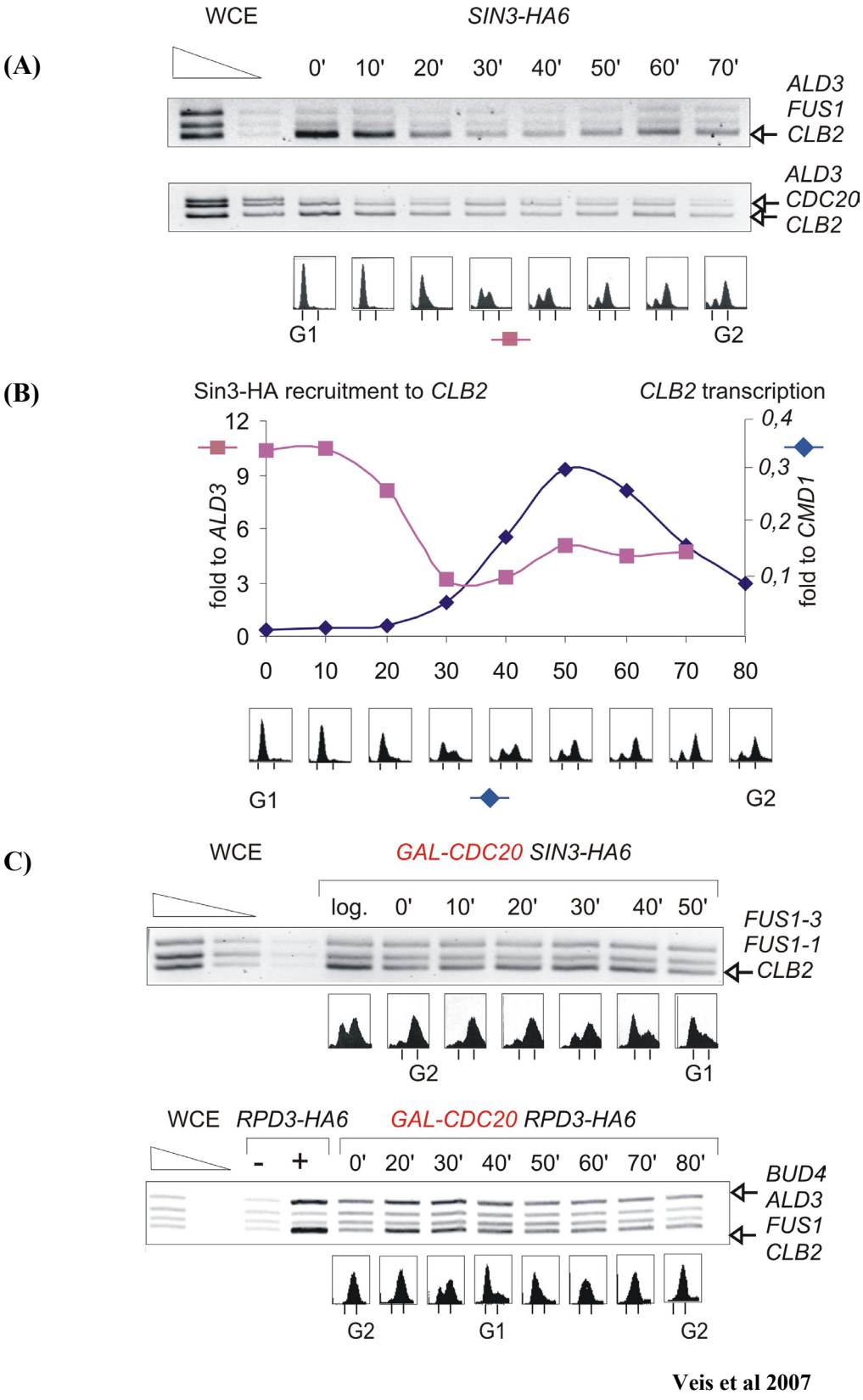
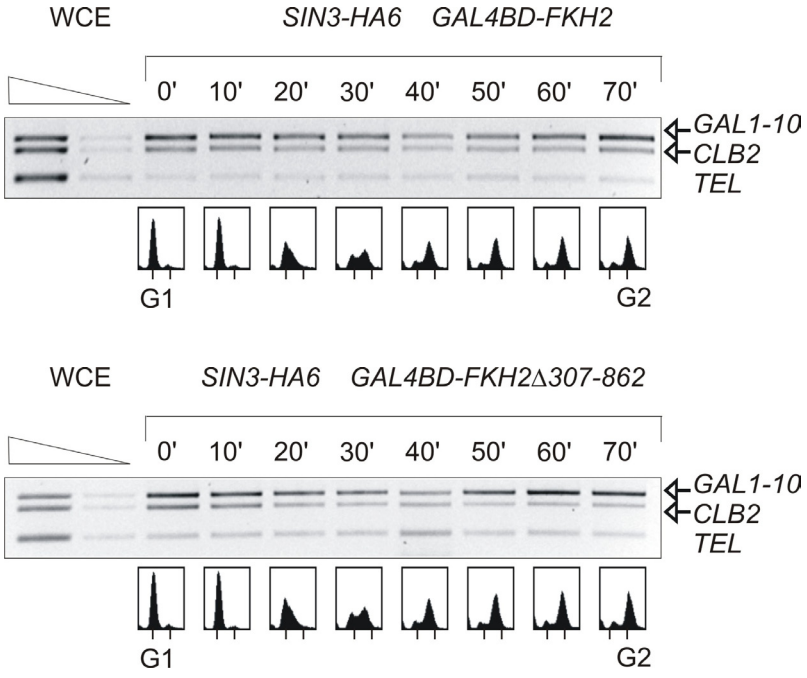


Figure 5. The binding of Sin3 to G2/M promoter oscillates during the cell cycle. (A) Time course of *SIN3-HA6* cells (JV305) released from a G1 arrest induced by α-factor. To arrest cells in G2 nocodazole was added after 35 minutes post release. HA-ChIP was performed at the indicated time points. **(B)** *CLB2* mRNA levels in wt (JV1) cells released from α-factor block. The transcriptional levels of *CLB2* are compared with quantified Sin3-HA ChIP data derived from Figure 2A. **(C)** Time course of *GAL-CDC20 SIN3-HA6* (MK196), respectively *GAL-CDC20 RPD3-HA6* (MK195) cells released from a Cdc20 depleted G2 arrest. HA-ChIPs were performed at the indicated time points. Positive and negative controls for Rpd3-HA recruitment were performed in logarithmic growing wt control strains (JV394, w303)

3.3.3 The N-terminal domain of Fkh2 recruits Sin3 to an artificial promoter in a cell cycle specific manner

As already mentioned and discussed above, Fkh2 is able to transfer the Sin3/Rpd3 complex to an artificial promoter region when fused to a foreign DNA binding domain such as the $Gal4_{DBD}$. To determine whether such a Sin3-Fkh2 interaction at the *GAL1-10* promoter is still cell cycle regulated, we looked in cells which were arrested in G1 with α-factor and subsequently released, while expressing the $Gal4_{DBD}$-Fkh2 fusion construct. ChIP data show that both events, the removal of Sin3 from the *GAL1-10* promoter in S-phase as well as its re-appearance in G2 phase, follow a regulatory pattern similar to the regulated binding of Sin3 to its native target promoters such as is the *CLB2* promoter (Figure 6). This pattern of cell cycle regulated Sin3 recruitment to the *GAL1-10* promoter could also be induced when a minimal version of Fkh2, consisting solely of the first 306 AA was fused to the $Gal4_{DBD}$.



Veis et al 2007

Figure 6. (A) Fkh2 presence at the *GAL1-10* promoter is sufficient to mediate cell cycle regulated recruitment of Sin3. ChIP analysis of a *SIN3-HA* strains (JV305) either expressing the full length Fkh2 fused to the DNA binding domain of Gal4 (pJV251) or its C-terminally truncated version (pJV287). Time course of cells released from a G1 arrest induced by α-factor. The synchrony of cultures and the appropriate arrests (G1 respectively G2) are followed by FACS analysis. Note: Strains used in Figure 2A, B, D, are *bar1* strains which exhibit a slight delay after release from α-factor block when compared to *BAR1* strains.

3.3.4 The C-terminal domain of Fkh2 is required for Sin3 binding to the CLB2 promoter.

According to our results obtained with the $GAL4_{DBD}$-Fkh2 fusion constructs, cell cycle regulated Sin3- Fkh2 interaction solely requires the N-terminal stretch of Fkh2 including its FHA domain. However, such conclusions are never fully conclusive as long as they were obtained purely in an artificial promoter context. Some other yet unknown modifiers, (positives and negatives) might be excluded from the artificial DNA region, thereby altering the experimental outcome significantly. Therefore we also assayed the effects of C-terminal truncations of Fkh2 on Sin3/Rpd3 binding to G2/M specific promoters. Surprisingly, in cells which expressed a C-terminally truncated version of Fkh2 (Fkh2ΔC), neither Sin3-HA nor Rpd3-HA could efficiently immuno-precipitate the *CLB2* promoter region (Figure 7C). One simple explanation was that the truncated version of Fkh2 does not bind DNA as efficiently as the full length version and is therefore replaced by its partial analogue Fkh1. Fkh1 is able to mediate activation via Ndd1 recruitment but fails to recruit Sin3. To preclude that the absence of Sin3 is a consequence of such a competition between Fkh2ΔC and Fkh1, the experiment was repeated in the absence of Fkh1, however, without any change in the result (Figure 7C). (Note: It can be virtually excluded that Fkh2ΔC does not bind DNA in *fkh1* cells, as cells depleted for both forkhead proteins exhibit strongly pseudohyphal growth and cannot be arrested in G1 with α-factor). Hence, although the C-terminus is not necessary for the interaction between Sin3 and Fkh2 at a heterologous promoter, it could play a crucial role in the loading of the repressor complex within certain promoter contexts.

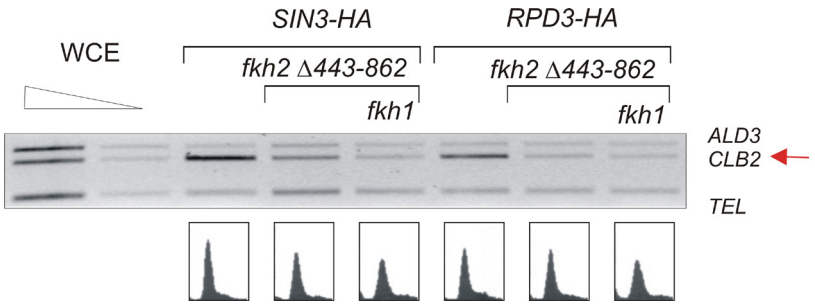


Figure 7. Sin3-HA and Rpd3-HA signals are reduced in G1 in *fkh2Δ443-862* cells and in *fkh2Δ443-862, fkh1* cells. HA-ChIP experiments were carried out in strains expressing either tagged Sin3 (MK257, JV410, JV414) or tagged Rpd3 (JV394, JV413, JV415). Cultures were arrested in G1 with α-factor, cell cycle stage was determined by FACS analysis. PCR was set up as described in previous figures.

3.4 The decrease of Sin3 recruitment correlates with an increase of histone H4 acetylation at the *CLB2* locus

Due to the above-described interactions between Sin3 and Fkh2, we expected to observe a difference in histone acetylation between wild-type cells and *sin3* mutants. Indeed, *SIN3* deleted, G1 arrested cells exhibited a significant increase in histone H4 acetylation at the *CLB2* promoter (Figure 8B). Interestingly, ChIP data derived from exponentially growing wild type cells did not show any H4 acetylation signals (Figure 8B). A possible explanation for this observation was that the assumed acetylation event is transient. Consequently, the fraction of cells exhibiting acetylated promoter regions might be too small to be detected in a sample of cells comprising all stages of the cell cycle. In order to test this hypothesis, we monitored the acetylation status of histone H4 during the transition through S phase, as Sin3 is absent at this stage of the cell cycle. Cells were released from pheromone arrest and samples for anti acetyl-H4 based ChIP analysis were taken. As before, no or very little H4 acetylation was detectable on the *CLB2* promoter in pheromone-arrested cells. However, at the time of Sin3 dissociation, a strong but transient acetylation signal appeared in the promoter region of the *CLB2* locus (Figure 8B). The transient appearance of the acetylation signal was confined to the *CLB2* promoter region, a region encompassing the multiple Mcm1 & Fkh2 binding sites of *CLB2* (depicted as *CLB2* in Figure 8B). PCR amplification of three other *CLB2* regions (Figure 8C) covering the *CLB2* untranslated region and the *CLB2* ORF, resulted in a constitutive signal with little variation from G1-phase to the beginning of S-phase (Figure 8C, time points 0'-20'). However, concomitant with the appearance of Ndd1 at the promoter (Figure 8E, time point 30'), histone H4 acetylation signals were lost from the whole *CLB2* locus (Figure 3D time points 30'-50').

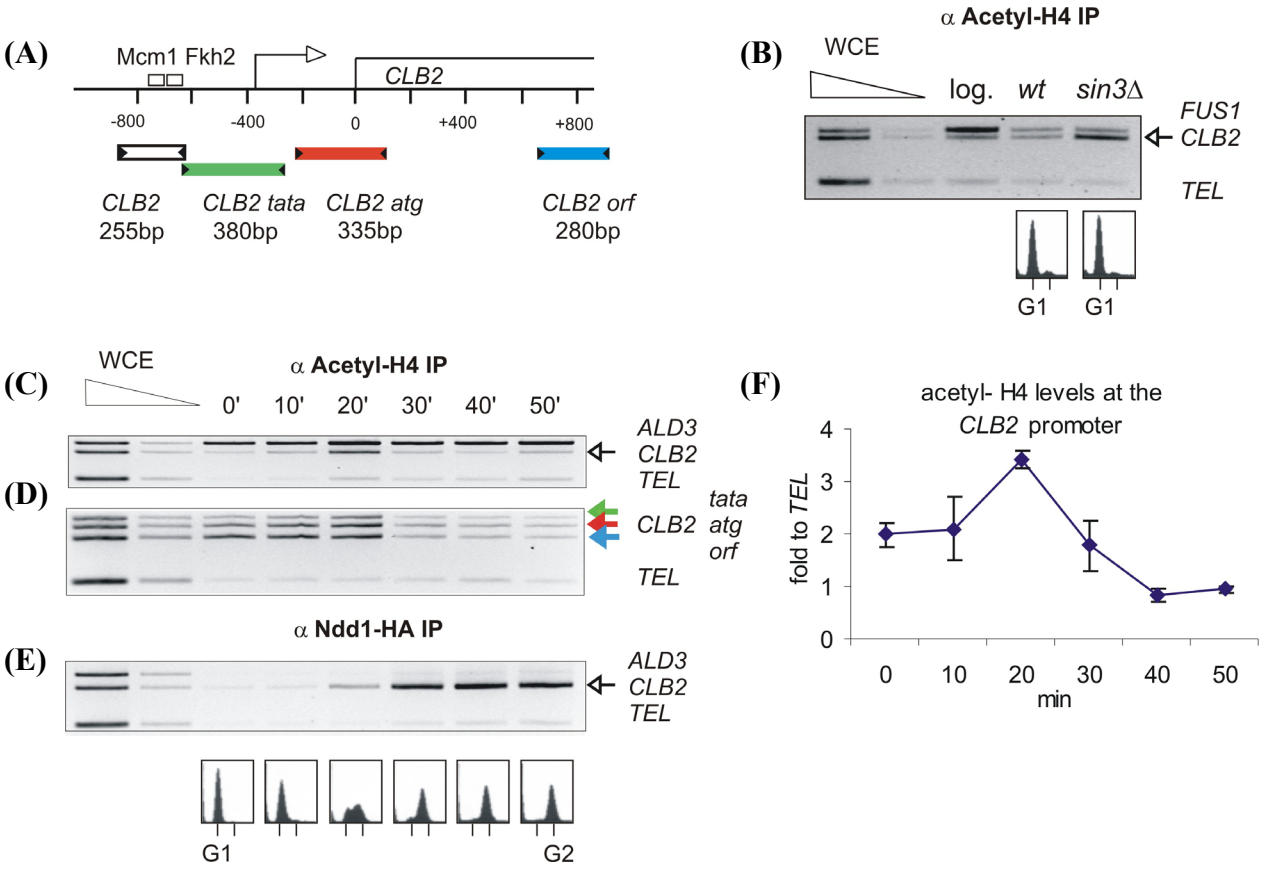


Figure 8. (A) Figure 3. Sin3 relief from the *CLB2* promoter leads to local H4-acetylation. (A) Scheme of the *CLB2* locus, indicating binding regions of Mcm1 and Fkh2 and 4 different DNA fragments amplified by the indicated primer pairs. **(B)** H4 histones at the *CLB2* promoter (*CLB2*) are acetylated in *sin3* cells. Chromatin IP was performed with antibodies directed against acetylated histone H4 in G1-arrested wt (JV1) and *sin3* cells (JV361). **(C)** At the *CLB2* region H4 histones are transiently acetylated in S phase. A wt strain was released from pheromone-induced G1 arrest and subjected to chromatin IP using α-acetyl-histone H4. **(D)** Chromatin regions downstream of the *CLB2*-promoter (*CLB2tata, CLB2atg* and *CLB2orf*) lose the Histone H4 acetylation signature after S-phase completion which correlates with Ndd1 recruitment. A *NDD1-HA* (MK155) strain was released from pheromone-induced G1 arrest and subjected to chromatin IP using α-acetyl-histone H4. **(E)** Ndd1-HA recruitment to the *CLB2* promoter region. Cell lysate from Figure 3D was subjected to chromatin IP using α-HA antibodies. Synchrony of cultures and cell cycle arrests were followed by FACS analysis. **(F)** Multiplex PCR data from three independent experiments was quantified with ImageQuant® software as described in Figure 1C.

3.4.1 Ndd1 function is not triggering Sin3/Rpd3 HDAC release from the CLB2 promoter.

Is Ndd1 involved in overriding Sin3/Rpd3 mediated promoter repression? In order to answer this, we first inquired whether cells depleted for Ndd1 could still remove the Sin3 complex from the *CLB2* promoter. For this purpose we analyzed the promoter occupancy of either tagged Sin3 or tagged Rpd3 in cells that expressed Ndd1 from a galactose inducible promoter. The cells were pre-grown in galactose containing medium, arrested in G1 by pheromone treatment and subsequently released into glucose containing medium. After progression through S-phase they arrested in G2 due to the lack of Ndd1. At this stage they displayed the typical elongated bud morphology of cells with low Clb-kinase activity (Figure 9). Still, Sin3/Rpd3 removal from the *CLB2* promoter occurred indistinguishably from wild type cells (Figure 9). Based on the assumption that Ndd1 was effectively depleted in these cells we suggest that Sin3 removal does not depend on Ndd1 activity. This finding is strongly supported by another observation, that in cells which were treated with hydroxyurea, a drug which inhibits ribonucleic reductase and thereby arrests cell in early S-phase, Sin3 is removed from G2/M promoter regions efficiently although no Ndd1 binding can be observed (for further information see chapter 4).

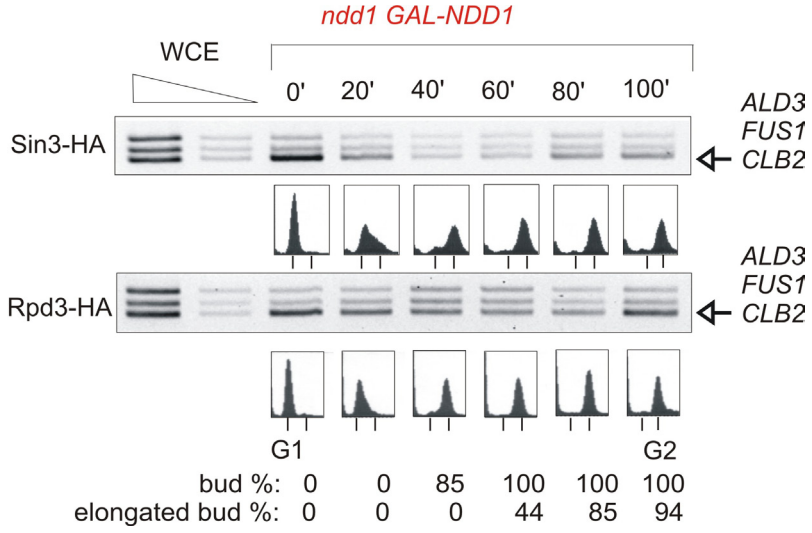


Figure 9. The Sin3/Rpd3 complex is released from the *CLB2* promoter in cells depleted for Ndd1 activity. ChIP-time course was performed with *SIN3-HA* (JV367) and *RPD3-HA* (JV396) strains, both deleted for *NDD1* and expressing Ndd1 under the control of a *GAL1-10* promoter. Cells were grown under galactose conditions, arrested in G1 with α-factor and released into glucose-containing medium. Samples for chromatin IP using α–HA antibodies were collected at the indicated time points. The synchrony of the culture is followed by FACS analysis. Impaired Ndd1 activity is reflected by elongated bud morphology and cell cycle arrest in G2.

3.4.2 Ndd1 is not required for the H4 acetylation event in early S-phase.

As mentioned above, Ndd1 binding is not involved in relieve of Sin3 from the *CLB2* promoter, however its presence at the promoter might still be a prerequisite for the already mentioned transient histone H4 acetylation event. We wanted to examine whether the histones at the *CLB2* promoter becomes acetylated when transcriptional activation does not take place because of the absence of Ndd1. In order to address this questions we used the strain described above, that expresses Ndd1 under a galactose inducible promoter. As shown in Figure 10, when cells were released from G1 arrest, histone H4 acetylation increases, irrespective of the presence of Ndd1. However, after completion of S-phase, Ndd1 depleted cells exhibited significantly higher levels of acetylated histone H4 when compared to cells expressing Ndd1 (Figure 10). Furthermore, in Ndd1 depleted cells acetylated H4 histones can still be detected throughout the genuine promoter region and the ORF region of the *CLB2* locus. There are two possible explanations for this observation. First, the strong reduction of acetylated H4 histone levels at the *CLB2* locus (which is accompanying its transcriptional activation) is due to a genuine Histone deacetylation activity. Second, the process of transcriptional activation itself triggers the eviction of nucleosomes from promoter and/or transcribed regions, thereby virtually leading to a lower abundance of acetylated histones. Thus, it can be speculated that Ndd1 is a prerequisite either for H4 histone remodeling or for increased H4 histone de-acetylation.

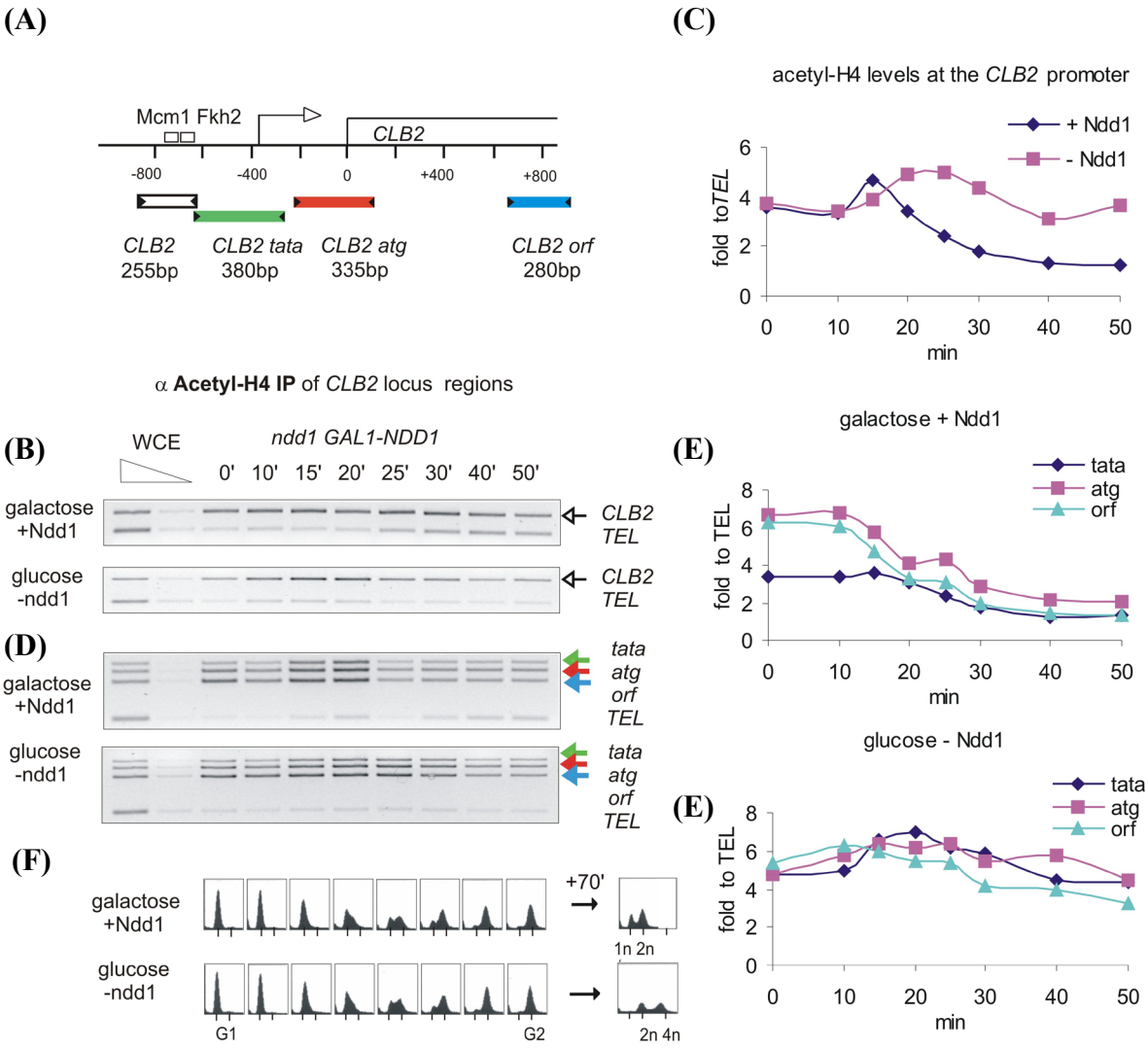


Figure 10. (A) Ndd1 is not required for the H4 acetylation event in early S-phase. (A) Scheme of the *CLB2* locus, indicating binding regions of Mcm1 and Fkh2 and 4 different DNA fragments amplified by the indicated primer pairs. **(B) The decrease but not the increase of acetyl-histone H4 signal at the *CLB2* promoter region in G2 requires Ndd1 presence.** An *ndd1, GAL1-10 NDD1* (JV323) strain was arrested with α-factor in G1 and released in either galactose (+Ndd1) or glucose (-Ndd1) containing medium. DNA was amplified by multiple PCR as described in Fig. 1. **(C)** Virtual values from Figure 11B were quantified using ImageQuant®. Values for the *CLB2* promoter region were calculated according to *TEL* signals as internal standard. **(D) The disappearance of the acetyl-H4 signal from the entire *CLB2* locus in G2 requires Ndd1 presence.** An *ndd1, GAL1-10 NDD1* (JV323) strain was arrested with α-factor in G1 and released in either galactose (+Ndd1) or glucose (-Ndd1) containing medium. **(E)** Acetyl-histone H4 ChIP signals were analyzed visually with ImageQuant® software. Values for the *CLB2atg, CLB2tata* and the *CLB2orf* regions were calculated using *TEL* signals as internal standard. **(F)** Cell cycle arrest, synchrony of cell culture and successful Ndd1 depletion was visualised by FACS analysis. NOTE: Cells depleted for Ndd1 show quatroploidy as they fail to undergo mitosis.

3.5 Ndd1 associated transcriptional activation triggers chromatin remodeling

Based on the findings described above and on previous observations at other promoters in yeast (Reinke and Horz, 2003), we favoured the scenario where the loss of acetylated histone signal at the *CLB2* promoter is a consequence of a nucleosome eviction and not the reflection of deacetylase activity. In order to prove this hypothesis, we performed ChIP experiments with antibodies directed against histone H4 regardless on its modification. These experiments showed that the histone H4 signal at the *CLB2* promoter decreases immediately after the time point where histone acetylation occurs, and that the signals remains low throughout S phase until G2 (Figure 11). Interestingly, other *CLB2* regions (*CLB2 atg*, *orf*) were more efficiently immunoprecipitated by histone H4, when compared to the *CLB2* promoter region at these stages of the cell cycle (Figure 11). Thus, it seems that histone acetylation is followed by the eviction of only these nucleosomes that are adjacent to the *CLB2* promoter region (*CLB*, *CLB2 tata*).

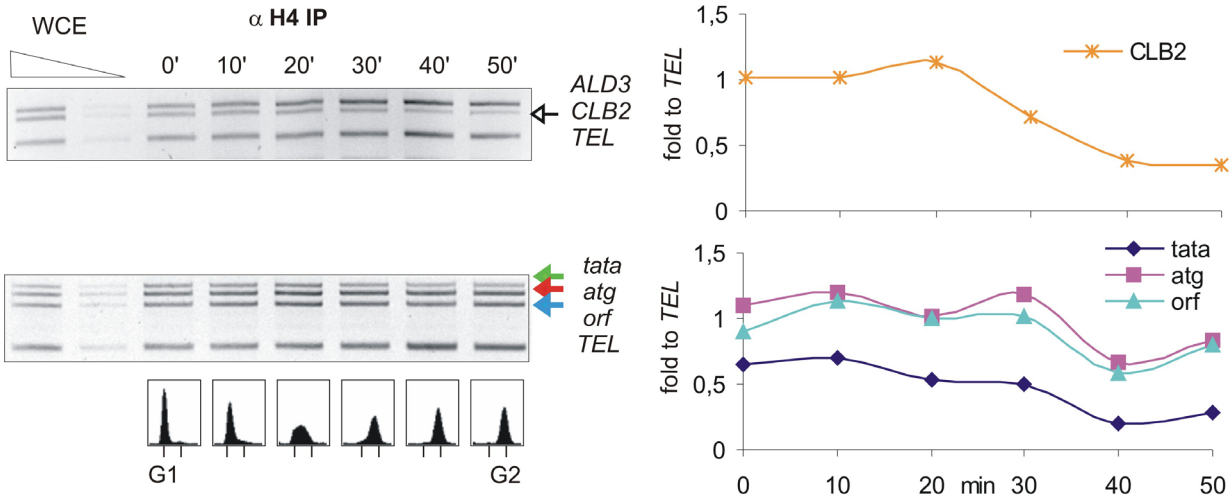


Figure 11. **H4 histones lose contact to the *CLB2* promoter region after S phase completion.** Samples of a pheromone-synchronized wt culture were withdrawn at the indicated time points and examined by chromatin-IP with α-H4 antibodies. The co-precipitated DNA was analyzed with two different PCR setups containing mixtures of primer pairs as indicated in the figure (**A:** *ALD3, CLB2, TEL*; **B:** *CLB2tata, CLB2atg*, *CLB2orf, TEL*). The presented data were normalized with ImageQuant® software using the *TEL* DNA band as internal standard. Note: Whereas the telomeric region (*TEL*) represents a negative control in α-acetyl histone H4 ChIP experiments, it serves as a positive control for histone presence.

3.5.1 Ndd1 recruitment is required for the recruitment of the histone remodeling complex SWI/SNF

Ndd1 neither antagonizes the function of the Sin3/Rpd3 HDAC, nor does it trigger the increase in acetylation of H4-histones, rather it seems to promote chromatin remodeling at *CLB2* promoters. In cells depleted for Ndd1, the increase in histone acetylation at the *CLB2* promoter in early S phase seems not to be affected, but early on we observe a significant difference in acetylated histone H4 abundance dependent on Ndd1 presence (see Figure 10). Our suggestion that transcriptional activation is accompanied by chromatin remodeling of the *CLB2* promoter was confirmed by measuring histone H4 (Figure 11). In order to determine whether Ndd1 is required for nucleosomes eviction from the *CLB2* promoter region, we measured H4-histones occupancy in the presence and absence of Ndd1 in cells progressing throughout the S-phase. As expected, H4-histones levels remained significantly elevated in the absence of Ndd1 (Figure 12B,C), suggesting inefficient remodeling. The question arose whether Ndd1 is involved in the recruitment a chromatin re-modifying complexes or whether it even possesses a genuine chromatin remodeling activity. To address this problem, we tested several Tap-Taq strains and identified Snf2, to be recruited to G2/M promoters in a cell cycle dependent manner (Syam Kumar personal com.). Snf2 is the ATPase core subunit oft the SWI/SNF chromatin remodeling complex (Smith and Peterson, 2005). Interestingly, the pattern of Snf2 recruitment tightly correlates with the presence of Ndd1 at the *CLB2* promoter region. We constructed a conditional Ndd1 strain expressing HA tagged Snf2 and investigated whether Snf2 recruitment requires Ndd1. The results presented in figure 12A clearly show that in the absence of Ndd1, Snf2 cannot be detected at the *CLB2* promoter. However, based on this observation we still cannot conclude that Ndd1 directly targets Snf2 to G2/M promoters, as one should always take into account that Ndd1 is a crucial prerequisite for transcriptional activation. Thus, absent or stalked transcriptional machinery might be the real cause for absent Snf2.

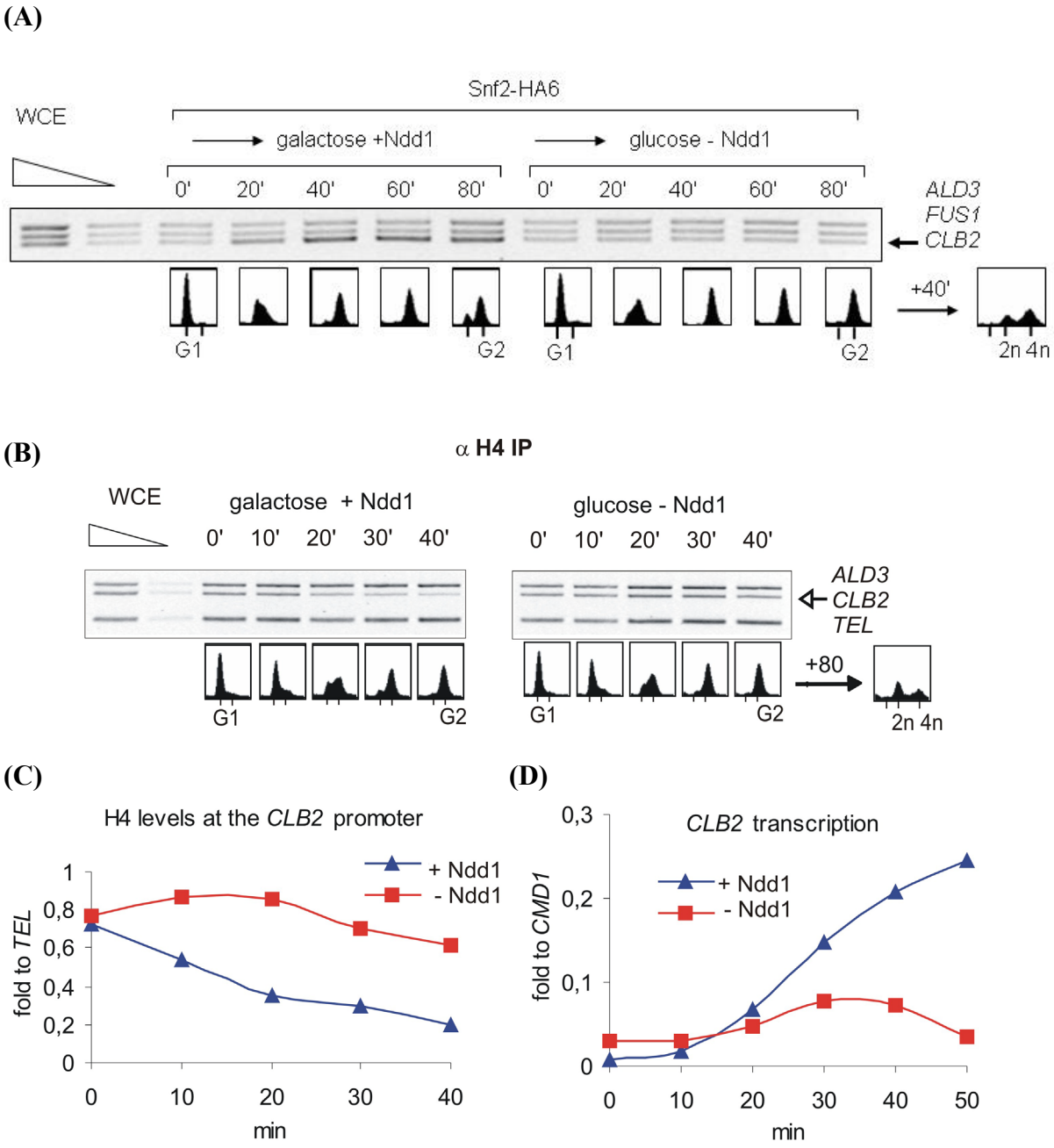


Figure 12. Snf2 recruitment to the CLB2 promoter requires Ndd1. A haploid *SNF2-HA6 ndd1 GAL1-NDD1* strain (JV 584) was constructed and arrested in G1 with α-factor. Subsequently cells were released in glucose and galactose media and aliquots were withdrawn for α-HA ChIP analysis. **(B) Promoter remodeling requires Ndd1.** An *ndd1 GAL1-NDD1* strain (JV 323) was arrested in G1 with α-factor and time course samples were subjected to α–H4-IP. Co-precipitated DNA was amplified with the indicated primer pairs. Signals for the *CLB2* promoter region are emphasized by an arrow. **(C)** Data from Figure 13 B were normalized with ImageQuant® software using the *TEL* DNA band as internal standard. Note: *ALD* and *TEL* signals represent positive controls for histone presence. **(D)** Samples for northern blot analysis of the cultures described in (B) were probed for *CLB2* transcripts and *CMD1* as reference.

3.6 The release of Sin3 is independent of B-type cyclins but requires G1 cyclins

As described in the introduction of this thesis, several cell cycle-specific Cdks become activated in rapid succession during G1/S transition. We wanted to investigate whether one of these cyclin associated kinase activities is required for Sin3 removal from the *CLB2* promoter after START. To assure that Sin3 removal after START is not a simple consequence of cellular growth after α-factor release we first tested a strain devoid of G1 cyclin expression. For this purpose we used a strain deleted for all G1-cyclin genes, but expressing *CLN1* from a galactose inducible promoter. We found that cells lacking Cln activity do not execute the removal of Sin3 from the *CLB2* promoter. These cells continued to gain in cell size but were unable to bud and initiate S phase. However, when *CLN1* expression was induced with galactose, Sin3 dissociated from the *CLB2* promoter at schedule (Figure 13A). To rule out that the persistence of Sin3 binding to the *CLB2* promoter in cells depleted for G1 cyclins is just a simple consequence of a prolonged pheromone arrest we also monitored the decrease in *FUS1* expression, which is dependent on pheromone signalling (Figure 13D). The transcriptional levels of *FUS1* decreased in G1-cyclin depleted cells with kinetics similar to those detected in cells expressing Cln1, indicating that the pheromone block was successfully inactivated.

The previous experiment indicated that Sin3 removal from G2/M promoters is indeed one of the START associated processes, yet it did not resolve the question whether any of the B-type cyclins was necessary for its execution. Therefore, we analyzed Sin3 binding in a *cdc34^{ts}* strain expressing a non-degradable *SIC1* allele under the control of a galactose inducible promoter (see introduction). *Cdc34^{ts}* cells were in G1 and subsequently released into galactose containing media at the restrictive temperature (37°C). Under this condition, cells started to bud but failed to replicate their DNA. However, Sin3 binding to the *CLB2* promoter decreased as cells started to bud (Figure 13B), proving that B-type cyclin dependent kinase activities are not involved in this event. The independency of Sin3 dissociation from B-type cyclin expression was further strengthened by time course experiments with cells conditionally inactivated for the Clb1-4 kinase (*clb1,2,4 Δ, clb2^{ts}*). In this strain, Sin3 was still removed from the *CLB2* promoter region in S phase (Figure 13E). We conclude that Sin3 removal from G2/M promoters is solely triggered by the rise in Cln kinase activity and independent of the activation of any of the Clb kinases.

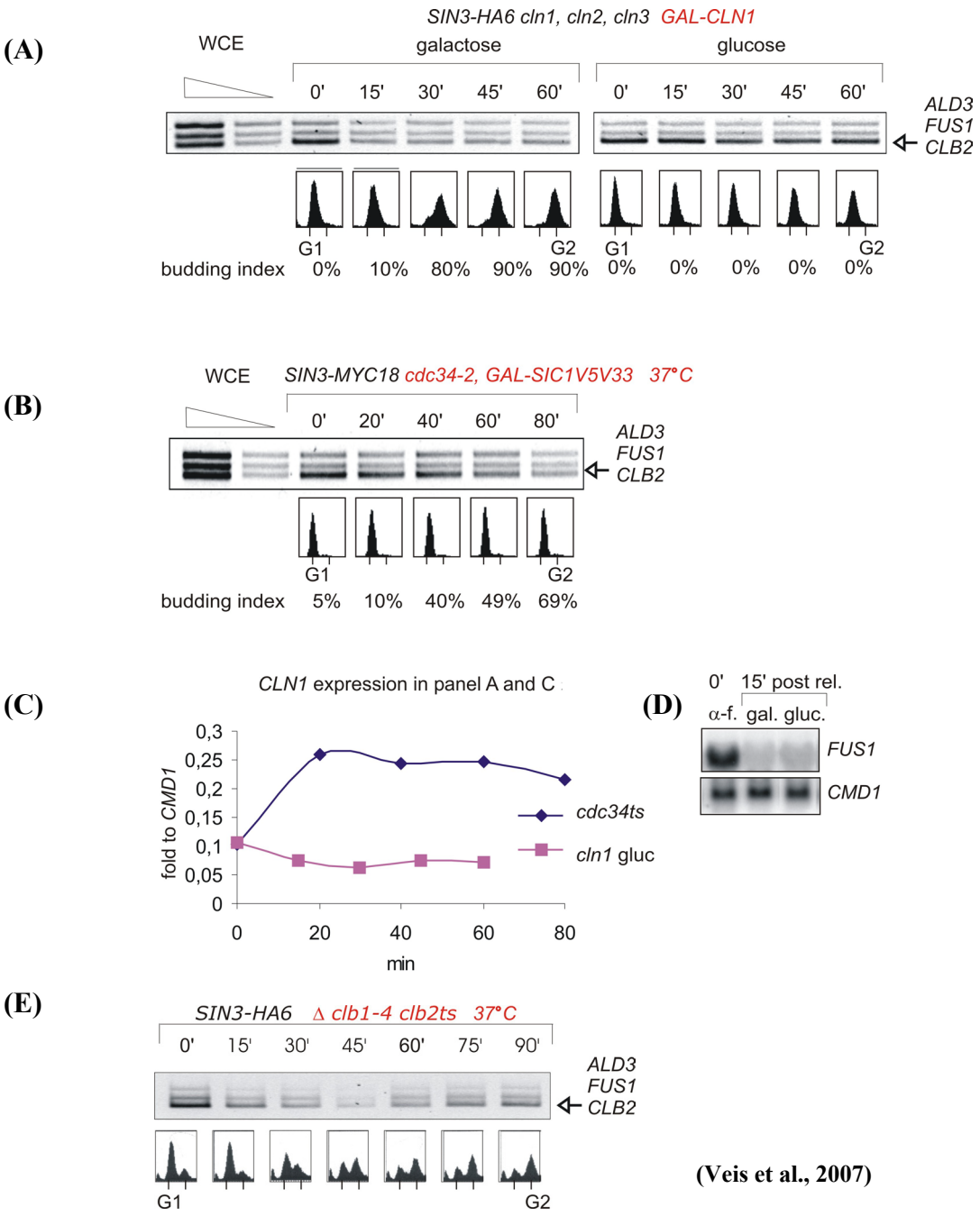


Figure 13. Sin3 removal from the *CLB2* promoter requires *CLN* activity (A) Sin3 dissociation requires cyclin activity. ChIP experiment with α-HA antibodies was performed in a Sin3-HA strain deleted for *CLN1, CLN2* and *CLN3,* expressing Cln1 under the control of the *GAL1-10* promoter (HEL404). Cells were grown in galactose-containing medium, arrested in G1, and released in either absence (right panel) or presence (left panel) of galactose **(B)** High Cln activity enables the dissociation of Sin3 from G2 promoters. ChIP-time course was made in a *cdc34*^{ts} strain carrying the non-degradable *SIC1V5V33A76* allele under the control of the *GAL1* promoter (JV515). Cells were grown under permissive conditions, arrested in G1 and released at 37°C in galactose containing medium. Samples for ChIP analysis using α-Myc antibodies were collected at the indicated time points followed by amplification of the DNA regions of interest with the indicated primer pairs. **(C)** Northern blot analysis of *CLN1* expression levels for two time course experiments showing similar FACS profiles but different budding indices (Figure 6A right panel, and Figure 6C). Cells were harvested simultaneously with cells used for ChIP analysis. **(D)** Cultures from Figure 14A were successfully released from the α-factor related cell cycle block. Northern blot analysis of α-factor dependent *FUS1* expression. **(E)** Sin3 removal from G2/M promoters does not require the activity of mitotic cyclins. Time course of a Sin3-HA strain (JV358) bearing a temperature sensitive allele of *CLB2* and deleted for the other mitotic cyclins (*CLB1, CLB3, CLB4)*. The cells were grown at permissive temperature, arrested in G1 and released into pre-warmed medium at 37°C. Cells were used for ChIP analysis with HA antibodies. DNA associated with Sin3-HA was amplified by PCR.

3.7 Quantitative phospho-protein analysis of Sin3/Rpd3 with mass spectrometry

Our data strongly indicated that there seems to be a direct interaction between the DNA binding factor Fkh2 and the Sin3/Rpd3 complex. Furthermore this interaction seems to be regulated in a cell cycle specific manner by G1 cyclin associated Cdc28 kinase. We wondered whether Sin3/Rpd3 itself might be a target of the kinase. Based on these observations we speculated that the Sin3/Rpd3 complex might be a target of Cdk. At first this hypothesis seems to be quite unlikely, because the Sin3/Rpd3 HDAC is involved in the transcriptional regulation of various gene clusters, of which the G2/M specific regulon represents only a small subset. Consequently, modification of one of the two core components of the complex might deal with the problematic how to restrict the thereby altered overall complex activity to specific promoters. On the other hand, both Rpd3 as well as Sin3 have minimal (S/T)P Cdc28 consensus sites (however, only 32% of 537 putative Cdc28 consensus sites inside the yeast proteome were found to be phosphorylated by the Cdc28 kinase (Ubersax et al., 2003)). Furthermore, Sin3 has been shown to co-immunoprecipitate with at least one of the G1 cyclins, but with none of the B-type cyclins (Ho et al., 2002). Third, we have shown that Sin3 interacts with the N-terminal domain of Fkh2. This region of Fkh2 includes the FHA domain, which as mentioned above is a phospho-recognition domain that preferentially binds phosphorylated proteins. Even more interesting is the fact that the interaction of Fkh2 and Sin3 remains cell cycle regulated even in the context of an artificial promoter system. In addition, the interaction between Fkh2 and Sin3 can be observed in *rpd3* strains, indicating that catalytically inactive HDAC is also recruited by Fkh2 to G2/M promoters. We therefore decided to purify Rpd3 and Sin3 via novel tandem tag purification method in order to map phospho-sites by mass spectrometry (MS). In a second step, we used quantitative mass spectrometry to follow the dynamics of phosphorylated Rpd3 throughout the cell cycle.

3.7.1 HIS-Biotin tandem tag purification

We considered two parameters to be precondition for a protein sample to be applicable for phosphor-site mapping as well as quantitative MS. First, the protein sample has to maintain its modifications throughout the purification process. Second, especially for quantitative MS a high protein yield is of great benefit. Particularly when experimental costs became a relevant factor and experimental bias should be avoided. Therefore, we chose denaturing conditions (8M urea or 6M GdmHCl) for our protein purifications to diminish the activity of cellular proteases and (more importantly) phosphatases. In addition we adapted a new tandem purification method, which is based on a so called HTB tag (Tagwerker et al 2006), for our purposes. This tandem purification tag is accessible to two purification methods, which are the poly-histidin/bivalent ion interaction and the biotin-avidin/streptavidin interaction, both of them working under fully denaturing conditions. We improved three aspects of the tagging plasmid (pCT468). First, the sequence including the HTB tagging cassette and the selection marker was extended by additional primer annealing sequences, in order to make the plasmid suitable for tagging primers used previously in our laboratory (Knop et al., 1999). Second, we introduced two additional TEV cleavage sites. Third, we extended the 6xHis tag to 12xHistidin tag, to increase binding efficiency of tagged proteins to the bivalent ion resin (Ni-agarose). We also optimised the original protein purification protocol, by introducing a buffer switch from GdmHCl to Urea during the first purification step (Wolfgang Reiter pers. com., Material & Methods). As depicted in Figure 14 we were able to purify up to 50 % of a tagged protein present in the starting lysate (A1) which made this method optimal for combination with quantitative MS (see next chapter).

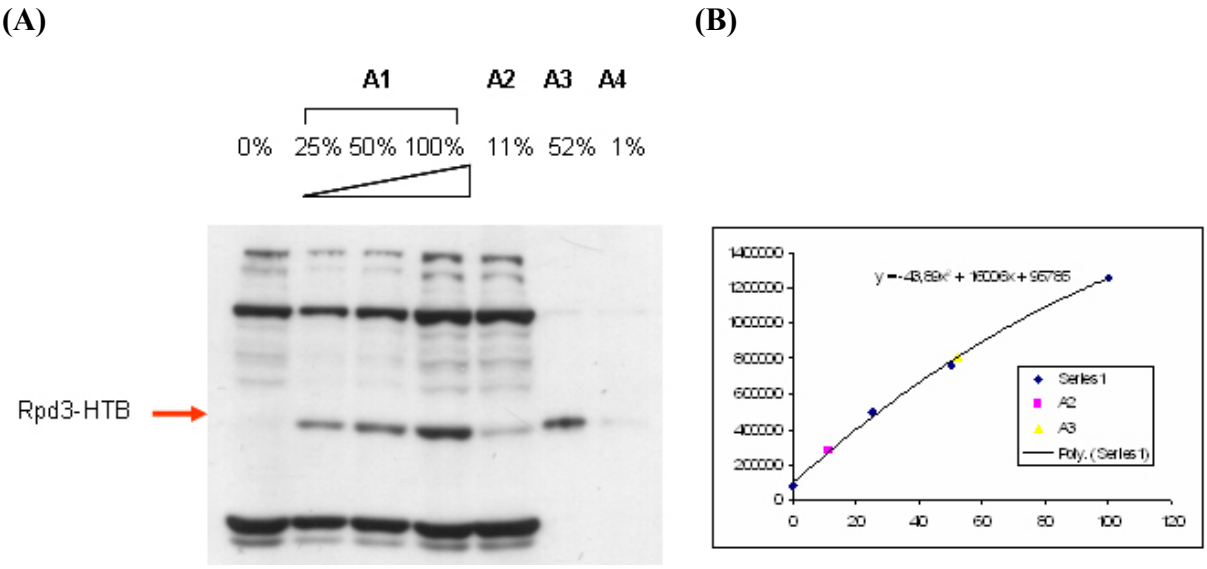


Figure 14. Calculating the yield of a HIS Biotin tandem Tag purification of Rpd3. (A) Western analysis of the purification of Rpd3-His-Biotin. Blot was incubated with horse radish peroxidas conjugated Streptavidin. Volumes of all protein samples were adjusted prior to loading to represent equal fractions of theoretical total protein. **A1:** lysate prior to first incubation. Serial dilutions were made in order to enable western quantification (A1=0 is represented by an untagged strain). **A2:** lysate after incubation with Ni2+ -Sepharose (=supernatant). **A3**: elution of the Ni2+ Sepharose beads. **A4**: supernatant after incubation with Streptavidin-Agarose beads. **(B)** Quantification with Image Quant of the dilution series of A1 was used to determine Rpd3-HTB abundance in A2, A3 and A4 relative to total amount.

3.7.2 SILAC analysis

In recent years mass spectrometry has became the favoured method in quantitative proteomics. Ongoing development has pushed this method from simple identification of sites, proteins and complexes to a powerful tool for quantitative measurement of protein levels and the extent of their modifications. The basis for this development was the introduction of different labelling techniques that allow internal standardization. One of the currently most popular approaches for quantitative MS is the so called "Stable Isotope Labelled Amino acids in cell Culture" method (SILAC). (Gruhler et al., 2005). The method relies on cells which are auxotroph for lysine and arginine. The cells grow in two different cultures supplemented with arginine and lysine which exclusively contain only one of the stable isotopes ^{12}C (light) or ^{13}C (heavy). Thus, after tryptic digest, otherwise identical peptides can be now distinguished in the mass spectrometer as they differ 6Da in mass. The ratio of their peak integrals correspond to the ratio of the two peptides in the sample. The big advantage of SILAC when compared to other

labelling methods is that all steps subsequent from the harvest of the cells until analysis in the spectrometer is identical for both isotopic peptides, thereby reducing the experimental bias significantly (Figure 15). Based on (Gruhler et al., 2005) we established a protocol that allows incorporation of the labelled lysine/arginine in our probes to more than 95%. To have comparable data with our previous results, which were all derived in the w303 genetic background we constructed an *argΔ lysΔ* W303 strain (JV579) (Wolfgang Reiter pers. com.; Material & Methods).

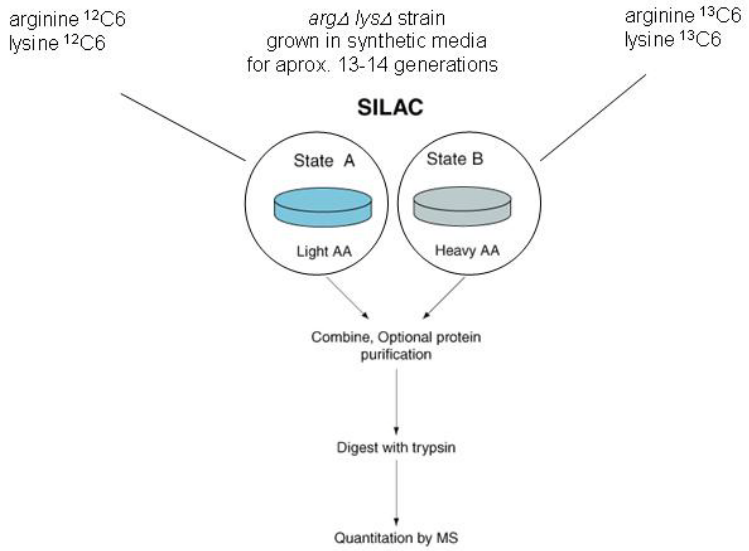


Figure 15. SILAC scheme

3.7.3 Threonine 391 of Rpd3 is phosphorylated but not in a cell cycle dependent manner

As mentioned above, we suggested either Rpd3 or Sin3 to be the target of the Cln kinase. Measurements of three independent Rpd3-HTB purifications detected multiple Rpd3 peptides giving an overall coverage of 40% of the total protein sequence (Figure 16). Fortunately, the peptide containing the presumptive Cdc28 kinase site was repeatedly detected. Furthermore, the respective peptide was found to be phosphorylated at threonine 391 (see Figure 16). In order to determine whether the phosphorylation pattern of Thr391 is altered during S-phase, we decided to perform a quantitative measurement of this phosphor-peptide applying SILAC. Both cultures, heavy and light were arrested by α-factor. Whereas heavy cells were harvested

immediately at the end of the arrest, the light culture was released into synthetic media and cells were harvested 40' post release when 99% of them have reached early G2 phase. Cultures were merged and Rpd3-HTB was purified for MS analysis (see Material & Methods). Unfortunately, we could not detect any significant change in the ratio of heavy to light abundances of the phospho-protein, suggesting that this phospho-site of Rpd3 remains unchanged during S-phase transition (Figure 16).

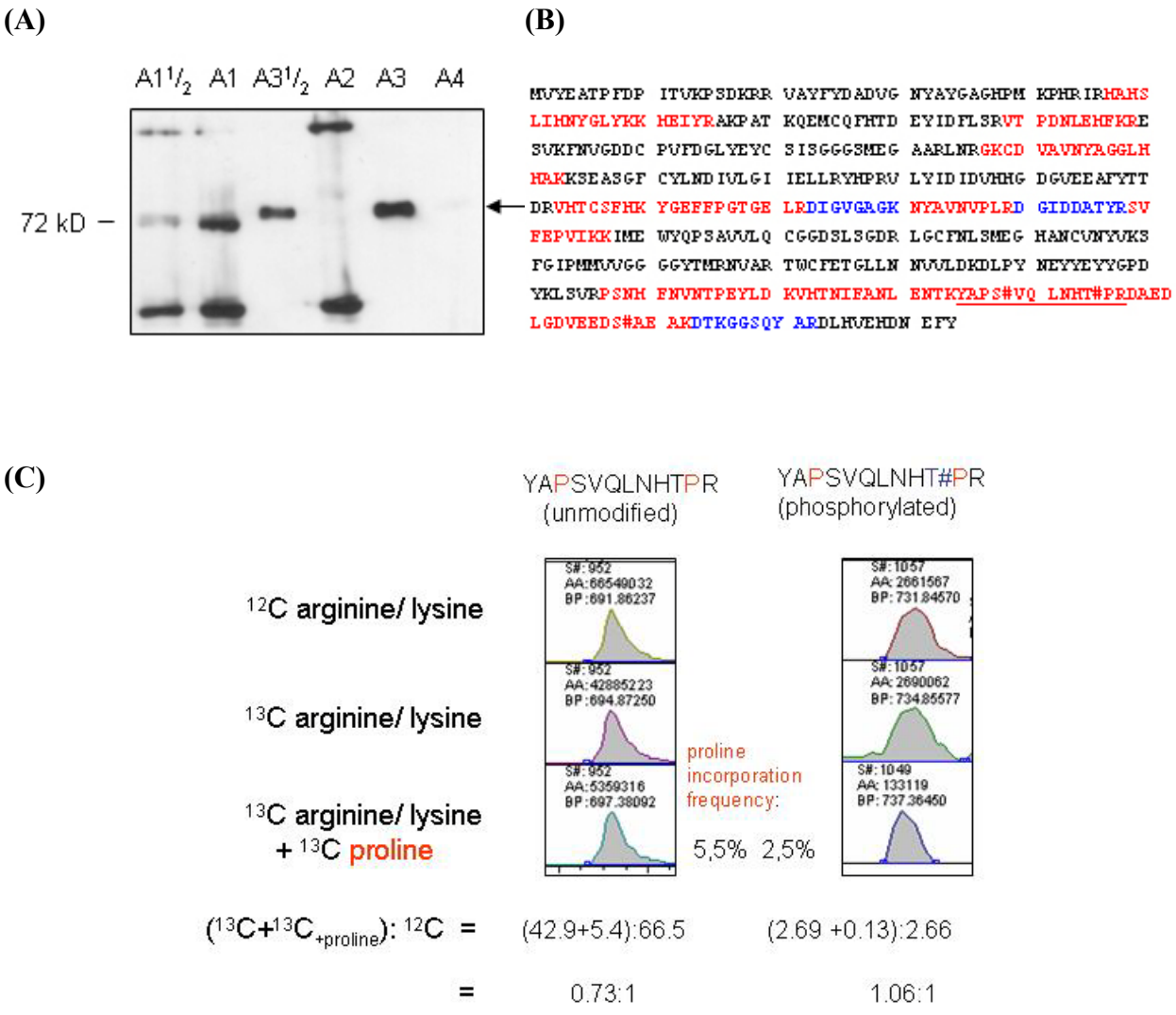
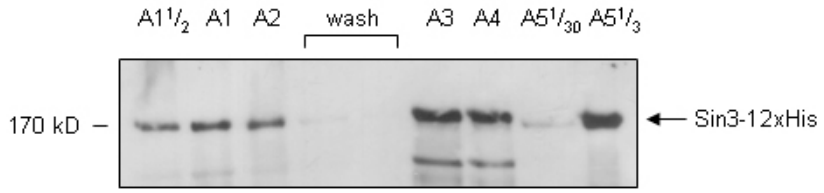


Figure 16. Thr 391 in Rpd3 is phosphorylated but not in a cell cycle dependent manner. (A) Rpd3-HTB purification from a SILAC strain (JV581). Blot was incubated with horse radish peroxidase conjugated Streptavidin. A1 to A4 analogous to Figure 14**. (B)** Sequence coverage of the Rpd3-SILAC experiment measured by FT MS gives 35% of the overall sequence and is outlined in red. Blue sequences signify peptides detected by previous MS measurements. The peptide including Ser 384 is underlined. **(C)** Ration of integrals of the light and heavy peptides YAPSVQLNHTPR of the phosphorylated and non-phosphorylated derived from the MS1 data from a MS-MS run. NOTE: The heavy peak (+6Da) has to be corrected by a second heavy peak where at least one of the two prolines is the side product of 13Carginine catabolism (+12Da). Peptides with two incorporated heavy prolines are not included in the calculation.

3.7.4 Threonine 304 of Sin3 is phosphorylated and is part of a highly conserved domain

Similar to Rpd3, the scaffolding subunit of the Sin3/Rpd3 HDAC, Sin3 was also tagged with the HTB (JV583, Wolfgang Reiter pers. com.). Although the yield of the Sin3_HTB purification did not exceed 10% (Figure 17A) a visible Sin3 protein band could be detected by comassie stain. Mass spectrometry analysis of this protein band gave a sequence coverage of 40% for Sin3. Herein we identified 4 residues to be phosphorylated. Interestingly all 4 phospho-sites (Figure 17B) were also identified in a large scale analysis of the yeast proteome (Albuquerque et al., 2008). Sequence alignment of Sin3 homologues from various species showed that threonine 304 is highly conserved during evolution (Figure 17C), suggesting that it might be functionally important. Threonine 304 is located in the close vicinity of the first paired amphipathic helix (PAH1) domain of Sin3 (amino acids 23-285) which is highly conserved from yeast to men.

(A)



(B)

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LPDSSASANQQVQHAQQHAQQQHEAQMHAQAQAQAQAQAQVEQQKQQQQFLYPASGYYGHPSNRGIPQQNLPPIGSFSPPTNGSTVHEAYQDQQ
HMQPPHFMPLPSIVQHGPNMVHQGIANENPPLSDLRTSLTEQYAPSSIQHQQQHPQSISPIANTQYGDIPVRPEIDLDPSIVPVVPEPTEPIEN
NISLNEEVTFFEKAKRYIGNKHLYTEFLKILNLYSQDILDLDDLVEKVDFYLGSNKELFTWFKNFVGYQEKTKCIENIVHEKHRLDLDLCEAFG
PSYKRLPKSDTFMPCSGRDDMCWEVLNDEWVGHPVWASEDSGFIAHRKNQYEETLFKIEEERHEYDFYIESNLRTIQCLETIVNKIENMTENEK
ANFKLPPGLGHTSMTIYKKVIRKVYDKERGFEIIDALHEHPAVTAPVVLKRLKQKDEEWRRAQREWNKVWRELEQKVFFKSLDHLGLTFKQADK
KLLTTKQLISEISSIKVDQTNKKIHWLTPKPKSQLDFDFPDKNIFYDILCLADTFITHTTAYSNPDKERLKDLLKYFISLFFSISFEKIEESLY
SHKQNVSESSGSDDGSSIASRKRPYQQEMSLLDILHRSRYQKLKRSNDEDGKVPQLSEPPEEEPNTIEEEELIDEEAKNPWLTGNLVEEANSQG
IIQNRSIFNLFANTNIYIFFRHWTTIYERLLEIKQMNERVTKEINTRSTVTFAKDLDLLSSQLSEMGLDFVGEDAYKQVLRLSRRLINGDLEHQ
WFEESLRQAYNNKAFKLYTIDKVTQSLVKHAHTLMTDAKTAEIMALFVKDRNASTTSAKDQIIYRLQVRSHMSNTENMFRIEFDKRTLHVSIQY
IALDDLTLKEPKADEDKWKYYVTSYALPHPTEGIPHEKLKIPFLERLIEFGQDIDGTEVDEEFSPEGISVSTLKIKIQPITYQLHIENGSYDVF
TRKATNKYPTIANDNTQKGMVSQKKELISKFLDCAVGLRNNLDEAQKLSMQKKWENLKDSIAKTSAGNQGIESETEKGKITKQEQSDNLDSSTA
SVLPASITTVPQDDNIETTGNTESSDKGAKIQ

(C) ClustalW 2.0.10 multiple sequence alignment

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Sin3 (yeast)          P22579   PAGVQPADAPDPSSNHADANDDNNNNENSHDEDADYRPLNVKDALSYLEQVKFQFSSRPD 240
Sin3A (homo sapiens)  Q96ST3   ---------------------------------QFQRLKVEDALSYLDQVKLQFGSQPQ 142
                                                                ::: *:*:******:***:**.*:*:

                      P22579   IYNLFLDIMKDFKSQAIDTPGVIERVSTLFRGYPILIQGFNTFLPQGYRIECSSNPDDPI 300
                      Q96ST3   VYNDFLDIMKEFKSQSIDTPGVISRVSQLFKGHPDLIMGFNTFLPPGYKIEVQTN--DMV 200
                               :** ******:****:*******.*** **:*:* ** ******* **:** .:*  * :

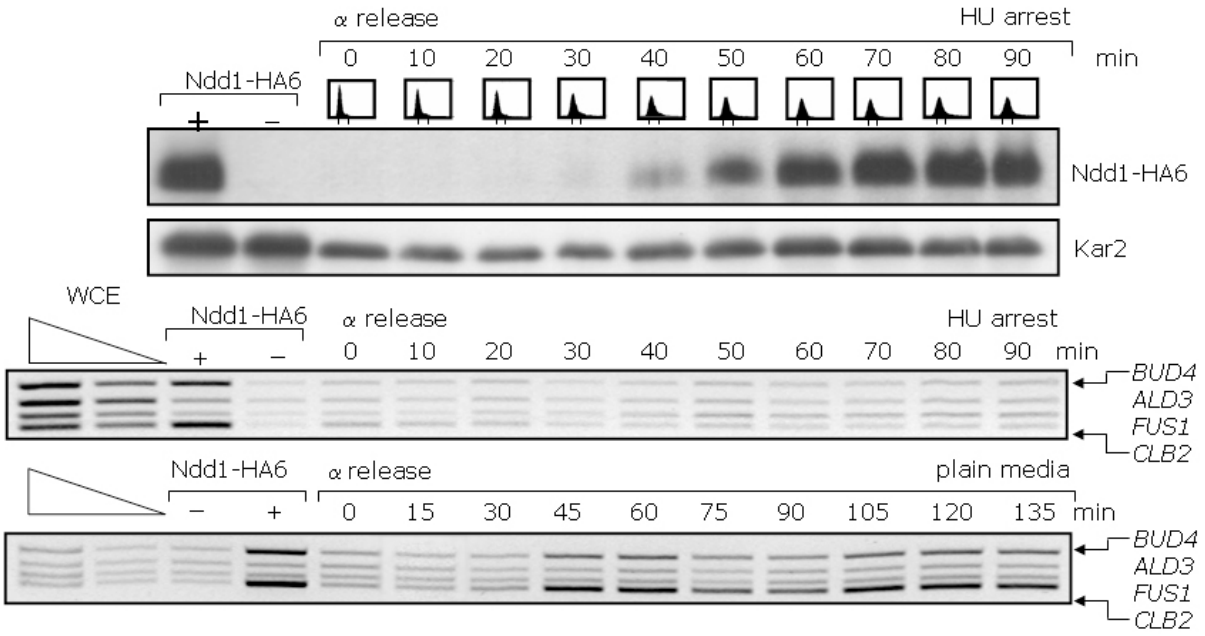
                      P22579   RVTTPMGTTTVNNNISPSGRGTTDAQELGSFPESDGNGVQQPSNVPMVPSSVYQSEQNQD 360
                      Q96ST3   NVTTPG----QVHQIPTHGIQPQPQPPPQHPSQPSAQSAPAPAQPAPQPPPAKVSKPSQL 256
                               .****       ::*.. *  .          .:...:..  *:: .  *...  *: .*
```

Figure 17. Threonine 304 of Sin3 is phosphorylated. (A) Western Blot analysis of Sin3-12HIS-Biotin purification (JV583). Detection with α-Penta-His HRP conjugate. A1-A4 corresponding to Figure 14. Protein sample after TEV cleavage is represented by A5. The dilution 1/30 reflects the decreasing volume after cleavage and corresponds to A1. **(B)** Sequence coverage of Sin3 is about 40%. Phospho-sites detected are marked in blue. Phospho-sites already published but not detected in our run are in magenta (Albuquerque et al., 2008). **(C)** Sequence alignment of the first aliphatic helix domain (PAH1) of Sin3 between yeast and human. The phosphorylated TP site is marked by a circle.

4 RESULTS II: NDD1 DEPENDENT GENE ACTIVATION CAN BE HALTED BY DNA DAMAGE

4.1 Ndd1 accumulates in S-phase arrested cells but is not recruited to G2/M promoters

The expression and activity of the transcriptional activator Ndd1 in the cell seems to be regulated at several levels. Its mRNA is transcribed in a cell cycle specific manner from late G1 to the onset of mitosis and largely correlates Ndd1 protein levels (thesis Helene Klug). Furthermore, several attempts to over express Ndd1 with the help of constitutive promoters such as GAL1pr and ADHpr failed (thesis Helene Klug), leading to the assumption that Ndd1 is also regulated on the level of translation or at the level of protein stability. As soon as Ndd1 protein levels are stabilized in the beginning of S-phase the protein becomes nuclear and binds to the promoter region of its target genes. This recruitment is mediated by Fkh2 and its phosphorecognition domain FHA which recruits the phosphorylated Ndd1 protein (Darieva et al., 2003; Reynolds et al., 2003). Thus the phosphorylation of Ndd1 might, besides its role in Ndd1 recruitment, also contribute for protein stability. Therefore, we were interested whether we can find any state (however transient under normal conditions) where Ndd1 is stable but unbound to the promoter. For this purpose we treated cells with the agent hydroxyurea which inhibits the ribonuclease reductase, thereby shortening the cellular pool on dNTP. Hydroxyurea treated cells that were released from a G1 arrest, arrest in early S-phase and are unable to replicate their DNA. However, these cells have successfully passed the START checkpoint as they are able to bud. Cells expressing a tagged version of Ndd1 showed that under these conditions Ndd1 accumulates in the cell (Figure 18A), becomes nuclear (Helene Klug thesis), but fail to bind its target promoters (Figure18B).



Helene Klug thesis

Figure 18. Ndd1 is not recruited to its target promoters in hydroxyurea treated cells. (A) Ndd1 protein levels accumulate in hydroxyurea arrested cells. Ndd1-HA6 expressing cells (MK161) were arrested in g1 by α-factor and released in 0,1M hydroxyurea containing media. Samples were withdrawn at indicated time points. Western blot analysis with α-HA antibodies and antibodies directed again Kar2 as inner control. Cell cycle arrest as well as cell synchronicity were confirmed by FACS analysis. **(B)** In hydroxyurea treated cells Ndd1 is not recruited to G2/M promoters. From the same culture like in A samples were withdrawn for ChIP analysis. Immunoprecipitated DNA was analysed by multiplex PCR with the indicated primer pairs. **(C)** Cells released in plain media show cell cycle dependent Ndd1 recruitment. (Courtesy Helene Klug)

4.1.1 Ndd1 cannot bind the FHA domain of Fkh2 in HU arrested cells

As discussed previously, Ndd1 is recruited to its target promoters by Fkh2 in a phospho-specific manner. We also showed that this interaction can be transferred into an artificial context by fusing Fkh2 to a second artificial DNA binding domain such as the DNA binding domain of GAL4. Using this construct, we wanted to investigate whether the inability of Ndd1 to bind G2/M promoters in hydroxyurea treated cells is based on its inability to interact with the FHA domain of Fkh2. Generally, there one can envision two simple mechanisms. Either the phosphorylation pattern of Ndd1 in hydroxyurea treated cells is different from S-phase Ndd1 so that the phospho-sites that are necessary for Ndd1 interaction with the FHA domain are absent. Alternatively, the FHA domain of Fkh2 is still able to bind Ndd1, but a yet unknown inhibitory mechanism prevents this interaction, by catalytic or steric hindrance. In order to restrict our analysis to the interaction of the FHA domain with Ndd1, we used a truncated version of Fkh2 (Fkh2aa1-306) that encompasses the entire FHA domain. As a negative control we used a similar construct where the FHA domain was mutated in its genuine phosphorecognition sites, in order to assure that any observed effects in the experiment are based on the recognition of phosphorylated Ndd1. After the release from α-factor, Ndd1 could be detected at the artificial promoter only when the intact FHA domain was present but not in the point mutant. When the same cultures were released into hydroxyurea containing media Ndd1 was neither able to bind its native target promoter *CLB2* nor the artificial *GAL1-10* promoter independently of the mutant or the wt FHA domain. These finding strongly suggest that the regulation of Ndd1 in hydroxyurea treated cells is mediated through its interaction with the FHA domain alone, as the absence of other regions in Fkh2 does not seem to have any effect on its recruitment.

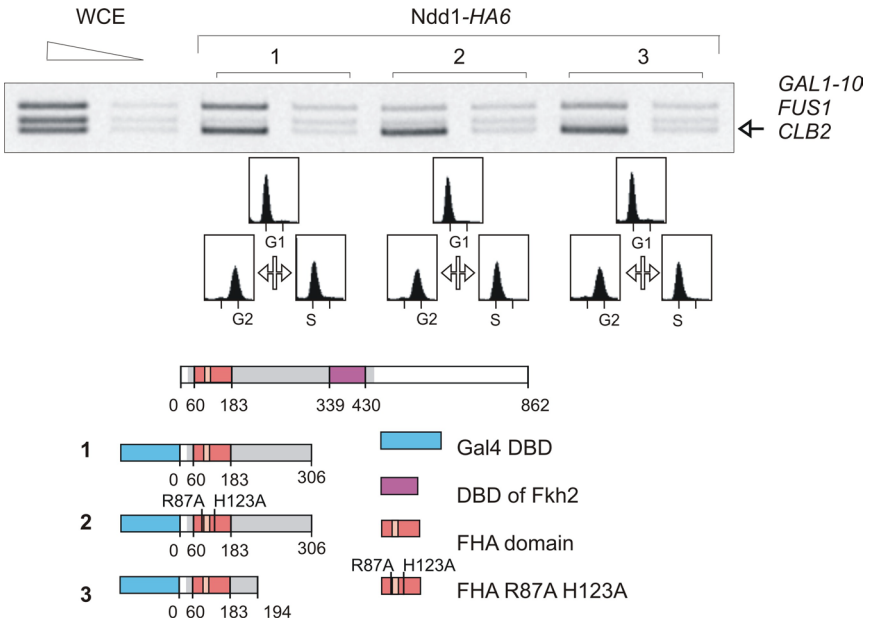
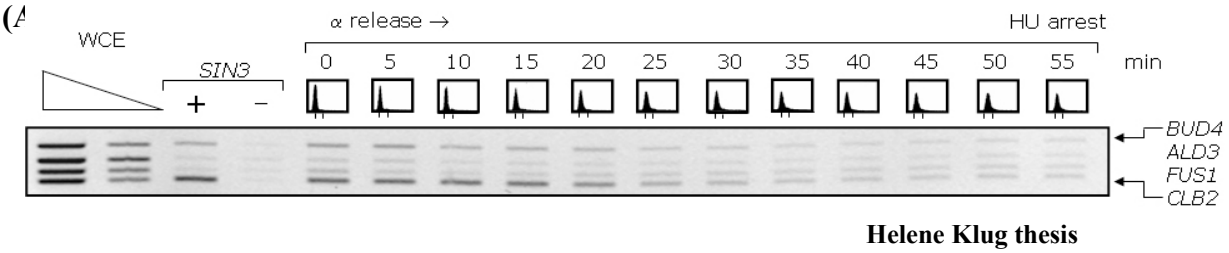


Figure 19. Ndd1 can not interact with the FHA domain in hydroxyurea treated cells. An Ndd1-HA6 (MK1551) strain expressing different N-terminal mutants of Fkh2 fused to the $GAL4_{DBD}$ (pJV287, pJV292; pJV402) was arrested in G1 by α-factor treatment and released in plain or in 0.1 M hydroxyurea containing media. Samples for ChIP analysis were withdrawn 50' post release. Cell synchronicity was confirmed by FACS analysis.

4.2 Sin3 dissociates from the promoter in S-phase arrested cells

Ndd1 absence at G2/M promoters in hydroxyurea treated cells might be also caused by simple steric hindrance by Sin3 which also binds at (or in close vicinity to) the FHA domain of Fkh2. Persistent Sin3 binding in hydroxyurea treated cells could be a reason for Ndd1 inhibition. To test this possibility, α-factor synchronized wt cells were released into hydroxyurea containing media and Sin3 associated chromatin was analyzed (Figure 20). The experiments clearly showed that in hydroxyurea treated cells Sin3 was still efficiently removed from G2/M promoters such as *BUD4* and *CLB2*. However, there was one noteworthy difference to cells released into plain media. Whereas under normal conditions Sin3 recuperate the *CLB2* promoter after S-phase completion (see Figure 5), in cells exposed to hydroxyurea Sin3 remain absent from chromatin.



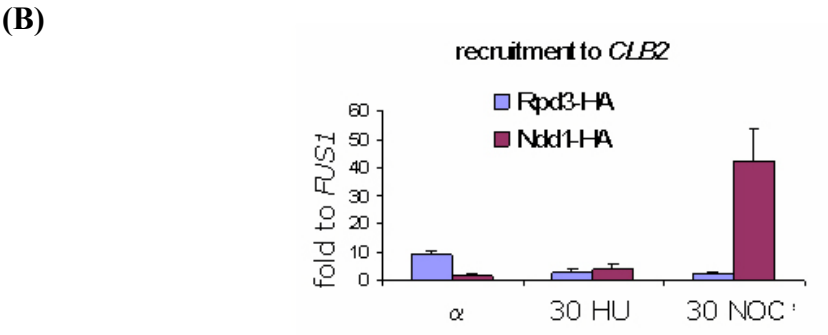


Figure 20. (A) Sin3 dissociates from the promoter in S phase arrested cells. Pheromone synchronized cells (w303) were released into fresh YEPD medium, supplemented with 0.1 M HU and samples were withdrawn at the indicated time points. Cell synchronicity was confirmed by FACS analysis Chromatin IPs were performed using α-Sin3 polyclonal antibodies. Promoter binding was visualized by Multiplex-PCR with the indicated primer pairs. Target promoters are highlighted by arrows. **(B)** Quantitative PCR of ChIP analysis of pheromone synchronised cells HA-tagged either for Ndd1or Rpd3. Cells were released either in HU containing media or in media containing nocodazole. (MK155) or Rpd3 (JV394).

4.2.1 S-phase arrest triggers de-repression of G2/M promoters

As soon as it became evident that Sin3 is released from G2/M promoters in HU arrested cells, we asked whether the state of the promoter, thus the absence of both, transcriptional activation as well as transcriptional repression, leads to moderate transcription levels. As shown previously, in cells which were deleted for *sin3* or *rpd3* we could show that transcript levels were elevated during α-factor arrest. However, in *sin3* and *rpd3* cells the absence of Sin3/Rpd3 HDAC activity is permanent and affects all stages of the cell cycle. Thus, the elevated transcription levels observed in G1 might have been a consequence of accumulative and/or adaptive effects. Furthermore, looking at *sin3* cells in G1 makes it impossible to distinguish between pre-mature S-phase specific transcription and inappropriate promoter shutdown at the end of mitoses. These arguments make clear why Sin3 removal is not necessarily succeeded by increased transcriptional activity. In order to compare de-repression and transcriptional activation in single experiment we measured *CLB2* mRNA levels in *cdc14*^{ts} cells synchronized by α-factor and released in hydroxyurea. Subsequently the very same cells were released from the S-phase block at restrictive temperature which causes cell cycle arrest at the mitotic exit. There were two interesting results from this experiment. First, cells released into hydroxyurea containing media show promoter de-repression which is three fold above the mRNA levels in the repressed state but still much less than in cells released from the hydroxyurea block. Second, although at the end of mitosis Ndd1 protein levels seem to decrease in the cell, Ndd1 remains bound to the *CLB2* promoter. Thus, it seems possible that there is an additional signal required for Ndd1 removal at mitotic exit.

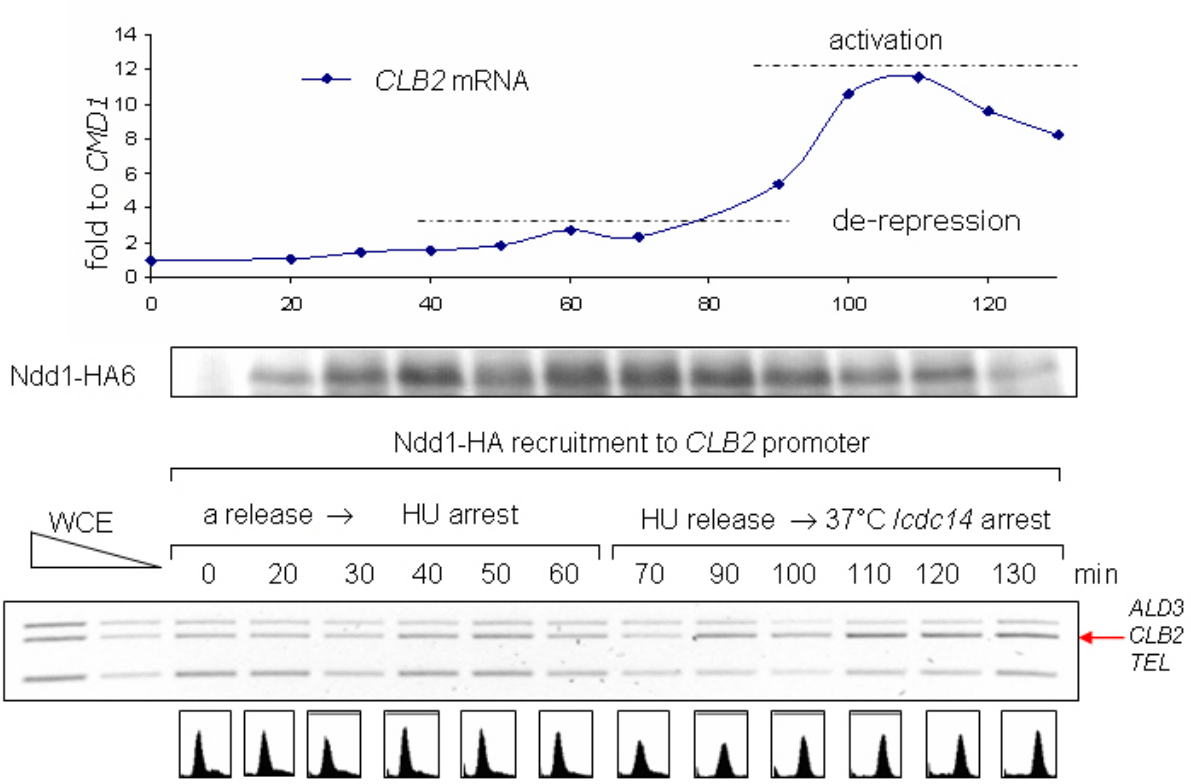


Figure 21. S-phase arrest triggers de-repression of G2/M promoters. Pheromone synchronized *cdc14-1* (HEL324) cells expressing Ndd1-HA were released into 0,1M HU containing media. After 70 min of arrest cells were collected again washed and released at restricted temperature. Samples for ChIP analysis, Northern and Western blotts were withdrawn at the indicated time points. Cell synchronicity was confirmed by FACS analysis. Chromatin IPs were performed using anti-HA antibodies. Promoter binding was visualized by Multiplex-PCR with the indicated primer pairs. Target promoters are highlighted by arrows. NOTE: In Cdc14 depleted cells Ndd1 is destabilized but remains bound to the promoter

4.3 S-phase checkpoint mutants partially re-establish Ndd1 recruitment to G2/M promoters in HU arrested cells

Cells treated with hydroxyurea arrest in early S-phase because cells cannot synthesise new dNTPs. As a consequence the replication fork stalls and the firing of late origins is prohibited. The signalling cascade which transduces the information from the stalled replication fork to its various downstream targets is named intra S-phase checkpoint (Chapter 2.1.4.2.). In the center of this network there are two partially redundant kinases, Mec1(ATR in mammals) and Tel1 (ATM in mammals) and their downstream effectors. One of the most important downstream targets of Mec1 (and Tel1) is the Rad53 kinase (Chk2 in mammals) which is responsible for many processes such as stabilisation of the stalled replication fork, inhibition of late origin firing and DNA repair. Because Ndd1 protein levels become elevated but Ndd1 is unable to bind to its target promoters in hydroxyurea treated cells, we wondered whether this special property of Ndd1 is downstream of the S-phase checkpoint machinery. We carried out a set of experiments in strains where at least one of the main S-phase checkpoint kinases was deleted and looked whether Ndd1 was recruited to the *CLB*2 promoter when cells were released from a α-factor block into hydroxyurea. We could show that under these conditions at least in the *mec1* mutant Ndd1 was efficiently recruited to the promoter and that transcriptional levels of *CLB2* mRNA were elevated when compared to the wt (Figure 22 B,D). However, the levels of Ndd1 recruitment as well as of *CLB2* expression in hydroxyurea treated *mec1* cells reached only 30% of the levels when the same cells were released in plain or nocodazole containing media (Figure 22 C). These results suggest that Mec1 might be at the root of Ndd1 inhibition in cells arrested in early S-phase. However, the absence of Mec1 is not sufficient to re-establish normal levels of Ndd1 binding and transcriptional activity that are observed in wt cells during S-phase progression. The later finding is less surprising, if one considers the fact that many factors like the Cdc5 kinase and all mitotic cyclins were described to play important roles in process of Ndd1 recruitment and stabilization (see discussion).

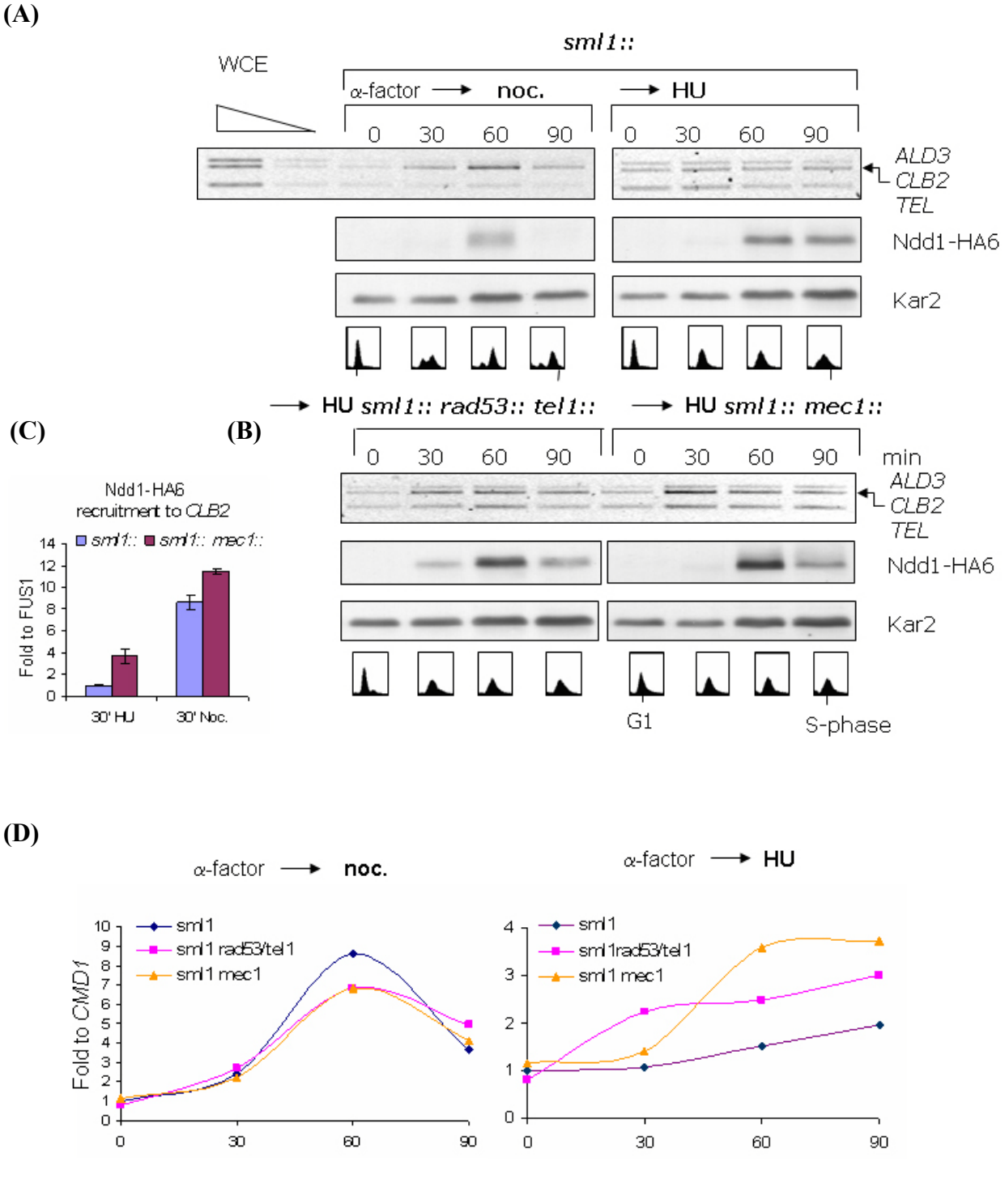


Figure 22. Ndd1 recruitment to the CLB2 promoter is partially re-established in S-phase checkpoint mutants. Pheromone synchronized cultures of the indicated strains (HEL476, HEL 428, JV471) were released into media containing HU **(A+B)** ore Nocodazole **(A)**. Samples for Western **(A+B)**, Northern **(D)** and ChIP **(A+B)** analysis, were withdrawn at the indicated time points. Cell synchronicity was confirmed by FACS analysis. Western and Chromatin IPs were performed using anti-HA antibodies. Promoter binding was visualized by Multiplex-PCR with the indicated primer pairs. Target promoters are highlighted by arrows. **(C)** Ndd1-HA recruitment to the *CLB2* promoter. Quantitative PCR of ChIP analysis of pheromone synchronised cells released into hydroxyurea or nocodazole containing media for the indicated time points**. NOTE:** All strains are deleted for Sml1 which is a inhibitor of ribonuclease synthesase.

5 DISCUSSION

5.1 G2/M promoters are activated in early S-phase by a step-wise mechanism including both, G1 as well as B-type Cyclins

The data presented in this thesis focus on the step-wise activation of the G2/M regulon in yeast cells. This mechanism includes the regulated removal of the transcriptional repressor Sin3/Rpd3 and the recruitment of the transcriptional activator Ndd1 and this transition from the repressed to the activated state is well reflected by the underlying chromatin.

In the first section I describe the Sin3 histone deacetylase complex as a crucial negative regulator of G2/M specific gene expression in yeast. While exploring the regulation of this repressor we uncovered previously unrecognized connections between the cyclin dependent kinase activities and the function of the different G2/M specific transcriptional regulators. Until now, most studies on the G2/M regulon have focussed either on a feedback loop involving the Clb2 kinase and the activator Ndd1 (Amon et al., 1993; Reynolds et al., 2003), or on the Clb5 kinase and its role in the modification of the carboxy-terminus of Fkh2 (Pic-Taylor et al., 2004). Recently, a positive feedback system was uncovered that is based on phosphorylation of Ndd1 by the polo kinase Cdc5 (Darieva et al., 2006). The *CDC5* gene is like *CLB2* a G2/M specific gene. The studies mentioned above focus on the mechanism by which already elevated expression levels of *CLB2* cluster genes during G2/M phase are maintained, but do not really explain the process of transcriptional initiation at the beginning of S-phase. This thesis provides new evidence that the modifications of Ndd1 and Fkh2 by mitotic and Cdc5 kinase respectively cannot be the sole connection between the cell cycle machinery and the timing of G2/M specific transcription. According to our data, Sin3 mediated chromatin deacetylation provides an additional level for cell cycle control. The reversal of G2/M specific promoter repression by the removal of the deacetylation complex actually seems to represent the first crucial step towards activation. Unexpectedly, this event happens prior to and independently of Ndd1 recruitment. It also does not require the function of any of the B-type cyclins. Instead, it seems to be part of the events orchestrated by G1-specific cyclins at the G1/S boundary. Thus, the initial transcriptional activation choreography at G2/M promoters is not conducted by a positive feedback loop. Rather, the previously proposed feedback mechanisms might just

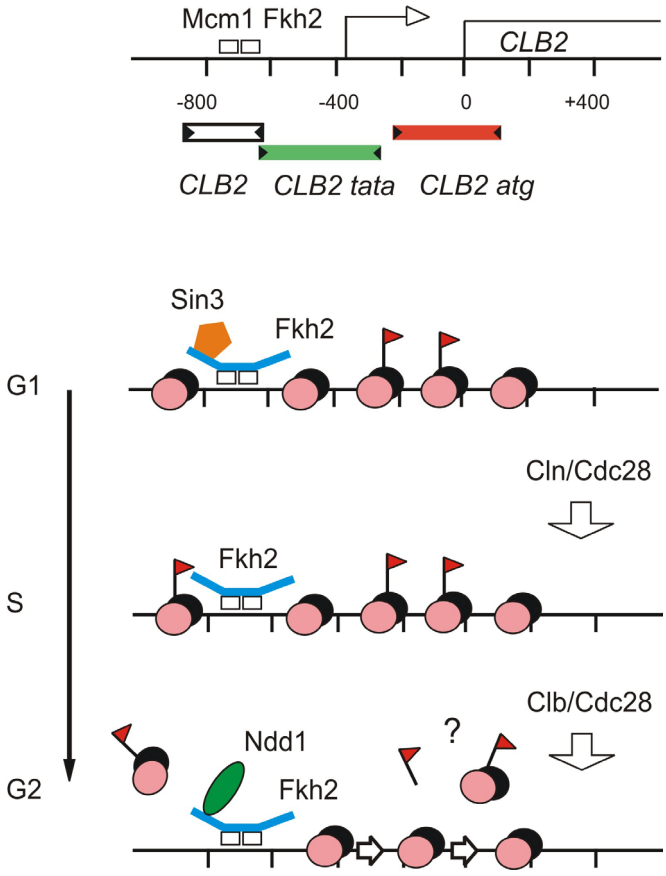
ensure that the expression of G2/M specific genes never decreases to levels that lead to premature re-replication in case of attenuated late cell cycle stages.

Another aspect of my work concerned the changes in the chromatin composition of the *CLB2* promoter during the switch from the repressed to the activated state. My data support a two-step model in which the H4 histones adjacent to the promoter region first become acetylated as a consequence of the removal of the Sin3/Rpd3 complex. In a second step, these histones are then expelled (see Model 1). It is only the second step that requires the function of Ndd1, implying that the factor is not part of the respective histone acetylase complex. In contrast, the transcribed region of the *CLB2* locus shows only a slight decrease in H4 histone binding although the histone acetylation signal seems to be entirely lost with similar kinetics as the nucleosome(s) are removed from the promoter. However, one cannot distinguish whether this event is a consequence of a de-acetylase activity or whether it reflects the selective eviction of acetylated histones (see Model 1). Recent work, using chromatin analysis based on micrococcal nuclease digestion patterns (Sherriff et al., 2007), unravelled the positions and the cell cycle specific remodeling of nucleosomes at the *CLB2* locus, and it is worthwhile to compare their results with our ChIP data. Their finding that the promoter region of *CLB2* including the UAS is poor on nucleosomes supports our contention that our ChIP data concerning this region truly reflect the modifications and the remodeling of mainly one nucleosome. A second interesting observation is the fact that the nucleosome patterns across the transcribed region of *CLB2* undergo several distinct changes during transcriptional activation which might be an indication for repositioning towards the 3´- end (Sherriff et al., 2007). The idea that Ndd1 is directly or indirectly involved in chromatin remodeling is further supported by the finding that the recruitment of the chromatin modifier complex Swi/Snf coincides with and is depending on Ndd1 binding to the *CLB2* promoter.

Recruitment of Ndd1 and thus initiation of transcription is independent on all B-type cyclins and transcription of the G2/M cluster genes occurs at schedule in these mutants (Helene Klug thesis). This idea corresponds well to recently published data showing that expression of cell cycle cluster gene mRNAs is cell cycle regulated even in the complete absence of B-type cyclin associated kinase activity (Orlando et al., 2008). The authors propose an oscillating network of transcriptional activators which is largely uncoupled from cyclin activity. The above mentioned facts were the starting point for the work of Helene Klug who tried to identify the cellular mechanism by which Ndd1 is stabilized after START and recruited to G2/M promoters. She found that when synchronised cells were arrested in early S-phase, Ndd1 is accumulated but not bound to its target promoters. Based on this

observation, the second part of my study deals with the question whether the intra S-phase checkpoint which is activated upon hydroxyurea treatment as cells arrest in early S-phase, is involved in the regulation of Ndd1 recruitment. We found strong evidence that this might be actually the case.

Our own as well as previously published data add up into a well orchestrated multi-step model which allows to tightly regulate G2/M cluster genes. However, there are many contradictions and uncertainties resulting from data presented in this thesis and data published previously by the community in the field. In the following sections I would like to address some of these, and discuss some possible future experiments which might help to resolve some of the question marks.



Model 1. Model of chromatin changes at the *CLB2* locus during the transition from repression to activation. Ovals represent nucleosomes and their putative positions at the *CLB2* locus as described by Sherriff et al. 2007. Nucleosomes with acetylated H4 histones are depicted with small flags. The model is based on results presented in Fig. 1 to 6 and reflects data generated by primer pairs amplifying 3 different regions of the *CLB2* locus (*CLB2*, *CLB2 tata*, *CLB2 atg*). Vertical arrows signify different kinase activities triggering the transitions from G1 phase to S phase and from S phase to G2 phase. The question mark signifies that in G2 the presence of non-acetylated H4 Histones might be a consequence of either histone de-acetylation (detached flag) or histone removal (detached oval) combined with histone remodeling (small horizontal open arrows).

5.2 The Sin3/Rpd3 HDAC negatively regulates G2/M promoters, but how is it regulated itself?

5.2.1 Is Sin3 a direct target of the Cln-kinase ?

Our deduction that Cln-kinases play an important role not only in the activation of SBF and MBF function (Skotheim et al., 2008) but also in the derepression of G2 specific promoters coinciding with the onset of S-phase is best illustrated by ChIP data comparing Sin3 binding in cells arrested by Cln depletion and cells arrested with high Cln kinase activity but low Clb2 kinase activity caused by inactivation of Cdc34. In cells depleted for Cln activity Sin3 persist at the *CLB2* promoter whereas in cells with peaking Cln activity Sin3 levels are decreased as cells pass START and budding becomes observable. Moreover, neither Ndd1 depletion nor Clb kinase deficiency prevents the dissociation of Sin3 complexes from G2/M promoters.

Thus, how do Cln kinases regulate the function of Sin3? Because Sin3 complexes are involved in many different processes (Silverstein and Ekwall, 2005), Cln kinase activity must be restricted to a small subset of Sin3 complexes that are relevant for cell cycle progression. One way how this could be achieved might be that factors mediating the recruitment of Sin3 to particular promoters are the target of the Cln kinase. Although several transcriptional co-repressors such as Ume6 or Ash1 have been found to be stably associated with the core members of the large Sin3 complex, we suggest that the recruitment of this complex to G2/M promoters is solely regulated via its interaction with Fkh2. Our data clearly suggested that Fkh2 is necessary and sufficient for Sin3 binding, as *fkh2* mutants fail to recruit Sin3 to G2/M promoters, whereas a Gal4-Fkh2 fusion protein targets Sin3 to the *GAL1-10* promoter. Furthermore, results obtained with Gal4DB-Fkh2 chimeras strongly suggest that the regulated recruitment of Sin3 is a property associated with the N-terminal part of Fkh2 and largely independent of the promoter context. Therefore, the C-terminal domain of Fkh2, which is missing in Fkh1, does not play only an essential role in Sin3 recruitment. The idea that Fkh2 directly interacts with the Sin3 complex is further supported by a global study on yeast protein complexes (Ho et al., 2002). More recently a second type of cell cycle specific promoters distinct to G2/M promoters was shown to be regulated by Fkh2 and its interaction with Sin3. In G1 the Ace2 dependent gene *CTS1* is actively repressed via Fkh2 in association with the Sin3/Rpd3 HDAC. The presence of Sin3 at the *CTS1* gene promoter

region is required in order to prevent Swi5 dependent trans-activation in vivo, as both transcription factors, Ace2 and Swi5 can bind to the promoter in vitro (Voth et al., 2007). Even more, genes like *EGT2* and *SIC1* which in wt cells are trans-activated by both proteins, Ace2 and Swi5 (Knapp et al., 1996; Toyn et al., 1997), lose their sensitivity towards Swi5 when Fkh2 bindings sites are introduced in their promoter region. However, in contrast to our results the authors claim that at Ace2/Swi5 dependent promoters as well as at the *CLB2* promoter both forkhead proteins Fkh2 and Fkh1 mediate the recruitment of Sin3/Rpd3 HDAC.

There is a large number of targets for Cln kinases in order to regulate the Sin3-Fkh2 interaction: it could be Fkh2 itself, an as yet unknown adaptor between Sin3 and Fkh2, or any component of the core Sin3 complex, thus the catalytic component Rpd3 or Sin3 itself. There are three findings which suggest that Sin3 might be a direct target of Cln kinase activity. A mass spectrometry analysis provided evidence that Sin3 co-precipitates specifically with Cln2, but not with B-type cyclins (Archambault et al., 2004). Moreover, a phosphorylated SPxxK motif has been identified within Sin3 during a screen for Cdc28 specific phospho-peptides in the yeast proteome (Ficarro et al., 2002). Third, there is growing evidence that at other parts of the genome Sin3 is removed from the chromatin at START and during early S-phase (Aalfs and Kingston, 2000; Vogelauer et al., 2002). Koch and co-workers have shown that Sin3 is binding to SBF bound promoters and is removed from these prior to START in a cell cycle dependent manner (Stephan and Koch, 2009). As a consequence one might wonder whether the removal of the Sin3/Rpd3 complex from SBF promoters is part of the recently described positive feed-back loop involving Cln1/2 (Skotheim et al., 2008).

To study this in more detail, we purified Rpd3 and Sin3 under denaturating conditions and identified several phosphorylation sites by mass spectrometry (Chapter 3.6). Some of the identified phosphorylation sites were S/T P sites which is the minimal consensus site for Cdc28 kinase activity. This consensus sites are very weak as only about 32% of yeast proteins which exhibit the even more restrictive consensus site (S/T)PX(K/R), are a true substrates for the cell cycle kinase (Ubersax et al., 2003). However, statistical evaluation of present data suggest that clustering of two ore more of weak (S/T)P consensus sites enhances the probability of a protein to be a true substrate for the kinase (Moses et al., 2007). Indeed there are two S/T P sites in close vicinity in Sin3 at the positions 304 and 316. Even more interesting is the fact that Threonine 304 is highly conserved from yeast to men and located adjacent to the first paired amphipathic helix domain (PAH1, amino acids 23-285 in yeast)

which is by far the best conserved PAH domain among Sin3 homologues and which was suggested to be functionally involved the recruitment of site specific transcription factors in mammals (Grzenda et al., 2009). Therefore, threonine 304 site might be highly relevant, and possibly part of an evolutionary conserved mechanism to regulate the activity of the HDAC. As quantitative phospho-proteomic analysis of Rpd3 indicates (Chapter 3.6), Rpd3 phosphorylation seems not to be altered in early S-phase which is not further surprising as we could show that Sin3 is still recruited to Fkh2 in Rpd3 deficient cells (Figure 3). For this reason it is of great interest to know, whether in *rpd3Δ* cells Sin3 is still recruited to G2/M promoters in a cell cycle specific manner. Surprisingly preliminary data indicate that Sin3 remains bound throughout the S-phase when is catalytic subunit Rpd3 is absent. In order to figure out whether threonine 304 in Sin3 is functionally relevant for its proper function we replaced it by an alanine substitution and expressed the *SIN3 T304A* allele from a galactose inducible promoter in a *sin3* strain. The yet available preliminary data suggest that this mutant is unable to recruit Rpd3 to the *CLB2* promoter. Whether this inability of Sin3 T304A to interact with Rpd3 is general feature of the complex or only restricted to certain cell cycle phases or promoters should and will be subject for further investigation.

5.2.2 Why are *ndd1* cells viable in *SIN3/RPD3* deletion mutants?

In a straight forward model Sin3 and Ndd1 constitute a pair of counter-actors in regard to transcriptional activity and the fact that *sin3Δ ndd1Δ* double deletion strains are viable seems little surprising. In other words, it is the absence of the repressor Sin3 at G2/M promoters at START which simply provides enough transcriptional output to surpass the chronic lack of the transcriptional activator Ndd1 and leads the cells through mitosis and thereby provides viability. But, on a closer look one can see that this first on mind coming explanation is little convincing. This is the case, because even in wild type cells Sin3 is removed successfully from G2/M promoters at START, independently on future events like Ndd1 presence or the abundance of mitotic cyclins (this thesis). In a consequence, as long as cells arrive to START there virtually should not be any difference between wild type cells and cells depleted for Sin3. However there is one important experiment to be done yet, in order to prove the idea described above. Wt and *sin3Δ* cells should be arrested in α-factor and re-leased in hydroxyurea containing media. As both should arrest in early S-phase in absence of Ndd1 and Sin3 at G2/M promoters there should not be any difference in mRNA levels of the G2/M cluster genes.

What thus might be at the root of the ability of *sin3* cells to bypass *ndd1Δ* lethality? We gained partial insight in this problematic as I realised that over-expression of Ndd1 is detrimental for growth in *sin3* or *rpd3* cells (data not shown). There are at least two different stages in the cell cycle where *SIN3* deletion might be very sensitive for Ndd1 over-expression. First in G1 where missing Sin3 presence and/or enzymatic activity might lead to precocious Ndd1 recruitment. Second at the end of mitoses where the already established Ndd1- Cdc28/Clb2 feedback loop might require Sin3/Rpd3 presence at G2/M promoters for successful promoter shut down. Especially the involvement of Sin3/Rpd3 in promoter shut down might be subject of further investigation as we do not know how it is regulated. ChIP data from several time-course experiments suggest that the Sin3/Rpd3 complex re-appears at G2/M promoters immediately after S phase completion and persists to bind the promoters throughout the G2 phase, without displaying its usual repressory function.

5.2.3 What triggers the re-recruitment of Sin3/Rpd3complex after S-phase completion?

In theory Sin3/Rpd3 recruitment could be triggered by decreasing Cln activity in G2, altering the very same phosphorylation sites which cause Sin3 relieve by high Cln activity. However, our data obtained from cells depleted for mitotic cyclins Clb1-4 contradict this theory (this thesis). Although, mutants depleted for all mitotic cyclins should exhibit elevated Cln levels in G2 (Koch et al., 1996), Sin3 is not only efficiently removed from G2/M promoters but it also reappears in G2, thus showing a similar oscillation pattern as in wt cells. Neither decreasing Cln nor the increasing Clb1-4 activity seems to be required for Sin3 binding in G2. The S-phase cyclins Clb5/6 might also be responsible for the Sin3 binding in G2. This idea is supported by the fact that in cells where all B-type cyclins, including Clb5/6, were inactivated, Sin3 was removed after G1/S transition and remained absent from the G2/M promoter. However the idea that Sin3 recruitment in G2 is regulated by the cell cycle kinase is particularly attractive there are many other alternative models left. To mention only one scenario, the positive feedback loop in late S-phase which involves the polo kinase Cdc5 could also account for Sin3 phosphorylation and recruitment. Regardless of the upstream mechanism, there is evidence that the Sin3 found at G2/M promoters during G2 is bound to the promoter via the FHA domain of Fkh2, suggesting that phosphorylation might play a role. We wondered why the ChIP signal derived via Sin3 is much less pronounced in G2 than in G1. Surprisingly, when Sin3 is targeted to the *GAL 1-10* promoter region via the FHA domain of the $Gal4_{DBD}$-Fkh2 fusion construct, the observed binding levels are significantly elevated in G2, indicating that in this artificial promoter context there is no difference in Sin3 abundance between G1 and G2 (Figure 6). A trivial explanation of this phenomenon could be that the impaired Sin3 levels observed at G2/M specific promoters in G2 are a simple reflection of the ongoing process of G2/M specific gene transcription, as the remodeled chromatin might be detrimental for an efficient immunoprecipitation of the respective DNA when targeted via Sin3.

5.3 Ndd1 is essential for the transcriptional activation of G2/M specific promoters, what are the requirements for Ndd1 stability and binding?

5.3.1 Does Ndd1 compete with Sin3 for the FHA domain of Fkh2?

We could show that the binding of Sin3/Rpd3 to *CLB2* cluster gene promoters requires the presence of Fkh2, and that Fkh2 alone is able to interact with Sin3/Rpd3 at a heterologous promoter such as *GAL1-10*. This is also true for the interaction between Fkh2 and Ndd1 (Reynolds et al., 2003), but we could not provide any conclusive evidence that there is mechanistic causality between Ndd1 recruitment and Sin3 relieve. However, although Sin3 relieve is not induced by Ndd1 recruitment, Sin3 displacement might be still a precondition for Ndd1 binding. This precondition seems to be likely as the minimal region of Fkh2 required for the interaction with Sin3 mainly consists of the N-terminal region including the FHA domain. The main difference between the recruitment of Sin3 and Ndd1 via this region is that Ndd1 requires intact phosphorecognition sites at the FHA domain, whereas Sin3 binding is not impaired when these sites are mutated (this study, data not shown). However, Sin3 might interact with the FHA domain equally well as Ndd1, and the fact that the phosphorecognition sites at the FHA domain are dispensable in the case of Sin3, could be explained by a enhanced affinity of the Sin3 complex to the overall structure of the domain. Therefore, it would be interesting to investigate whether Ndd1 and Sin3 are able to bind Fkh2 simultaneously. If it turns out that both factors have to compete for one binding site, the repressive activity of the Sin3/Rpd3 complex at G2/M promoters might not only be a consequence of its genuine enzymatic function, but also to be at least partially due to spatial hindrance of Ndd1 recruitment. In order to determine simultaneous binding it would be either necessary to co-immuno-precipitate both factors or to use ChIP assays where Sin3 would be targeted to a heterologous promoter by Ndd1.

5.3.2 How does the intra S-checkpoint prevent Ndd1 recruitment?

In Chapter 4, I provide evidence that the intra S-phase checkpoint machinery interfere with Ndd1 recruitment. Treatment with hydroxyurea leads to arrest in early S-phase as cells are not able to synthesize fresh dNTPs and thus fail to replicate DNA (see introduction). Under these conditions Ndd1 is stabilized but is absent from its target promoters. However, when the central kinase of the intra S-checkpoint pathway, Mec1, is disrupted, Ndd1 becomes at least partially recruited to its target promoters again. Both Ndd1 recruitment as well as the transcriptional activity reaches only 30% of the levels that can be observed when cells were released in plain media. There are at least three different ways how these impaired levels could be explained. First *mec1* cells are much more sensitive to hydroxyurea treatment than wt cells because they cannot stabilize their replication forks. In a consequence most of them fail to successfully undergo next mitosis and arrest or die (Loy et al., 1999) (unpublished data). This increased toxicity might help to explain reduced levels of immunoprecipitated DNA, under the assumption that a considerably large amount of *mec1* cells start to disassemble their DNA associated complexes or increase proteolysis of Ndd1. This idea is somehow supported by the fact that in *mec1* cells which have been released in hydroxyurea containing media Ndd1 levels peak 60 minutes post release to decrease significantly afterwards, whereas wt cells keep on accumulating Ndd1 throughout the entire time of the arrest. Therefore it would be interesting to know whether the Fkh2-Mcm1 ternary complex remains unaffected in *mec1* cells during hydroxyurea treatment (as in wt cells, Helene Klug thesis). Second, in *mec1* deleted strains the impaired intra S-phase DNA damage checkpoint might cause increased cross-talk and instability of the DNA damage checkpoint network, as Mec1 function can be partially bypassed by Tel1. Because of this instability, Tel1 might rescue the *mec1* deletion in some cells, and prevent Ndd1 from recruitment. Interestingly, the *mec1 tel1* double mutant shows less Ndd1 recruitment than the *mec1* single mutant (data not shown). However, these data should be treated with care, as *mec1Δ tel1Δ* cells are even more sensitive to hydroxyurea then each one of the single mutants. Third, *mec1Δ* cells could still recruit Ndd1 to its target promoters but this recruitment could be transient. An increasingly transient recruitment of Ndd1 could occur in the case when cells fail to establish one or both of the presumptive positive feedback loops (via Cdc5 or Clb2). Recent a findings show that in hydroxyurea treated cells the Cdc28 kinase is associated predominantly to Clb5/6 and not to mitotic cyclins (as these seem to be targeted for degradation), support this

hypothesis (Keaton et al., 2007). When transient events are measured by ensemble- methods like ChIP the mean signal will always become reduced, depending on the duration of the signal and on the synchronicity of cells. To prove that Ndd1 recruitment in hydroxyurea treated *mec1* cells occurs in each cell but is transient, single cell analysis would be of great benefit.

What might be the downstream targets of the Mec1 kinase, that are relevant for the prevention of Ndd1 recrzuitment? The most prominent target of Mec1 is the Rad53 kinase (see introduction) which distributes many but not all of the signalling mediated by Mec1 to downstream targets. One of these targets is the kinase Cdc7 which phosphorylates the Mcm2-7 complex and thereby allows the replication origin to fire. When the intra S-phase checkpoint is activated, phosphorylation by Rad53 inhibits the activity of Cdc7 and thereby prevents the firing of late replication origins until DNA damage is resolved. For this reason we wanted to know whether conditional Cdc7 mutants would show the same response as cells treated with hydroxyurea. Indeed temperature sensitive mutants of Cdc7 accumulated Ndd1 which was not bound to its target promoter (data not shown). However, when cells depleted for Cdc7 were allowed to fire their origins by expression of the *MCM5-bob1* allele (Ogi et al., 2008), Ndd1 was successfully recruited to its target promoters (Syam Yelamanchi personal communication). This result clearly shows that firing of replication origins, and not the enzymatic activity of Cdc7, is the prerequisite for Ndd1 recruitment.

In general, there are two ways how activation of S-phase checkpoint might prevent Ndd1 recruitment. Ndd1 could be a direct substrate of the Mec1/Rad53 kinase pathway or Ndd1 is not a direct target and a third and yet unknown factor is activated and prohibits the Ndd1-Fkh2 interaction as it blocks their interaction sites by steric hindrance. The first view fits to the fact that the Mec1 kinase preferentially phosphorylates an evolutionary conserved (S/T)Q sequence (Traven and Heierhorst, 2005), such as is also present in Ndd1. The second hypothesis is supported by the fact many downstream targets of Mec1 (such as Rad53 and Dun1) have FHA domains (Lee et al., 2008) which are similar to the FHA domain of Fkh2. When Mec1 becomes activated it phosphorylates Rad53 at two adjacent residues. These two phospho-sites are recognised by the FHA domain of Dun1 which leads in a subsequent step to Dun1 phosphorylation and activation. Interestingly the FHA domain of Dun1 recognises two phospho-sites in Rad53, a mechanistic requirement which is very similar to the Fkh2-Ndd1 interaction (Lee et al., 2008). Thus, one might envision a mechanism where activated Dun1 binds to Ndd1 via its FHA domain and thereby prevents Ndd1 interaction with the FHA domain of Fkh2.

The fundamental difference between both models is that whereas in the first case Ndd1 would become modified in the second it wouldn't. Although, theoretically modification of Fkh2 could also play a role, there is strong evidence that this is not the case, as the FHA domain when fused to a GAL4 DNA binding domain shows the same pattern of Ndd1 recruitment upon hydroxyurea. Therefore, it would be very interesting to determine the phosphorylation status of Ndd1 during hydroxyurea treatment.

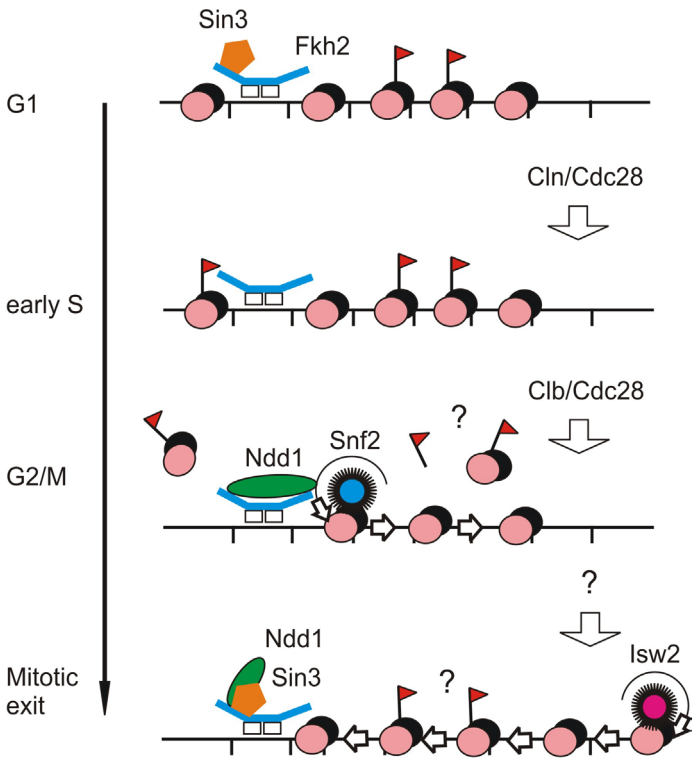
5.3.3 How does Ndd1 trigger transcriptional activation?

Our results could clearly show that the resolution of Sin3 - Fkh2 interaction at the G1/S transition is conducted by a remarkable increase of Histone H4 acetylation. Currently, we do not know yet, whether Histone acetylation occurs as a simple consequence of Sin3/Rpd3 removal or if the recruitment of the respective acetylase is also regulated by cell cycle or other signals. However, in cells which were pre-synchronised with α-factor and released in hydroxyurea media no H4 acetylation could be detected at the *CLB2* promoter although under these conditions Sin3 is absent from the promoter. We also do not know yet which acetylase might be involved. Preliminary data suggested that Esa1 might be the H4 acetylase of interest, as e*sa* ^{ts} mutants show decreased H4 acetylation levels at restrictive temperature, at this specific stage of the cell cycle (unpublished data). Unfortunately we were not able to prove Esa1 presence at the promoter. We think that transient binding of Esa1 might be detrimental for ChIP analysis. Interestingly, in *esa* ^{ts} mutants Ndd1 was still efficiently recruited to the *CLB2* promoter (data not shown). Thus, either Ndd1 recruitment is independent on histone acetylation or Esa1 is not the only acetylase involved.

Data presented in this paper strongly support the idea that Ndd1 triggers chromatin remodeling as it is required for the recruitment of the Swi/Snf2 chromatin re-modifying complex. However, the question remains whether Ndd1 possess any intrinsic catalytic activity or whether it only acts as a recruiting factor for chromatin re-modifying factors. It is also possible that Ndd1 is involved in transcriptional initiation via the recruitment of the transcriptional machinery. Thus it should be determined whether Snf2 can be recruited and whether chromatin remodeling takes place at G2/M promoters, when Ndd1 is present but transcription can not be initiated because of a conditional Pol II allele (Nonet et al., 1987). There are data which suggest that Ndd1 most likely acts in the direct recruitment of the

transcriptional machinery as Ndd1 trans-located to an artificial promoter triggers transcriptional actovation. Interestingly this ability of Ndd1 to act as a transcriptional activator is highly dependent on its spatial proximity to the underlying chromatin, as Ndd1 which was grafted directly to the binding domain of Gal4 was 100 times more efficient than Ndd1 which was recruited by a Gal4BD-Fkh2 fusion construct (unpublished data). Here again it should be determined whether Snf2 can be found in this specific promoter context or not.

There is an second genetic connection between Ndd1 and chromatin modifiers. Cells deleted for *ISW2* and *ISW1* can bypass *ndd1* lethality (personal observation, (Sherriff et al., 2007). Even more interesting, it was recently shown that *isw*2 and *isw1* mutations affect the distribution of nucleosomes across the *CLB2* locus and that *CLB2* mRNA levels are increased in these mutants (Sherriff et al., 2007). From the distribution of nucleosomes in *isw2Δ* cells can be concluded that Isw2 is involved in promoter repression. To determine when and where Isw2 is recruited to G2/M promoters is particularly difficult because wild type Isw2 can not be annotated to specific loci at DNA via ChIP, most probably because of the high dynamics of its chromatin remodifying activity. However the catalytic inactive mutant of Isw2 was already successfully immunoprecipitated with specific DNA loci (Whitehouse et al., 2007), and could be used to determine the time point in the cell cycle when Isw2 influences G2/M promoters.



In our current view after START Ndd1 re-modifies the *CLB2* locus via or in parallel with the Swi/Snf complex. At the end of mitosis when G2/M promoters are shut down Isw2 might help to establish the repressive state of chromatin together with the re-appearance of Sin3. Whereas Sin3 as well as Ndd1 and Snf2 can be detected solely at the promoter region, from where they seem to influence the whole downstream locus, it is quite probable that Isw2 acts differently, as recent findings suggest that Isw2 is often recruited to the 3' ends of ORFs (Whitehouse et al., 2007) (see model).

5.4 Fkh2 serves as a platform for both, the Sin3/Rpd3 HDAC and Ndd1, whether and how does its modification influence the recruitment and activity of these two?

5.4.1 What is the role of the C-terminal domain of Fkh2 in transcriptional repression and activation?

Genetic data strongly suggest that the C-terminal domain of Fkh2 is involved in transcriptional repression and thus in viability and morphology of yeast cells (Hollenhorst et al., 2000; Hollenhorst et al., 2001; Zhu et al., 2000). These observations also fit to our finding that a full length Fkh2 is required for Sin3 recruitment to the *CLB2* promoter. However there are no convincing data which could explain how the C-terminal region influences transcriptional regulation of G2/M specific genes. In vitro experiments claim that two sites in the C-terminus of Fkh2 become exclusively targeted by the Clb5/6 associated kinase activity (Pic-Taylor et al., 2004). Unfortunately, our in vivo data are much less convincing, as we were not able to measure any changes in Ndd1 recruitment and/or in the onset of transcription, in the respective Fkh2 site mutants or in cells deleted for the respective cyclins (Jiri Veis diploma thesis, Helene Klug thesis). Even more confusing, it has been previously claimed that in the absence of the C-terminus of Fkh2 transcriptional activation of the *CLB2* cluster occurs independently on the absence or presence of Ndd1 (Reynolds et al., 2003), thus rendering the cell independent of this otherwise essential protein. In a suggested model, Ndd1 and the C-terminal domain of Fkh2 constitute a pair of antagonising co-actors, and the elimination of both re-establishes the wild type pattern of transcription and viability. Our data contradicts this model fundamentally, as we were not able to show any involvement

of the C-terminus of Fkh2 in transcriptional activation. In our hands the *Fkh2 1-584* mutant was not able to survive Ndd1 depletion (Figure 1), although the same mutation was referred to be an efficient *ndd1* lethality suppressor (Reynolds et al., 2003). This disagreement might be due to the fact that we introduced *FKH2* mutations in diploids, to avoid selection steps in haploid cells, which usually opens a window of opportunity for second site mutants and/or epigenetic modifications. Especially epigenetic changes seem to occur with high frequency in conditional *ndd1* mutants (unpublished data). Because of this, we prefer a model in which the C-terminal domain of Fkh2 mediates G2/M promoter repression by acting as a anchor for Sin3/Rpd3, to an alternative one where it works as a direct counter-actor of Ndd1.

The already mentioned finding that cells deleted for the C-terminus of Fkh2 are not able to bypass the lethality of Ndd1 depletion contradicts with the assumption that these cells do not recruit Sin3 to the *CLB2* promoter in vivo. One might believe that such cells should excel higher Clb2 levels thereby promoting transcriptional activation of the complete G2/M cluster. However, there are at least three different ways to resolve this antilogy. First, the above described C-terminal dependency was investigated solely at the *CLB2* promoter, and the gained insights might not be true for different *CLB2* cluster genes promoters, even though all of them are targets of Ndd1. Especially, the promoter of the *CDC5* gene was not included into the study although it recently became evident that the Cdc5 kinase is part of a positive feedback loop which contains Ndd1 and acts prior to the positive feedback loop annotated to Clb2. Second, the ability of *sin3* cells to bypass the Ndd1 dependency might be based on secondary effects, independently on its activity on Clb2 cluster genes in general. In a third view, the ambiguities of the Fkh2 C-terminus is explained by a possible involved in two opponent processes, such as are promoter repression and promoter de-repression or activation. Therefore, excision of the C-terminus alone would not have equal effects on the cell like the depletion of Sin3.

6 MATERIAL AND METHODS

6.1 Yeast

6.1.1 Cell culture

6.1.1.1 Growth conditions

Yeast pre-cultures were grown in the appropriate selective media or in YEP media containing either 2% glucose or 1% raffinose and 1% galactose. Cells were diluted (OD_{600} = 0.1- 0.2) and grown to OD_{600} = 0.6 in rich media. To induce cell cycle arrest, *bar1Δ* strains (at OD_{600}=0.4) were treated once with α-factor (1 µg/ml final concentration) during 1 replication time. To arrest *BAR1* strains, α-factor (0.5 µg/ml) was added 4-5 times (depending on the replication time of the strain) in intervals of 25 min. This results in a total concentration of 2-2.5 µg/ml. When mentioned in the figure, additional synchronization of cells released from α-factor block was performed by the addition of nocodazole (5 µg/ml final concentration). In order to induce cell cycle arrest in *GAL1-10 CDC20* strains, cells were grown in raffinose containing media for 2h. In time-course experiments, arrested cells were harvested by filtration, washed once with the respective media, and released in permissive and/or restrictive conditions. In experiments where cells were shifted from galactose to glucose conditions, 2% glucose was added 15' prior release, in order to suppress galactose driven promoters. The *cdc34^{ts} GAL1 SIC1V5V33A76* strain was grown in glucose containing media and arrested at 27°C with α-factor for 1 replication time. Cells were shifted to 37°C for 50 min (in order to adapt to higher temperatures) and subsequently harvested by centrifugation, washed twice with pre-warmed media, and released at 37°C in galactose/raffinose containing media .

6.1.1.2 SILAC media and growth conditions

based on (Gruhler et al., 2005)

Yeast SILAC strains were inoculated in YEP glucose media and grown over night. Next day ON cultures were diluted to = 0.1 and grown until OD_{600} = 1-2 in YEP glucose. The exact OD_{600} value was determined and the dilution factor for inoculation into SILAC media was calculated (e.g. 50µl of an OD_{600} = 1 culture which was inoculated into 50ml of SILAC

media should give an OD_{600} = 0,001). The appropriate amount of cells (usually 50-500µl) was removed from the YEP glucose culture and centrifuged 1' 4000rpm in an eppendorf fuge. YEP glucose supernatant was removed (!!) and cell pellet was taken up in a SILAC master media (consisting of SILAC plain media, 2% glucose and 100X SILAC drop out). The virtual inoculation OD_{600} was in the range of 0,0005 to 0,005. The master media was mixed well and splited into two equal parts, each one supplemented with either 100X arginine/lysine 12C or with arginine/lysine 13C stock solutions. Both cultures were grown ON for 14 to 18h (. Next day OD were measured and replication time was calculated. The replication times were 1h 40' for BY strains and 1h 30' for w303 strains with little differences between 12C and 13C, usually 13C being the slower. The overall difference in OD should not exceed 10%. When the slower culture reached OD_{600}=1 cultures were harvested simultaneously by centrifugation or filtration and frozen in liquid nitrogen. The more dense culture was taken up in the appropriate larger amount of buffer before mixing both cultures 1:1 thereby establishing the right ratio of cells.

SILAC plain media (1000ml)
(autoclaved):

Bifco Yeast nitrogen base	8g
Tyrosine	0,055g
Adenine	0,055g
Uracil	0,055g

100X U-13C6 L-Arginine:HCl (5ml)
(filter sterilized):

Cambridge Isotope Laboratories
25mg / 5ml H2O

100X U-13C6 L-Lysine: 2HCl (5ml)
(filter sterilized):
Cambridge Isotope Laboratories
25mg / 5ml

SILAC drop out (500ml)
(filter sterilized):

Histidine	0,5g
Iso-leucine	3,0g
Leucine	3,0g
Methionine	0,5g
Phenylalanine	3,0g
Threonine	2,5g
Tryptophane	2,0g
Proline	1,0g

6.1.1.3 FACS analysis

1ml of yeast culture (approximately 10^7 cells/ml or A_{600}=0.4-1.0) was transferred into a 1,5ml Eppendorf tube. For fixation and permeabilization, the cells were harvested, resuspended in 1 ml 70% ethanol and incubated (with shaking) for 1 hour up to overnight. (optional: cells were washed with 1ml 50mM TRIS/Cl pH7.5) and resuspended in 0,5ml 50mM TRIS/Cl pH7.5 containing 10µl of 10mg/ml RNAse A. Cells were incubated by shaking for 4 hour up to overnight at 37°C. Cells were resuspended in 0.5 ml FACS (200 mMTRIS/Cl pH7.5, 211mM NaCl, 78mM $MgCl_2$) buffer (optional cells were washed once with FACS buffer) containing 55µl of 0.5mg/ml propidium iodide. Aggregated cells were separated by sonication: (5 times 5 pulses power level 40%) 20-30 µl of the sonicated propidiumiodide cell-suspension was added into 1ml 50mM TRIS/Cl pH7.5 containing 5µl of 0.5mg/ml propidium iodide stock solution. Subsequently, 10 000 cells were measured for fluorescence using a Becton Dicinson FACScan according to the manual.

6.1.2 Strains

Strains used in this study:

Table I. Yeast strains

Strain	Genotype	Source
W303 1a	*MAT***a** *ho ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3-52 ssd1*	Rodney Rothstein
S1104	*MAT***a** *ssn6:: Kan*MX	Ramon Serano
K2771	*MAT***a** *cln1:: cln2:: cln3::LEU2* p*GALpr-CLN1*	Kim Nasmyth
K2957	*MAT***a** *ndd1:: LEU2 p[GAL1-10 NDD1 CEN URA3]*	Kim Nasmyth
K5875	*MAT***a** *cdc34-2^{ts}* p*GALpr-SIC1V5V33A76-HA1*	Kim Nasmyth
K7428	*MAT***a** *pGAL1-10 CDC20*	Kim Nasmyth
MK71	*MAT*α *sin3::HIS3*	David Kadosh
MK155	*MAT***a** *NDD1-HA6::HIS3*	Koranda et al. 2000
MK161	*MAT***a** *bar1:: NDD1-HA6::HIS3*	Koranda et al. 2000
MK195	*MAT***a** *pGAL1-10 CDC20 RPD3-HA6::HIS3* (from K7428)	Veis et al. 2007
MK196	*MAT***a** *pGAL1-10 CDC20 SIN3-HA6::HIS3* (from K7428)	Veis et al. 2007
MK257	*MAT***a** *SIN3-HA6::TRP1*	Veis et al. 2007
MK 259	*MAT*α *ndd1::LEU2 p[GAL1-10 NDD1 CEN URA]*	this study
MK267	*MAT***a** *SIN3-HA6::TRP1 fkh2::HIS3*	Veis et al. 2007
MK581	*MAT***a** *RPD3-HA6::TRP1*	this study
JV1	*MAT***a** *bar1::*	Veis et al. 2007
JV305	*MAT***a** *bar1:: SIN3-HA6::HIS3*	Veis et al. 2007

JV323	*MAT***a** *ndd1::KanMX p[GAL1-10 NDD1 CEN URA3]*	Veis et al. 2007
JV330	*MAT***a** *ndd1::KanMX p[GAL1-10 NDD1 CEN URA3] fkhΔ4572-862-Myc9::HIS3*	this study
JV331	*MAT***a** *ndd1::KanMX p[GAL1-10 NDD1 CEN URA3] fkhΔ4572-862-HA6::HIS3*	this study
JV332	*MAT***a** *ndd1::KanMX p[GAL1-10 NDD1 CEN URA3] fkhΔ4572-862::HIS3*	this study
JV333	*MAT***a** *bar1:: ndd1::KanMX p[GAL1-10 NDD1 CEN URA3] fkhΔ443-862::LEU2*	this study
JV343	*MAT***a** *ndd1::KanMX p[GAL1-10 NDD1 CEN URA3] fkhΔ585-862::HIS3*	this study
JV345	*MAT***a** *ndd1::KanMX p[GAL1-10 NDD1 CEN URA3] fkhΔ585-862-HA6::HIS3*	this study
JV349	*MAT***a** *RPD3-HA6::TRP1 sin3::HIS3*	this study
JV350	*MAT***a** *SIN3-HA6::TRP1 rpd3::HIS3*	this study
JV361	*MAT***a** *bar1:: sin3::HIS3*	Veis et al. 2007
JV363	*MAT***a** *bar1:: rpd3::HIS3*	Veis et al. 2007
JV367	*MAT***a** *ndd1::KanMX p[GAL1-10 NDD1 CEN URA3] SIN3-HA6::HIS3*	Veis et al. 2007
JV394	*MAT***a** *RPD3-HA6::HIS3*	Veis et al. 2007
JV396	*MAT***a** *ndd1::KanMX p[GAL1-10 NDD1 CEN URA3] RPD3-HA6::HIS3*	Veis et al. 2007
JV471	*MAT***a** *bar1::URA3 sml1::HIS3 rad53::HIS3 tel1::HIS3 [nat.pr. NDD1-HA6 CEN URA3]*	
JV509	*MAT***a** *SIN3-HA6::TRP1 fkh1::HIS3*	Veis et al. 2007
JV515	*MAT***a** *SIN3-MYC18::TRP1 cdc34-2^{ts}* p*GALpr-SIC1V5V33A76-HA1* (from K5875)	Veis et al. 2007
JV579	*MAT***a** *CAN1 arg4::KanMX lys1::KanMX*	this study
JV581	*MAT***a** *CAN1 arg4::KanMX lys1::KanMX RPD3-12HIS-3TEV-Bio::hph*	this study
JV583	*MAT***a** *CAN1 arg4::KanMX lys1::KanMX SIN3-12HIS-3TEV-Bio::hph*	this study
HEL324	*MAT***a** *cdc14-3 p[nat.pr. NDD1-HA6 CEN URA3]*	this study
HEL404	*MAT***a** *SIN3-HA6::TRP1 cln1:: cln2:: cln3::LEU2* p*GALpr-CLN1* (from K2771)	Veis et al. 2007
HEL428	*MAT***a** *bar1::URA3 sml1::HIS3 [nat.pr. NDD1-HA6 CEN URA3]*	this study
HEL476	*MAT***a** *bar1::KanMX sml1::KanMX mec1::HIS3 [nat.pr. NDD1-HA6 CEN URA3]*	this study

6.1.3 Transformation techniques

Yeast cells were transformed using the one-step transformation method (Chen et al., 1992). To achieve high transformation efficiency, the lithium acetate method was used (Schiestl and Gietz, 1989).

High efficiency transformation

▪ inoculate a fresh 2-3mm colony in 25 ml on culture ▪ dilute in 50, 150 resp. 300ml ▪ medium OD 0.2 - 0.3 ▪ 3h 30° ▪ pellet cells 3’ 2000 rpm (Heraeus) ▪ wash with 20 to 50 ml dH2O ▪ 3’ 2000rpm ▪ re-suspend cells in freshly prepared TE/LiAc (if starting from a 300ml cultures: 1,5ml TE/LiAc 100mM LiAc/ 1xTE), ▪ add 1 (plasmid) to 10 (linear) µl DNA and

10 µl Salmon Sperm DNA to a freshly prepared 600 µl PEG/ LiAc (0,5ml 10xTE, 0,5ml 1M LiAc, 4 ml 50% PEG 3350) solution. ▪ add 100 µl Yeast/TE/LiAc solution-vortex ▪ incubate30' on 30° shaking ▪ add 70 µl DMSO mix well DO NOT VORTEX ▪ 15' 42° ▪ 1-2' on ice ▪ spin down 4000 rpm 3' remove supernatant ▪ add 0,5 ml 0,5 X TE ▪ plate 200 to 500 yl ▪ (recombination) resp. 1:10, 1:100 dilutions (plasmid DNA)

6.1.4 Genomic tagging/deletion

1) The genomic ORF of *SIN3*, *RPD3* and *SNF2* was tagged at its C-terminus with a DNA fragment encoding 6HA-epitope tags using a PCR-based strategy and the *His3MX* gene (pGA2256) as marker (Knop et al., 1999). the resulting strain was checked by western blotting (YJV305).

2) Replacement of the C-terminal end of *FKH2* (YJV115, YJV147 from bp 1713 of the ORF to 411 bp downstream of the ORF) by 6 HA epitopes or by a NheI restriction site, has been done in the same way using plasmids pGA2256 (HA6-HisMX) and pGA2260 (NheI-HisMX). These strains were checked with control primers.

3) The *Δ443-862* C-terminal deletions of *FKH2* (JV333) was constructed using a plasmid where the *FKH2* reading frame was disrupted by the *LEU2* gene flanked by Stop- codons. DNA fragments obtained by cutting these plasmids at both sites of the marker gene were gell eluted and used for homologous recombination. Recombinants were checked using control primers situated inside of the *LEU2* gene and outside of the transforming DNA fragment.

4) The *BAR* gene was disrupted in several strains using pGA2200 cut with EcoRI and SalI. transformants (*bar::URA3*) were tested for α-factor arrest. The *URA3* gene is flanked by homologous regions and can be flipped out growing cells on FOA media.

5) The SILAC w303 strain was constructed by amplifying arg4::KanMX lys1::KanMX disruption cassettes out of the SILAC BY strain (JV439). Mata and Matα w303 wt like strains (K699, K700) were transformed with one of these cassettes. Subsequently, in the *arg4::KanmX* strain the *can1-1* locus was reconstituted by transforming the strain with a 300bp fragment of the functional *CAN1* allele and selecting the cells for viability on synthetic media. *CAN1* is an arginine permease and *can1-1 arg4::* strains are unviable on synthetic media as thay can not take up arginine. *CAN1* locus repair was confirmed by PCR analysis and viability assay. The *arg4::KanmX CAN1* strain was crossed together with the *lys1::KanMX* strain and backcrossed once with w303 wt to confirm co-segregation of viability and the *CAN1* locus.

6.1.4.1 Colony PCR

▪Take a pipette tip of a fresh colony directly after transformation and transfer into 19 µl H_2O in a PCR tube.▪ Do not homogenise by pipetting ▪ Boil for 40´´ at 600W in microwave▪. Add 6 µl Master-mix containing of:

- 2,5 µl 10x *Erich* Buffer
- 0,5 µl dNTP 10mM
- 0,5 µl Primer upstream ORF or tagging region 10pM
- 0,5 µl Primer downstream ORF or tagging region 10pM
- 0,5 µl Primer internal of newly introduced sequence 10pM
- 0,4 µl Tag Pol. 5U/µl

▪ Run PCR 30x 1´elongation time. ▪ Only fragments shorter than 2000bp can be detected.

6.1.4.2 Primers used for genomic tags and deletions

The following primers have been used:

1)

SIN3- tag-up	5′ AACGACTGGGAATACTGAATCTTCAGACAAGGGGGCTAAGATTC AATCCGGTTCTGCTGCTAG 3′
SIN3-tag-low	5′ ATGCGTAATGAAAGTGGCGAACAGCTAATAAGGACCAGACTTCC TCCCTCGAGGCCAGAAGAC 3′
SIN3-del-up	5′ CTTTGTTACTTCCCTCCCTCCACAACAGGCTACGCTATTGTCCG GTTCTGCTGCTAG 3′
SIN3-check-low	5′ ACACCGTCTTCAATCATGGC 3′

2)

RPD3- tag-up	5′ CGCAATATGCGAGGGACCTACATGTTGAGCATGACAATGAATTCT ATTCCGGTTCTGCTAG 3′
RPD3-tag-low	5′ TTCTTCGAAACGTATGGGACGCGGTTGATGTATGAACGGAACGCA TCCCTCGAGGCCAGAC 3′
RPD3-check-low	5′ TTATCAACAGCGGTGGGACG 3′

3)

SNF2- tag-up	5′ TGAATCTTTCACAGATGAAGCGGACTCGAGCATGACAGAAGCGA GTGTATCCGGTTCTGCTGCTAG 3′
SNF2-tag-low	5′ CGGCACGTTATACTAGAGATATTAAACATCCCAACTCGGTTAAT GGGTACCCTCGAGGCCAGAAGAC 3′
SNF2-check-tag	5′ GAGGGTTCAGTAGTATACG 3′

4)

FKH2(*1-1713*)- tag	5′ AAGCTCCCAATTCAAATGCTAACTTAAATCAAAACAATATGAAA GAATACTCCGGTTCTGCTGCTAG 3′
FKH2-d-SC	5′ TGAAAGAATACAAAGAGTCACTTCATCCGCCTGCAATATCAATA TCACAATCCGGTTCTGCTGCTAG 3′
FKH2-tag-low	5′ CCCAGGAGACGCTTTAGCGGGCAGTTTCAGGTTCTAGGAGCGTT CTCCGCCTCGAGGCCAGAAGAC 3′
FKH2-ch-low	5′ GAAAATCACCCACTTGGGGAC 3′
FKH2-ch-up	5′ GTGGTAGCACCGCCAATGCC 3′

5)

*ARG*kanup	5′ GCGGCCGCCTCATTGGCAGAATCC 3′
*ARG*kandown	5′ CCCTGAAACGCTTGAG 3′
*LYS*kanup	5′ GCGGCCGCCCTGCGATTTCAGCGA 3′
*LYS*kandown	5′ GTTCTTGCTGGGAAATG 3′
*CAN1*_fw	5′ GTTCTTCAGACTTCTTAACTCCTG 3′
*CAN1*_re	5′ ACCTGTACCAATAGTACCACCAAG 3′

6.2 DNA techniques

6.2.1 Cloning

Standard DNA cloning techniques were used according to *Sambrook et al., 1989* and *Ausubel et al., 1995*. Gel extraction was performed using QIAEX gel extraction kit. Ligations of DNA fragments were either performed with the DNA Rapid Ligation Kit for 30’ at room temperature, or with T4-DNA ligase at 16°C over night. To prevent self ligation DNA vectors were usually de-phosphorylated with Shrimps Phosphatase for 10’ at 37°C and phosphatese activity was inhibited after 20’ at 65°C. Competent E.Coli were transformed and plasmid was recovered according to the protocols.

6.2.1.1 Competent E.Coli

▪ Grow over night culture in 20ml 2xTY. ▪ Dilute the culture in 400ml 2xTY to a concentration of OD_{600} = 0,1. ▪ Grow the culture till OD600= 0,4. ▪ In the meantime prepare 200ml of sterile 0,1M $CaCl_2$, and 5ml of sterile 50 mM $CaCl_2$/15% glycerol, store at 4°C). ▪ Centrifuge cultures in 8 pre-chilled tubes 4000rpm/5min at 4°C. ▪ Resuspend the pellets in 25ml 0,1M $CaCl_2$ and pool in 4 tubes (work at 4°C). Leave on ice for 30 min (coldroom). ▪ Centrifuge 4000rpm for 8min at 4°C. ▪ Resuspend the pellets in a total volume of 3,5ml 50mM $CaCl_2$/15%glycerol. ▪ Aliquot 200µl to 500µl in eppendorf tubes and freeze immediately in liquid nitrogen. ▪ Store at –80°C. ▪ To test the cells transform 1µl of 1:100 and 1:10 000 dilutions of a control plasmid.

6.2.1.2 E.Coli transformation

▪ 70µl of competent E.Coli are thawn on ice, 10 µl of DNA ligation are added. ▪ 20' on ice. ▪ 7' heat shock at 37°C. ▪ Addition of 1ml 2xTY medium. ▪ 1h shacking at 37°C. ▪ Centrifugation 4000rpm and plating on selective medium.

6.2.1.3 Mini Prep (Quiagen)

▪The pellet of the over night culture (3-5 ml) is deluted in 0,3 ml of P1 in 0,3ml P1 (pellet dilution buffer, including RNAse). ▪ Addition of 0,3ml P2, invert, 5' RT. ▪ Addition of 0,3ml P3, invert, 5' ice. ▪ 10' 13000rpm 4°C. ▪0,8ml supernatant transferred in new vial, 0,56ml isopropanol, vortex. ▪ 30' 13000rpm 4°C, ▪ dry pellet for 15' in speed vac. ▪ disolve pellet in up to 80 □l 0,5xTE

6.2.1.4 Midi Prep

▪grow 50ml TY Amp over night culture ▪ spin down in 50ml Falcon tube for 3´ at 4000rpm, RT ▪ wash with 10ml Sol I (4ml 0.5M EDTA, 5ml 1M Tris pH 8, 9ml 20% glucose, fill up to 200ml with dH_2O) ▪ spin down again 3´,4000rpm, RT ▪ resuspend in 5ml Sol I ▪ add 10ml Sol II (176ml dH_2O, 4ml 10M NaOH, 20ml 10% SDS), mix gently ▪ 5´ on ice ▪ add 7.5ml Sol III (120ml 5M KOAc, 23ml HOAc, 57ml dH_2O), mix normally ▪ 20´ on ice ▪ spin down for 10´ at 4000rpm, 4°C ▪ filter through Miracloth in new Falcon tube ▪ add 20ml of isopropanol (RT), mix ▪ spin down for 10´at 4000rpm, 4°C ▪ pour out supernatant, leave pellet to dry a little ▪ dissolve pellet in 350µl of dH_2O ▪ add 200µl 5M LiCl ▪ 20´ on ice ▪ spin down again for 10´ at 4000rpm, 4°C ▪ transfer supernatant into new eppendorf ▪ add 1 volume isopropanol ▪ spin 10´ at 14000rpm, 4°C ▪ wash with 70% EtOH (-20°C) ▪ dry pellet ▪ dissolve in 150µl to 400µl of TE (or 0.5xTE)

6.2.1.5 Primers used for cloning

1)

The following primers were used to clone truncated versions of Fkh2 into pGBT9. They all exhibit additional restriction sites, so that the PCR products could be ligated directly into the desired plasmid. These sites are indicated by the primers names. RI stands for EcoRI, N for NotI and P for the PstI restriction site. *FKH*2-17M-Pst can be only used for PCR reactions with the *FKH2ΔC-MYC3* (pMK372) gene as template, which already exhibits two NotI restriction sites flanking the *MYC3* gene.

2)

The following primers were used in order to modify pCT468 (Wolfgang Reiter pers. com.) in order to create pWR 159

1)

R1 Fkh2	5′ TTTGAATTCATGTCCAGCAGCAATTTTAACG 3′
Fkh2-N-Pst1	5′ TTTCTGCAGTTAGCGGCCGCCGTTGTTGATAA 3′
Fkh2-13N-Pst1	5′ TTTCTGCAGTTAGCGGCCGCTTGCTACTGAGGAT 3′
Fkh2-9N-Pst1	5′ TTTCTGCAGTTAGCGGCCGCTGATGGTATTTGG 3′
R1-2-Fkh2	5′ TTTGAATTCAAGAACATGGCGACGGAAAT 3′
Fkh2-6-N-Pst	5′ ATTTGTATAGAACACGGCGGCCGCTAACTGCAGAAA 3′

2)

HBTchangeup	5′ GCGGCCGCTATCCGGTTCTGCTGCTAGGGGTTCACATCAT 3′
HBTchangelow	5′ GCGGCCGCCCTCGAGGCCAGAAGACTCGACACTGGATGGC 3′
EcoRI-HTB	5′ GCGAATTCGCGGCCGCTATCCGGT 3′
XbaI-BIO	5′ GAGTCTAGAACCGCCGCACTAGCTC 3′
HIS-XbaI	5′ GGTTCTAGACTCTGATTGAAGTACCAG 3′

6.2.1.6 Plasmids

Plasmids used in this study are listed in Table II.

Table II. Plasmids

Plasmid	Genotype	Source	
pAS40	YCPlac33	*NDD1-HA3* nat.pr.	Veis et al. 2007
pJV251	pGBT9	*GAL4BD-FKH2*	Veis et al. 2007
pJV287	pGBT9	*GAL4BD- fkh2 1-306*	Veis et al. 2007
pJV292	pGBT9	*GAL4BD- fkh2 1-443*	this study
pJV292	pGBT9	*GAL4BD- fkh2 1-306 R87A H123A*	Veis et al. 2007
pJV301	pGBT9	*GAL4BD-fkh2 156-306*	this study
pJV403	pGBT9	*GAL4BD-fkh2 1-194*	this study
pJV405	pGBT9	*GAL4BD-fkh2 57-194*	this study
pJV408	pGBT9	*GAL4BD-fkh2 57-306*	this study
pWR159	YCPlac33	12HIS-3TEV-Biot::hph	this study

6.3 Isolation and analysis of RNA

based on (Cross and Tinkelenberg, 1991)

Yeast cultures were collected at an OD_{600}= 0.6, the RNA was isolated and 18 µg RNA was subjected to Northern blot. . The DNA fragments that were used as hybridization probes were labelled by random priming. For detection of *SWI5* transcript, a 1.5 kb *SalI* fragment from C2847 (Manfred Koranda thesis,), for *CLB2*, a 380bp *EcoRV* fragment from pUS5 (Manfred Koranda thesis), and for *CMD1*, a 300bp fragment derived from a genomic PCR amplification. The protocol was modified according to Veerle de Wever.

*CMD1*up	5′ GTCCTCCAATCTTACCGAAG 3′
*CMD1*low	5′ TTACAAGTAGAATCCATTTAGATAACAAAGCAGCG 3′

Detailed protocol:

Culture harvest

▪take FACS probes ▪ collect 25ml culture in a precooled (on ice, 2-3 hours in the coldroom is enough, filed with 25ml of autoclaved H2O) Falcon (50 ml)tube ▪ Centrifuge the culture 2' (when harvesting pseudohyphal cells, at least 5') 4000 rpm 4°C, ▪ Cold room : pour off supernatant (SN), re-suspend in 0,8ml pre-cooled DEPC H_20 ▪ Transfer sample to a pre-cooled 2 ml safe lock eppendorf (use a cleaned (EtOH) pipette) ▪ Spin 30" 14000 rpm 4°, take off SN (pipette) ▪ Freeze (-20°C) or use immediately for extraction

RNA - extraction: (use gloves and work on ice)

▪ Thaw samples on ice ▪ Add 200 µl extraction buffer & re-suspend (keep eppendorfs on ice !) ▪ Add 200 µl PCI solution) ▪ Add glass beads (1/2 to 2/3 of total liquid volume is enough) ▪ Vortex in the cold room for 20' at maximum speed (check if your save lock eppendorf. is o.k.) ▪ Spin 15' 14000 rpm 4°C ▪ Take off the upper/aqueous layer and transfer to a new pre-cooled eppendorf ▪ (take about 160 µl - experience does it – but do NOT touch the interface) ▪ (from now on, you can use a 1,5 ml ep) ▪ add an equal volume of CI, mix by flicking (you can add the CI before and pre-cool it ▪ together with the eppendorf, to proceed more rapidly) ▪ spin 10' 14000 rpm 4°C ▪ take off the upper layer (about 130 µl) & transfer to a new precooled eppendorf (including 130 µl CI), mix by flicking ▪ spin 10' 14000 rpm 4°C

RNA precipitation

▪ take off upper layer (about 100 µl) & transfer to a new pre-cooled eppendorf ▪ Add 1/20 volume 4M NaAc (pH +/- 4,2) ▪ Add 2 volumes EtOH 100% (do not !! make a master mix out of EtOH and NaAc), mix by flicking ▪ Put in the –20°C for approx 30' ▪ Spin 10' 14000 rpm 4°C ▪ take off the supernatant ▪ add 150 µl 80 % EtOH (just add gently, do not mix in any way) ▪ centrifuge (2' 14 000 rpm 4°C) ▪ take off SN (use the 200 µl pipette and take as much as possible) & put the opened eppendorfs in the 37°C room for approx 15' until they are dry ▪ re-suspend in 50 µl DEPC H2O (depending on the pellet size, experience does it …)

RNA concentration measurement- (immediately after RNA precipitation)

▪ split the sample in two parts by transferring 25 µl in a new pre-cooled eppendorf tube (leads to more correct RNA concentration). ▪ Store the other 25µl -20°C (storage at -80°C caused sometimes degradations) and continue with the transferred 25µl samples (keep them on ice) ▪ measure nucleotide content and purity in a 2,5µl /1000µl dilution of the sample at $A_{260/280}$ (A_{260} = 1 corresponds to 40µg/ml RNA concentration)

Sample preparation (should be performed immediately after RNA measurement)

▪ In order to achieve a final concentration 18µg RNA per slot (18µg/20µl), add to the residual 22,5µl RNA sample corresponding amounts of DEPC H2O, FGRB, Formamide and Formaldehyde (freshly prepared FFF master mix) ▪ should give a final concentration per 20µl sample: 10µl formamide, 3,5µl formaldehyde., 2µl 5XFGRB) ▪ denaturate RNA by incubating it for 15'at 65°C ▪centrifuge for 5" and store on ice ▪ add the respective amount of sterile RNA loading buffer ▪ store at -20°C (samples stay liquid and can be used for aproximatly 6 months)

RNA Gel

▪ Incubate combs and trays overnight (or at least for 6h) in a 0,1M HCl ▪ For the Gel :▪ Dissolve 1,47 g agarose in 80,6 ml DEPC-H2O DEPC-H2O (do not cool it down, just wait about 5') ▪ Under the hood : Add 26 ml 5X FGRB first and mix (final concentration : 1M) ▪ Add 23,4 ml formaldehyde (37% stock solution), mix (final concentration : 2,2 M) ▪ cast gel – leave for approx 30' at RT▪ run gel in 1X FGRB (dilute the 5X FGRB in fresh H_20)▪ pre run : approx 20-30V for minimum 30' ▪ load the samples directly from the freezer ▪ run :approx 60 – 80V ▪ turn gel and electricity lines approx every 30 minutes

Northern blot

▪ Transfer to nylon membranes was performed over night as described in *F M Ausubel et al. 1991* using 20x SSC as buffer ▪ To check the transfere, the membranes were washed to remove the rests of agarose, UV fixed and stained in a solution of 0,05% methylene blue in 0,5 M NaAc, pH 5.5.

Hybridization

▪ After pre-hybridisation of the membrane for about 6h at 65°C fresh hybridisation buffer containing radioactive probe is added. ▪ Pre-hybridisation is performed over night and the membrane is washed 2X at room temperature and 2X at 63°C with washing buffer for 15' until background radioctivity is removed. ▪ Membranes are wrapped in kling film and exposed in a PhosphoImager exposure cassette from 4h to over night. Data were visualised with PhosphoImager 840 and Image Quant.

Preparation of radioactive probes

▪ Cut linearized DNA band out of low melting agarose gel and place it in an eppendorf the weight of the agarose should not exceed 20 µg ▪ Aliquots can be stored at - 20°C. ▪ fill up to 26,5 µg/µl with dH2O ▪ add 10 µl of random primer buffer (STRATAGENE Prime-It® II kit was used, containing 9er primers) ▪ Denaturate DNA probes by boiling it for 5' at 95°C ▪ Add 10 µl dATP buffer and 1 µl klenow enzyme. ▪ Add in the hot lab 2,5 µl alphaP32 dATP and incubate at 37°C for 30' ▪ add 50 µl TE to increase your working volume and to stop the reaction ▪ put the total probe over a sephadex column to remove un-incorporated nucleotides and spin it through with the quick spin bottom (2000 rpm) ▪ take out the flow-through with a pipet and tansfer to an eppendorf. ▪ Denaturate the probe by placing the eppendorf tube at 95°C for 2' 30" and add immediately to the pre-hybridisation buffer.

Sephadex columns

▪ take a 1 ml syringe, take out the plunger, introduce a small piece of Whatmann paper on the bottom and fill the syringe with Sephadex resin in TE, until the resin is 5-10 mm from the top. Place the whole in a plastic test tube and then in a 50 ml Falcon tube▪ quick spin to 2000 rpm, discard the flow through.

Extraction buffer : (filtersterilize - store at room temperature)

50 mM Tris pH 7 – 7,4
130 mM NaCl
5 mM EDTA
5% SDS

PCI : 25:24:1 (store at 4°C) (RNA : acidic pH – DNA : neutral pH)
CI : 24 : 1 (store at 4°C)

DEPC H20 : 0,1% DEPC in H20 (autoclaved)

FGRB 5X :

0,1 M MOPS pH 7,0
40mM NaAc
5mM EDTA pH 8,0

for 500 ml :

dissolve 10,3 g MOPS in 400 ml (DEPC-H2O - 50mM NaAc) or add the NaAc later to the DEPC H2O, to a final concentration of 40 mM
adjust the pH to 7,0 with 2N NaOH
add 5 ml 0,5 M EDTA pH 8,0
adjust to 500 ml with DEPC H2O
filter sterilize – store at room temperature, keep away from light.

RNA Loading Buffer :
50 % glycerol
1 mM EDTA pH 8,0
0,25 % bromophenolblue
0,25 % xylene cyanol FF

Prehyb buffer :
0,5 M Sodium phosphate buffer (19.3 g/l NaH2PO4 + 64g/l Na2HPO4 results in a pH of about 7.2)
7% SDS
1mM EDTA pH 8

(one can make stock solutions of 1M Sodium Phosphate buffer, 20% SDS and 0,5 M EDTA pH8)

Hyb buffer :
the same as the prehyb buffer, but with the denaturated radioactive DNA probe added.

washing solution :
0,5 X SSC, 0,1 % SDS

stripping solution :
0,1X SSC, 0,1% SDS

6.4 Proteins

6.4.1 Antibodies

6.4.1.1 Antibodies used in ChIP analysis

α-HA: 12CA5 hybridoma cells (Egon Ogris); α-Myc: 4A6 (Upstate); α-H4: ab10158 (Abcam); α-acetyl-H4: 06-866 (Upstate); α-Sin3:sc-17637 (Santa Cruz)

6.4.1.2 Antibodies used in Western Blott analysis

α-HA: 12CA5 hybridoma cells (Egon Ogris); α-Myc: 4A6 (Upstate); HRP-penta His conjugate (Qiagen); Streptavidin-HRP conjugate (21126: Pierce Biotechnology)

6.4.2 Yeast Protein Extract in 8M UREA

▪ 10 ml of logarithmically growing cells ($OD_{600:}$0,6-1,0) (resp. 1ml ON culture) were harvested by 30'' 12000rpm ▪ pellet was taken up in 400 µl UREA buffer (for 50ml: 24g urea, 3ml 5M NaCl, 2,5ml 1M Tris/HCl pH8, 2,5ml 1M Na-PO4 pH8, 250µl Nonident P-40) ▪ Tranfer into screw cap tubes together with 400µl beads ▪ Fast Prep FP120 3x 45'' power level 5,5▪ Spin 10' 12000 rpm. Take of 100µl of supernatant and add 100µl 0f 2X SDS loading buffer (5ml: 200µl Tris pH6,8 1M, 2,4g urea, 2,5ml SDS10%, 1µl EDTA (0,5M pH8), 50µl β-mercapto EtOH bromphenolblue) ▪ 3-5' 95°C

6.4.3 Yeast TCA lysate

▪ 25 ml of logarithmically growing cell (OD_{600}: 0,6-1,0) were harvested and pellet weight was determined▪ pellet was taken up in 200 µl 2M NaOH in 5% b-Mercaptoethanol / 100 mg pellet▪ 10 min on ice ▪ After addition of 40 mg 50% trichloro acetic acid /100 mg pellet, samples were vortexed for 30 sec and incubated for another 10 min. on ice▪ The samples were than spun down for 3 min at 4000 rpm, washed with 1M Tris, pH 7.5and pellet volume was determined again. . ▪ The samples were resuspendet in 100 µl SDS sample buffer (4ml H_2O, 1ml 0.5M Tris pH 6.8, 0.8ml 100% Glycerol, 1.6ml 10% SDS, 0.4ml β-Mercaptoethanol, 0.1% Bromphenolblue) . ▪ After 5' at 95°C and centrifugation supernatant was transfered and 5 µl used for small gel. ▪ The rest was stored -20°C.

6.4.4 Yeast Protein Extract by TRIzol

Protocol is based on (Ficarro et al., 2002) and on Invitrogen TRIzol protocol

Prepare a 40 ml O/N culture in *Silac full media*

▪ Inoculation of 40 µl of a logarithmic growing culture OD(~ 0,6) in 40 ml Silac medium gives with a respective replication time of $1^{50'}$ of BY strains in Arg, Lys supplemented synthetic media a density of OD1 in about 19 to 20h.

Break-up of the cells

▪ 40ml stressed respectively unstressed cells of OD1(≈10^7 cells/ml) are harvested by centrifugation (1' Heraeus full speed, pour off supernatant, transfere with residual liquid to eppendorf)
- Remove the remaining liquid by centrifugation 12000x g, 1' and freeze in liquid nitrogen
- Resuspend in 1,5 ml TRIzol reagent (to be added under the hood as it represents a mixture of Phenol and Guanidinsisothiocyanat), pool the respective stressed and unstressed ^{12}C and ^{13}C fractions (pool in 10ml Greiner tubes),
- Split up into two Screw tubes together with about 300ml sterile glass beads each (the architecture of the experimant should be maintained! e.g. 80ml of culture is broken up in 4 tubes)
- Homogenize with three consecutive sessions of 45s each in a Fastprep FP120 shaker (4°C, power level 6)
- Hole the bottom of the screw cap tube with the tip of a sharp thin razor blade and transfer the liquid to a 1,5ml eppendorf tube by centrifugation at 2000rpm in a Heraeus fuge at 4°C for 1' (Inserted in a precooled 50ml Greiner tube). / Alternatively: remove 600µl of the supernatant without beads with pipette (4°C)
- Remove insoluble material from the homogenate by centrifugation at 12000 x g for 15 at 4°C
- Remove the supernatant (2 x 700µl) in a fresh eppendorf
- Incubate for 5' at RT
- Add Chloroform 2 x 140 µl and shake 15'' vigorously by hand
- Incubate for 2'-3' at RT
- centrifugation at 12000 x g for 15 at 4°C
- Phase-separation occurs
- The aqueous phase 420 µl contains exclusively RNA,
- Organic phase contains DNA and Proteins

- **RNA purification (optional)**

- Add 700µl Isopropanol to the aquous phase, mix by hand
- 10' RT
- 10' 12 000 x g 4°C
- Wash with 2 x 150µl 80 % EtOH (0°C)
- 2' 12 000 x g 4°C
- Remove supernatant, dry at 37°C (do not dry too much)
- Resuspend in 2 x 50 µl DEPC H_20, store at - 20°C

- **DNA precipitation**

- Remove the remaining aqueous phase overlaying the interphase and the organic phase
- Add 2 x 225µl of 100% EtOH, and mix by inversion
- Incubate for 2-3' at RT
- Sediment DNA by no more than 2000 x g for 5' at 4°C
- Transfere the phenol-ethanol supernatant into a new 2ml eppendorf

- **Protein precipitation**

- Add 2x 1.125 ml of Isopropanol
- Incubate for 10' at RT
- Sediment Protein by 12000 x g for 10' at 4°C
- Protein wash (to be repeated 3x), the 3rd wash step should be used to transfer protein pellet into an 1,5ml eppendorf, be very careful not to lose any protein
- Add 2x 1 ml 0.3M guanidine hydrochloride in 95% EtOH
- Incubate for 20' at RT
- Centrifuge 5' at 4°C 7500 x g

- **Protein wash (1x)**

- Add 2x 1 ml 95% EtOH
- Incubate for 5' at RT
- Centrifuge 5' at 4°C 7500 x g

- **Re-dissolving the protein pellet**

- Vacuum dry for 1-5' prevent the pellet of getting dry
- Add 1ml 8M Urea, NH_4HCO_3
- incubate 1h by RT shaking
- cool the probe on ice 10'
- 5 x 2'' sonication power level 5
- Sediment by 10 000g for 10' at RT
- when pellet to big (90% of the original pellet should be resolved repeat previous 4 steps

6.4.5 His-TEV-Biotin Tandem Tag Purification

based on (Tagwerker et al., 2006a; Tagwerker et al., 2006b)

- **(harvest)** transfere 2 x 100 ml yeast culture (JV 581, w303, Rpd3-HTB; OD_{600nm}=1 to 1,2) grown in *SILAC synthetic media* in 4 x 50 ml precooled plastic tubes
- Spin for 2' at 4000 rpm in Heraeus fuge 4°C
- Discard supernatant, transfer together with residual liquid in 4 precooled 1,5 ml Eppendorf
- Spin for 20'' at 13000 rpm in eppendorf fuge 4°C
- Remove supernatant and freeze in liquid nitrogen
- Thaw on ice and re-suspend both pellets in 4x 1ml (respectively more ml, when OD600 values exceed 1 in order to achieve ^{12}C to^{13}C equilibrium) of freshly prepared **Buffer I (Guanidium buffer stock solution including Complete tabs and PMSF)**
- Mix 2ml of ^{13}C with 2ml of ^{12}C cultures and split up in 10 screw cap tubes including 500µl beads (RT)
- Break up cells by 3 x 45'' in Fast Prep FP120
- power level 4,5 at 4°C
- Hole the scew cab tubes and transfere the lysates to 1,5ml eppendorf tubes by spinning at 2000 rpm in Hereaus fuge 2' (inserted in a 50ml tube)
- Spin 15 000 x g for 15' 4°C
- Pool supernatants for total 4ml(aliquots for western: [**A1=**. Add 450µl 100% EtOH, fuge 10' 15000rpm 4°C, remove supernatant take up in 50µl Buffer II and 50µl of 2X SDS-β–MercaptoEtOH and freeze at -20°C)
- Incubate lysate in a 15ml Tube at RT with 400µl Ni2+ Sepharose slurry for 5-6h (pre- equilibrated by washing slurry 1x 20' with 1ml of Buffer I at RT)
- Spin 3000 rpm 20'' and remove supernatant (keep **A2 =** 50µl/2 ml; treat exactly like A1)
- Wash beads with 4-6 ml **Buffer II (UREA pH8-8,5)** 4'RT 300 rpm 1' (keep aliquot from supernarant for western 50µl/1ml)
- Wash 2 times with 4-6 ml **Buffer III (UREA; pH 6,3-6,5)** 4'RT, spin 3000 rpm 20'' (unifie wash supernatants and keep aliquot for western; 50µl), use the second washing step to transfere the beads into a 1,5ml Eppendorf tube
- Elute with 0,666 ml **Buffer IV (UREA; pH 4,3; EDTA; was in reality pH 3,8)** 10'RT, spin 3000 rpm 20''
- Repeat elution step 2 times and unifie all 3 eluates (to a total of 2ml)
- Spin the elutae 20'' 3000rpm to remove residual Sepharose Beads. Transfer the eluate into a new 2ml eppendorf tube.
- Adjust pH8 by adding 30µl 2M NaOH to 1ml of eluate [**A3=** 20µl/1 ml. Add 20µl of 2X SDS-β–MercaptoEtOH and freeze at -20°C)
- incubate ON (10-12h) with 80µl pre-equilibrated Streptavidin-Agarose beads [by washing slurry 20-30' in 1ml **Buffer Buffer II** at RT]
- spin 3000 rpm 20'' to remove supernatant (**A4=** 50µl/1 ml. Add 20µl of 2X SDS-β–MercaptoEtOH and freeze at -20°C)
- wash 6 times 4' with 0,5 ml Buffer VI **(UREA; Tris;)**
- deliver to mass spektrometry unit

(!!sterilize all buffers !!)

Buffer I (10 m):

GdmHCl pH 8,8 6M
1M Tris/HCl pH8 50mM
TritonX100 0,10%
NaF 5mM
prepare freshly:
100mM PMSF 1mM
complete (1MiniTab/10ml)

Buffer II (50ml):

urea 8M (24,00g)
5M NaCl 300mM (3ml)
1M Na-PO4 pH8 50mM (2,5ml)
Tween 10% 0,01% (50,00µl)

Buffer III (=Buffer II pH6,3-6,5):

Buffer II 10ml
2M HCl 150µl
Buffer IV (Elution Buffer):

Buffer IV(=Elution Buffer pH4,0-4,3):

BufferII 10ml
2M HCl 260µl
0,5MEDTA pH8 200µl

Buffer VI (10ml):

Urea 4,8g
5MnaCl 0,4ml
1Mtris/HCl pH8 1ml

6.4.6 TEV cleavage

- wash 6 times 4' with 0,5 ml TEV Buffer, spin at 4000 rpm
- cleave with 150µl cleavage buffer for 1,5h at 30°C rotating
- spin 4000 rpm and remove the supernatant
- wash beads 1' at RT with 2X SDS-β–MercaptoEtOH buffer
- spin at 4000 rpm, remove the buffer and ad to the first supernatant
- run a NuPAGE Gel at 200V
- stain 2h, de-stain H2O over night and deliver to mass spectrometry

TEV cleavage buffer	150	µl
TEV protease	0,75	µl
H2O	142	µl
DTT	1,5	µl
20XTEV buffer	7,5	µl

6.4.7 Western blot

Western-blot analysis was performed as described by (Piatti et al., 1996).

▪ All protein samples were separated on 6-10% poly-acrylamide gels in Tris running buffer (0.375 M Tris, pH 8.8, 0.1% SDS).▪ The stacking gel contained 4% acrylamide in stacking buffer (0.125 M Tris, pH 6.8, 0.1% SDS). ▪ The electrophoreses was performed at 20 mA per gel. ▪ The proteins were transferred onto nitrocellulose membrane in a semidry transblot cell (BioRad). The apparatus was run for 1.5 hours at 1.2 mA/ cm2 using Tris running buffer (0.375 M Tris, pH 8.8, 0.1% SDS). The membrane was afterwards treated with Ponceau S, to check thr protein transfer. ▪ to block unspecific protein binding the membrane was incubated in 10 ml 1X PBST (8 g/ l NaCl, 0.2g /l kCl, 1.15 g/l Na_2PO_4, 0.1% Tween20) containing 4% milk powder for 1.5 hour. ▪ Then the primery antibody was added (10 µl of monoclonal α-HA in PBST/ Milk) and incubated for another hour. ▪ After 3 times washing with 1XPBST the membrane was incubated for 30 min with the secondery antibody in 20 ml PBST (0.7 µl of α-mouse antibody coupled to horse radish peroxidase). ▪ Detection was performed using the Amersham life Science Company ECL+ staining system. After incubation with the reaction agent, the membrane was wrapped in cling film and exposed to light sensitive film.

6.4.8 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation PCR assays were performed as described (Cosma et al., 1999) with minor modifications. The cell extracts were sonicated using a Virsonic 100 cell disrupter (VirTis). The gels were photographed using Gel Doc 2000 (Biorad).

ChIP detailed protocole

1st day

▪Dilute over night culture of 5 ml selective medium in 50 ml YEPD (experiments with nocodazole arrest) or selective medium (normal Chip) to a conc of OD_{600} 0.2.
▪ Harvest the cultures at exactly OD_{600} 0.6, add 1,5 ml Formaldehyd and shake for 10' at RT. ▪ Add 3 ml 2,5M glycine (unbuffered) and incubate for additional 10' at RT shaking and store them at 4°C until all cultures are finished.
▪ Centrifuge for 2' 4000 rpm in 50 ml tube and wash pellet 4x with 15-25 ml 4°C TBS (20 ml 1M Tris pH7.5, 30 ml 5M NaCl in a total of 1l H_20),4x 2' 4000 rpm.
▪ Discard the buffer and remove the rest with pipet.

▪ you can store the pellet over night at –20°, (however, I never did this)
▪ Resuspend the pelet in 600µl lysis buffer (freshly prepared and stored at ice), and transfer the suspension in srew cap vials
▪ Put 4 vials in aplastic tube and shake vigorously 8-10 min, and check under the microscope if about 70% of the cells are lysed.
▪ Hole the vial with hot needle in the coldroom and fuge them in eppendorf.
▪ Sonicate the samples 2x 30'' and 1x10'', leave them on ice.
▪ Centrifuge 10' full speed at 4°C.
▪ Transfere suppernatants in new vials and remove 1/50 volume of any one sample to an additional vial (represents WCE).
▪ Add 30 µl of ready to use anti body susupension (not in WCE, store WCE at ice).
▪ Incubate for 1,5 h at 4°C pivoting.
▪ Wash the magnetic beads using the magnetic tray:
2x with 1ml lysis buffer
2x with 1ml lysis buffer + 360 mM NaCl
2x with 1ml washing buffer
1x with 1ml 4°C 1XTE
▪ Spin down 3' 300rpm, and discard the suppernatant completeely.
▪ Add 50µl elution buffer (TE+1% SDS) and incubate for 10' at 65°C shaking.
▪ Spin 5'' 13000 rpm give in the magnetic tray and transfer 30 µl in a new vial.
▪ Add 30 µl elution buffer to the vial with magnetic beads and repeat the elution.
▪ Add to the 60 µl sample 240 µl of elution buffer and incubate over night at 65°C.
▪ Add 240 ml ml of elution buffer to the WCE sample (which persisted all the time at ice) and incubate at 65°C over night.

2nd day

▪Add to the samples 301µl of the following mastermix (280µl TE, 6µl glycogen, 15µl proteinase K).
▪ Incubate 1.5h at 37°C.
▪ Add 600µl PCI, vortex and centrifugate 30' 13000rpm.
▪ Remove upper layer, transfere to a new vial, repeat extraction with 600µl PCI, centrifugate 20' 13000 rpm.
▪ Remove upper layer, transfere to a new vial, add 600µl CI, vortex and centrifugate 5' 13000rpm.
▪ Remove upper phase in a new vial add 5M NaCl to a final concentration of 200mM (20µl in 500µl sample), vortex.
▪ Add 600 µl isopropanole, vortex and incubate for 15' at –20°C.
▪ Fuge 10'13000 rpm at 4°C
▪ Remove supernatant and dry the pellet in the speed vac.
▪ Dissolve the pellet in 30µl TE (do not add any RNAse).
▪ Store the pellets at –20°C.

Lysis buffer mastermix 50 ml:

10 ml	Hepes 250mM
1,4 ml	NaCl 5M
100 µl	EDTA 0,5ml
500µl	TritonX100
50 mg	Deoxycholic acid
500µl	PMSF
1000µl	Complete (1 tablet in 1ml)
15mg	Benzamidine

Washing buffer 20ml:

200 µl	Tris 1M pH8
1 ml	LiCl 5M
40 µl	EDTA 0,5ml
100µl	NP 40
100 mg	Deoxycholic acid

6.4.8.1 Multiplex PCR

1µl of sample was mixed with 49µl of the following master-mix

10X master mix:

400µl H_2O

50µl *Erich* Buffer

4-20µl Primer pair I 10pM/µl (e.g. *TEL* 4µl)

4-20µl Primer pair II 10pM/µl

4-20µl Primer pair III10pM/µl

10µl dNTP 10mM

0,5 µl Taq pol. 5U/µl (e.g. Fermentas Dream Taq)

following primer pairs were used:

CLB2	up	GGAAATAGCCGCCAAAAGAC
	low	CTGAAACTCTATGCCCATGC
CLB2tata	up	GCTAGCATCTCATAGCATGG
	low	ATTGAATAGCTCGCAGTAGG
CLB2atg	up	GCGGTTCTTTGATTGAGCATC
	low	GTTAGGGCTGGATTATTCGC
CLB2orf	up	GAAGGAAGATCTCTATCAGC
	low	TTCATCTTCCGTACATGCAC
BUD4	up	CGTCGTGCATGCTTCTGTAC
	low	CAAATCTCTCGAAGTGCCTC
FUS1	up	CATGTGGACCCTTTCAAAAC
	low	TGATGGCTTATATCCTGCTC
GAL	up	AGAGCCCCATTATCTTAGCC
	low	CGCATTATCATCCTATGGTTG
TEL	55	GCGTAACAAAGCCATAATGCCTCC
	56	CTCGTTAGGATCACGTTCGAATCC

Cycles:

1 x 94°C/5min, 53°C/1min, 72°C/1,5min
25-30 x 94°C/1min, 53°C/1min, 72°C/1,5min
1 x 72°C/10min

6.4.8.2 Real time PCR:

ChIP samples were diluted in 30µl of 1XTE and stored at -20°C. From these 40µl of an 1/10 dilution in high grade purity water (Sigma) were prepared (4µl Sample + 36µl H20). In an 96 dwell plate 6 times [(FUS1RT+CLB2RT) x triplicate] 5µl of the sample were pipetted. and overlayed with 20 µl of the appropriate master-mix solution (FUS1 or CLB2 see below). Standard curves were prepared with the help of an 1/10 diluted (see above) WCE dilution series (1:10, 1:40, 1:160, 1:640). The 96 dwell plate was run on an Eppendorf Master-cycler, and the obtained date were analyzed with the help of Eppendorf Realplex.

n samples n	mastermix (n+ 5%) µl	20µl µl	
48	645,12	12,8	H_2O Sigma
48	126	2,5	25mM $MgCl_2$
48	126	2,5	10x buffer II + KCl (Ampli Tag, Fermentas)
48	25,2	0,5	dNTP (10mM)
48	25,2	0,5	Pr mix (10pM/µl)
48	50,4	1	SYBR Green (1:1000)
48	10,08	0,2	Tag (5U/µl) Dream Tag (Fermentas)
48	1008	**20**	total volume per template
		5	Template DNA (1:10)

primers (designed with the help of ***PrimerPy***)

CLB2RT	up	GCGACCGAATCAGGAAAAG
	low	GCCCATGCTATGAGATGCTA
FUS1RT	up	GACGACAACAACTGTGCTGA
	low	CTGAGCCGCCACATTAGAAA

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8 CURRICULUM VITAE

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