Cell cycle regulated transcription and genome integrity in yeast

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Declaration

I, Anastasiya Kishkevich confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Cell cycle is composed of G1, S and G2 phases and Mitosis and is governed by waves of transcription. G1/S transcriptional wave is involved in cell cycle commitment.

In my thesis, I investigate the role of histone acetylation in G1/S transcriptional regulation. In *S. cerevisiae* histone deacetylase Rpd3 and histone acetyltransferase Gcn5 are recruited to G1/S target gene promoters and are implicated in regulation of G1/S transcription. Here I show that acetylation of histones at G1/S promoters is cell cycle regulated. However, deletion of *RPD3* or *GCN5* does not lead to loss of transcriptional regulation, but only results in mild de-repression of transcription in G1 and S phase in *rpd3*Δ cells and not full activation at G1/S transition in *gcn5*Δ cells.

In the thesis, I present my work and that of our collaborators from Oxford, where established that histone methyltransferase Set2-dependent H3K36me2 and H3K36me3 are necessary for activation and maintenance of G1/S transcription in response to replication stress caused by hydroxyurea and short bleomycin treatment in *S. pombe*.

Here I also describe a study performed with our collaborators from Ben-Gurion University of Negev and Duke University. The G1/S transcriptional network in distant yeast species is regulated by homologous proteins, but varies considerably in size. In budding yeast Swi4 and Mbp1 DNA binding components recognize specific SCB and MCB DNA motifs in G1/S target promoters. However, in distantly related yeast species only MCB motif and one transcription factor are present. We establish that Swi4 is the likely ancestral DNA binding domain and a MCB-like motif the likely DNA binding sequence, and the SCB motif representing an optimised sequence for Swi4 binding.

Deregulation of G1/S transcription is found in all cancer types. I describe an approach to investigate dependencies of fission yeast with deregulated G1/S transcription as a model of cancer development.

Impact Statement

The work in the chapter on the role of histone acetylation and deacetylation is based on previous studies, which showed that HDAC Rpd3 and HAT Gcn5 are recruited to G1/S target promoters and linked these enzymes to transcriptional repression and activation genome-wide in budding yeast. In my thesis, I am investigating the direct role of Rpd3 and Gcn5 in regulation of G1/S transcription, which has not been established. Based on this study a manuscript will be prepared for publication.

In the second chapter, for the first time the direct involvement of histone methyltransferase Set2 in activation of G1/S transcription as a part of the cellular response to replication stress in fission yeast has been showed. The results of this study are a part of a manuscript Pai, C.C., Kishkevich, A., Deegan, R., … de Bruin, R.A.M, Carr, A.M. & Humphrey, T.C. “Set2 methylation of histone H3K36 suppresses replication stress through MBF-dependent transcription”, which is *in press* in *Cell Reports*.

The results, presented in the third results chapter, are the part of functional analysis, that have been recently published in *PLOS Genetics* journal (Adi Hendler, A., Medina, E., Kishkevich, A., Abu-Qarn, M., Klier, S., Buchler, N., de Bruin, R. & Aharoni, A. Gene duplication and co-evolution of G1/S transcription factors specificity in fungi are essential for optimizing cell fitness. *PLOS Genetics, 13* (5), e1006778). This case study, provides an insight on how G1/S transcription has expanded during evolution and on one of the mechanisms of transcriptional network evolution via expansion.

The fourth results chapter describes the approach to identify and validate dependencies of fission yeast with deregulated G1/S transcription. Hits identified in the synthetic genetic array will be further validated by other members of the de Bruin group. The genetic system with controlled expression of G1/S transcription co-repressor Nrm1, which I have been developing, will be utilised for identification of processes, which become crucial for cell survival with deregulated G1/S transcription. The pathways identified in these studies can be then investigated in human cells to find new potential targets for anti-cancer therapy.

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Abbreviations

ATM – Ataxia telangiectasia mutated

CDK – cyclin dependent kinase

ChIP – Chromatin Immunoprecipitation

DBD – DNA binding domain

DDR – DNA damage response

DMSO – Dimethyl sulfoxide

DSB – Double strand break

DTT – Dithiothreitol

EDTA – Ethylenediaminetetraacetic acid

HAT – Histone acetyltransferase

HDAC – Histone deacetylase complex

HR – Homologous recombination

HU – Hydroxyurea

NHEJ – Non-homologous end joining

qPCR – Quantitative polymerase chain reaction

RS – Replication stress

RT-qPCR – Reverse transcriptase quantitative polymerase chain reaction

SGA – Synthetic Genetic Array

SDS – Sodium dodecyl sulphate

ssDNA – Single-stranded DNA

TF – transcription factor

wt – wild type

1. INTRODUCTION

1.1. Regulation of transcription.

1.1.1. General principles of transcriptional regulation.

DNA is the genetic code, which carries information about all the proteins required for cell functioning. This information is converted into proteins via transcription and translation. Transcription allows copying of genetic information from DNA to RNA. This is carried out by a certain class of multiprotein complexes: RNA polymerases, which build mRNA complementary to DNA. Transcription is tightly regulated by transcription factors (TFs). TF complexes recognise and bind regulatory sequences at promoter regions and this binding facilitates recruitment of other transcriptional activators and polymerases. Then mRNA is processed and translated into amino acid sequence, which in turn is folded and modified to form a functional protein.

In order for transcription to take place, transcription factors and polymerase must have access to DNA. In eukaryotic cells, DNA is organised into chromatin, composed of nucleosomes around which DNA is wrapped. Each nucleosome consists of 8 histone proteins (two molecules of each histone H2A, H2B, H3 and H4) around which 147 bp of DNA is wrapped. The tight interactions between histone-histone and histone-DNA result in DNA compaction and folding to make the genome fit into the nucleus, but makes DNA not accessible for the transcriptional machinery (reviewed in Horn & Peterson, 2002). To overcome this issue a number of chromatin remodelling and modifying enzymes can open up this structure, which provides another level of transcriptional regulation. Chromatin remodelling enzymes alter nucleosome position, composition and structure in an ATP-dependent manner (reviewed in Saha *et al*., 2006). Chromatin modifying enzymes affect chromatin structure via covalent histone posttranslational modifications, such as acetylation, methylation, phosphorylation, ubiquitination and SUMOylation. Posttranslational modifications alter chromatin properties. Addition of covalent attached groups can change the charge of histone proteins and nucleosomes can be shifting along DNA. These modifications can also prevent contacts

between histones from different nucleosomes, which in turn prevent chromatin compaction, making DNA more accessible for the transcriptional machinery. Moreover, acetyl and other groups serve as a base for binding of chromatin remodelling enzymes and transcription factors.

In conclusion transcription factors and chromatin remodelling and modifying enzymes are involved in transcriptional regulation. Therefore, understanding how chromatin structure is linked to transcriptional regulation will provide insight into how transcription is controlled.

1.1.2. Regulation of G1/S cell cycle transcription.

The cell cycle is composed from four stages, which are characterised by specific events. These phases are Gap 1 (G1), Synthetic (S) and Gap 2 (G2) phases and Mitosis. During S phase the entire genome is replicated once and during Mitosis the replicated DNA is segregated into the two new daughter cells. The Gap phases separate these two events.

Progression through the cell cycle is governed by cooperative work of cyclins and cyclin-dependent kinases (CDKs). Accumulation of specific cyclins at distinct phases of the cell cycle provides the CDK activity required to progress into the next phase. Cyclin accumulation is driven by cell cycle-dependent gene expression, which in turn is regulated by CDK activity, forming an interdependent regulatory network that drives cell cycle progression.

In most Eukaryotes, cell cycle regulated gene expression can be grouped into three main waves, which coincide with transition points during the cell cycle: G1-to-S, G2-to-M and M-to-G1 (reviewed in Bähler, 2005). Activation of the G1-to-S phase transcriptional wave drives the G1-to-S transition and commits a cell to enter a new cell cycle. The G1/S transcriptional network includes genes that encode for proteins involved in DNA replication, DNA damage repair, cell cycle regulation and many others. G1/S transcription is activated upon phosphorylation of transcriptional inhibitors by CDKs, which releases them from transcriptional activators. Activation of G1/S genes initiates a positive feedback loop, via accumulation of additional cyclins, which in turn, further activates G1/S transcription (Skotheim *et al*., 2008). Activated genes

govern cell cycle progression and make the cell enter S phase, which commits the cell to a cell division cycle. Upon progression into S phase transcriptional repressors accumulate and bind promoters of G1/S genes and inactivate transcription. These repressors were found to be G1/S targets themselves and thus form an auto regulatory negative feedback loop. As a result, transcription peaks at the G1-to-S transition, involving both positive and negative feedback loops to ensures tight regulation of the cell cycle progression (Bertoli *et al*., 2013b).

The mechanism of G1/S transcriptional regulation is conserved from yeast to human. In human cells, G1/S transcription is regulated by E2F family of transcription factors which includes 8 members: E2F1, E2F2 and E2F3 are activators and E2F4, E2F5, E2F6, E2F7 and E2F8 are repressors (Ivey-Hoyle *et al*., 1993; Beijersbergen *et al*., 1994; DeGregori *et al*., 1995; Hijmans *et al*., 1995; Cartwright *et al*., 1998; Di Stefano *et al*., 2003). Another group of proteins, involved in G1/S transcriptional regulation, are dimerization partner proteins (DP-1) and pocket proteins (Rb family) (Helin *et al*., 1993; Hijmans *et al*., 1995). In G0 and early G1 phase, E2F4 and probably E2F5 repressors together with pocket proteins p130 and p107 (both belong to Rb family) bind to G1/S promoters to repress transcription. At the same time activators E2F1, E2F2 and E2F3 are inhibited by Rb protein. Hyper-phosphorylation of Rb in a cyclin E-CDK-dependent manner is required to remove this inhibition (Narasimha *et al.*, 2014). Activator E2Fs replace inhibitors E2F4 and E2F5 and G1/S transcription is activated. Since G1 cyclins are targets of E2F they facilitate further transition to S phase via positive feedback loop. In S phase G1-cyclin/CDK (cyclin E-CDK2) and S-cyclin-CDK (cyclin A-CDK2) phosphorylate p27, the S phase cyclin-specific inhibitor, and p27 is degraded. With the progress to S phase, CDK2 activity increases. This increased CDK2 activity allows initiation of DNA replication. At the same time, activator E2F1-E2F3 are phosphorylated by cyclin A-CDK2 and leave gene promoters, thus providing a negative feedback loop. An additional negative feedback loop relies on E2F6, E2F7 and E2F8 repressors, which are also E2F targets. In contrast to E2F4 and E2F5, these three repressors do not require pocket proteins and therefore are able to repress transcription when pocket proteins are phosphorylated (at S phase and after) (reviewed in Dimova & Dyson, 2005; Bertoli *et al*., 2013b).

Recent studies revealed that E2F6 has a dominant role in repressing G1/S transcription during S phase (Bertoli *et al*., 2013b).

1.1.3. Regulation of G1/S transcription in budding and fission yeast.

In yeast, the transition from G1 to S phase, referred to as Start, is crucial for the cell cycle commitment to the same extent as in human cells. While the mechanism of G1/S transcriptional regulation is conserved, the transcription factors involved share no sequence homology between yeast and human.

In budding yeast *Saccharomyces cerevisiae* G1/S transcriptional network is regulated by the SBF (Swi4-Swi6 cell cycle box binding factor) and MBF (*Mlu1*-box binding factor) transcription factor complexes (Andrews and Herskowitz, 1989a; Andrews & Herskowitz, 1989b; Nasmyth & Dirick, 1991; Ogas *et al*., 1991; Verma *et al*., 1992; Koch *et al*., 1993) and encompasses around 300 genes (Iyer *et al*., 2001). SBF and MBF are heterodimer complexes, each composed of homologous DNA binding domains Swi4 and Mbp1, respectively, and the activation domain Swi6 (Nasmyth & Dirick, 1991; Moll *et al*., 1992; Verma *et al*., 1992; Andrews & Moore, 1992; Koch *et al*., 1993). While the temporal pattern of transcription induced by SBF and MBF is the same, the mechanism of regulation is quite different (Fig.1A): deletion of SBF DNA binding subunit Swi4 results in loss of activation of SBF-targets at G1/S transition, while in cells lacking the MBF DNA binding subunit Mbp1 transcription is no longer repressed outside of G1 (de Bruin *et al*., 2004). Swi4 recognises SCB elements *CACGAAA* and binds to target promoters in G1 phase. SBF is inhibited by the transcriptional inhibitor Whi5 and to a lesser extent Stb1. Whi5 is phosphorylated by Cln3/CDK, dissociates from SBF and is exported from nucleus (Dirick *et al*., 1995; de Bruin *et al*., 2004; Costanzo *et al*., 2004; de Bruin *et al*., 2008; Traversa *et al*., 2013). G1 cyclins Cln1 and Cln2 are among targets of SBF and also phosphorylate Whi5, providing a positive feedback loop of transcriptional regulation. This positive feedback loop facilitates progression into S phase. Stb1 is also phosphorylated by Cln1-2/CDK and released from promoters (de Bruin *et al.*, 2008). Upon transition into S phase SBF is phosphorylated by Clb/CDK and released from promoters, which results in

transcriptional inactivation (Siegmund & Nasmyth, 1996). In contrast MBF is a transcriptional repressor required for repression of MBF-dependent targets outside of G1. The DNA binding component Mbp1 binds MCB elements *ACGCGT* during the entire cell cycle (Verma *et al*., 1992; Koch *et al*., 1993). In early G1 phase MBF together with Stb1 represses MBF dependent transcription. Accumulation of Cln3/CDK leads to MBF/Stb1 phosphorylation and inactivation and allows G1/S transcription (de Bruin *et al*., 2008). In S phase, transcription is repressed via the binding of co-repressor Nrm1 to MBF (de Bruin *et al*., 2006). Nrm1 is a target of MBF and accumulates when MBF-dependent transcription is activated and therefore constitutes a negative feedback loop.

In fission yeast *Schizosaccharomyces pombe* transcriptional network, activated upon G1/S transition, involves only around 80 genes and is regulated by a single hetero-trimeric transcription factor complex Res1-Cdc10-Res2 (Lowndes *et al*., 1992; Tanaka *et al*., 1992; Miyamoto *et al*., 1994; Ayte *et al*., 1995; Baum *et al*., 1997; Aligianni *et al*., 2009). Res1 and Res2 are DNA binding subunits like Swi4 and Mbp1 in budding yeast, while Cdc10 is an activation subunit (Swi6 in budding yeast). Res2 is responsible for repression of transcription outside of G1, and deletion of *res2* results in constant G1/S transcription throughout the whole cell cycle. Res1 is required for activation of transcription upon transition and in the absence of Res1 transcription is not activated (Baum *et al*., 1997). Since the Res1-Cdc10-Res2 complex recognises MCB elements at target promoters it is also called MBF (Fig.1B). MBF is bound to its target promoters throughout the cell cycle. At the G1 to S phase transition transcription is activated and co-repressors Nrm1 (de Bruin *et al*., 2006) and Yox1 (Aligianni *et al*., 2009), both MBF targets, are also expressed. Nrm1 and Yox1 bind MBF and facilitate transcriptional repression in late S phase via an autoregulatory negative feedback loop.

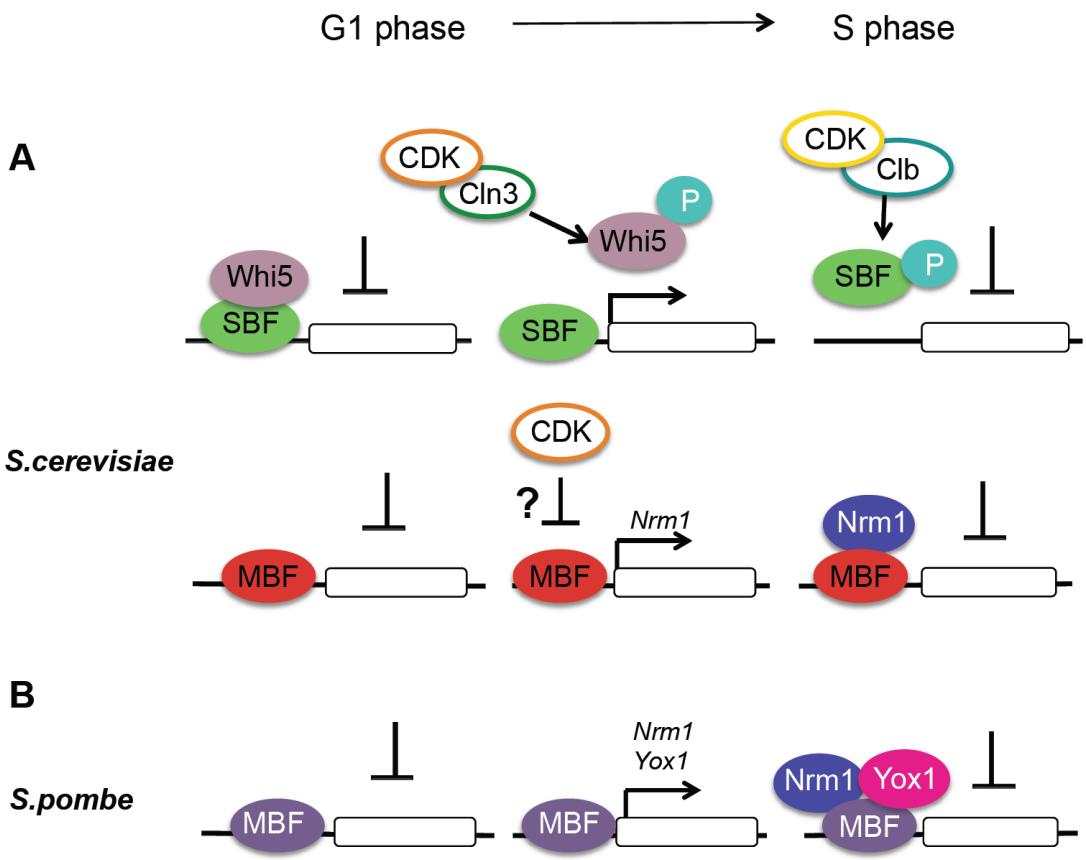


Figure 1. Schematic representation of G1/S transcriptional regulation in budding yeast *S. cerevisiae* (A) and fission yeast *S. pombe* (B). Description is in the text.

1.2. Histone posttranslational modifications and transcriptional regulation.

1.2.1. The role of histone posttranslational modifications in transcriptional regulation.

In all eukaryotic cells, DNA is tightly wrapped around nucleosome. Nucleosomes are composed of eight histone proteins, two molecules of each H2A, H2B, H3 and H4. The complex of DNA and histones, chromatin, is highly dynamic. While DNA is wrapped around octameric core, the N termini tails are not bound and subjected to various reversible covalent modifications, such as acetylation, methylation, phosphorylation, ubiquitylation and SUMOylation, and this list is still growing. These modifications result in changes in chromatin structure, which in turn affect gene expression.

Modifications of histone tails affect nucleosome-intrinsic interactions between nucleosome and DNA by changing charge. This leads to nucleosome mobility and shifting along DNA facilitating transcription. Posttranslational modifications also interfere with nucleosome-intramolecular interactions and prevent chromatin from folding into chromatin fibres and nucleosome arrays and obstructing transcription. Finally, modifications of N-terminal histone tails are recognised by certain effector proteins (extramolecular interactions), such as chromatin remodelling enzymes and transcription factors. All these effects of posttranslational modifications on histones facilitate proper transcriptional regulation (reviewed in Ruthenburg *et al*., 2007).

The first biochemical evidence of a possible role of histones in regulating transcription were provided by Allfrey and colleagues in early 1960s, when they were able to show that histones “inhibited” transcription in calf thymus nuclei and removal of histones resulted in increased rates of transcription (Allfrey *et al.,* 1964).

One of the most well studied histone posttranslational modification is acetylation of lysine residues in histones tails. In general histone acetylation is a mark of actively transcribed genes. Addition of acetyl moieties to histone tails alters the physical properties of histones, making DNA more accessible for effector proteins and recruitment of the transcription machinery to facilitate gene

expression. Thus, histone acetylation plays a role in transcriptional activation. In line with this, studies in *S. cerevisiae* show a correlation between histone acetyltransferases (HATs) Gcn5 and Esa1 recruitment and increased transcriptional rates (Robert *et al*., 2004; Pokholok *et al*., 2005). At the same time deletion of histone deacetylases (HDACs) Rpd3 and Hda1 leads to an increase in genome-wide transcription levels (Bernstein *et al*., 2000; Fazzio *et al*., 2001; Robyr *et al.*, 2002). Correlation between acetylation and gene transcription was established in higher eukaryotes as well. Actively transcribed genes are highly acetylated at histones H3 and H4 in *Drosophila* (Schübeler *et al*., 2004) and in maize (Zhang *et al*., 2015).

Another well-studied histone modification is methylation of lysine and arginine residues. Histone methylation of arginine residues is strongly associated with transcription activation, while the role of lysine methylation in transcriptional regulation depends on the specific residues modified and chromatin context. Methylation of histone H3K36 is conserved from yeast to human and is involved in transcriptional regulation. In yeast, the methyltransferase Set2 is responsible of all mono-, di- and tri-methylation of H3K36, while in human cells SETD2 is responsible of only tri-methylation of H3K36 (Wagner & Carpenter, 2012). In both yeast and human cells Set2/SETD2 interacts with RNA pol II and facilitates transcriptional elongation (Kizer *et al*., 2005). In budding yeast, tri-methylation, H3K36me3, positively correlates with transcriptional activation (Pokholok *et al*., 2005). However, methylation of H3K36 by Set2 is involved in repression of *GAL4* transcription (Landry *et al*., 2003), but this seems to be an exception rather than the rule. In fission yeast H3K36me2 is present at actively transcribed genes (Morris *et al*., 2005), while genome wide studies established that H3K36me3 is required for transcriptional repression of certain sets of genes (Suzuki *et al*., 2016).

Phosphorylation is involved in numerous processes within the cell, including transcription. Phosphorylation of histones occurs on serine and threonine residues and has strong link with regulation of proliferative genes (reviewed in Rosetto *et al*., 2012).

Ubiquitylation of histone is similar to methylation, where the effect on transcription depends on specific residues: ubiquitylation of histone H2B is

connected to transcriptional activation, while ubiquitylation of histone H2A – transcriptional repression (Cao & Yan, 2012).

Histone marks do not act in isolation. Single modifications can recruit other histone modifying enzymes and initiate a cascade of modifications. Methylation of histone H3K36 is required for recruitment of the deacetylase Rpd3S complex to prevent aberrant transcription initiation (Keogh *et al.*, 2005). Another example is histone H2B monoubiquitylation, which is necessary for recruitment and binding of histone methylation complex COMPASS (Dover *et al*., 2002). Thus, histone modification cross-talk provides another level of complexity in the regulation of transcription.

1.2.2. The role of histone acetylation and deacetylation in transcriptional regulation in yeast.

The balance of histone acetylation/deacetylation is maintained by opposing activity of HATs and HDACs. While actively transcribed genes are hyperacetylated, hypoacetylation is a common feature of repressed genes.

Gcn5 is a HAT subunit of the transcription co-activator complex SAGA (Grant *et al*., 1998; Sterner *et al*., 1999). SAGA plays important role in transcriptional initiation and elongation. SAGA is required for global genome regulation, and stress response genes are preferentially activated by SAGA (Huisinga & Pugh, 2004). ChIP-on-chip studies showed, that Gcn5 is recruited to actively transcribed genes. Moreover, Gcn5 is recruited to promoters upon gene activation, supporting the idea of active genes being hyperacetylated (Robert *et al*., 2004; Pokholok *et al*., 2005). Gcn5 is responsible of acetylation of a large number of lysine residues on both histone H3 and histone H4: H3K9, H3K14, H3K18, H3K27, H4K8 and H4K16 (Vogelauer *et al*., 2000; reviewed in Sterner & Berger, 2000; Robert *et al*., 2004; Pokholok *et al*., 2005).

HDACs are conserved from yeast to human, and therefore yeast is a good model organism to study function of histone deacetylases. Currently three classes of HDACs are identified in budding and fission yeast. Class I includes Rpd3, Hos2 and Hos1 in *S. cerevisiae* and Clr6 and Hos2 in *S. pombe*. Class II is composed of two HDACs in *S. cerevisiae* (Hda1 and Hos3) and one HDAC in

S. pombe (Clr3). Class III (or sirtuins) includes Hst1, Hst2, Hst3, Hst4 and Sir2 in budding yeast and Hst2, Hst4 and Sir2 in fission yeast (reviewed in Ekwall, 2005).

Rdp3 is a catalytic subunit of two HDACs: Rpd3L and Rpd3S (Shevchenko *et al*., 2008). In both these HDACs Rdp3 acts together with Sin3. Deletion of Rpd3 results in more than two-fold up-regulation of around 170 genes (Bernstein *et al*., 2000). The list of these up-regulated genes significantly overlaps with genes up-regulated in *sin3*Δ mutants. Moreover, the same genes are up-regulated upon treatment with the HDAC inhibitor trichostatin A, indicating that the increase is caused by a deficiency in histone deacetylation. A number of studies provided more evidence on Rpd3 involvement in transcriptional repression. Studies on global recruitment of Rpd3 have established that Rpd3/Sin3 complex is recruited to certain subsets of genes: specifically, cell cycle, meiosis and sporulation genes (Robyr *et al*., 2002; Robert *et al*., 2004).

Deletion of another HDAC, Hda1, also results in an increase in the transcription levels of certain groups of genes. These genes are involved in drug transport, detoxification, stress response, cell wall function and carbohydrate metabolism. Moreover, the increase in transcription correlates with increase in acetylation levels of H3 lysine 18 specifically, but also H3K9 and H2BK16 (Vogelauer *et al*., 2000; Robyr *et al*., 2002). Overall, there is strong evidence in yeast that HDAC activity is involved in transcriptional repression.

1.2.3. The role of histone acetylation and deacetylation in G1/S cell cycle transcription.

As described above G1/S transcription is tightly regulated by multiple transcription factors including activators, inhibitors, repressors and co-repressors. Several studies have suggested that the transcriptional regulators affect G1/S transcription via changes of the chromatin state at G1/S target promoters with a central role for histone acetyltransferases and deacetylases.

The role of histone acetylation in regulation of G1/S transcription is suggested by the correlation between increase in E2F transcription and levels of histone H3 and H4 acetylation in human cells (Taubert *et al*., 2004). Retinoblastoma protein Rb that inhibits the activator E2Fs and thus is involved in repression of G1/S transcription at G1 phase, interacts with histone deacetylases complex HDAC1. The Rb protein is thought to bring HDAC1 to E2F to enable repression of G1/S target genes. Mutations that interfere with Rb-HDAC1 interaction or inhibition of HDAC1 activity lead to insufficient repression of G1 transcription (Brehm *et al*., 1998; Luo *et al*., 1998; Magnaghi-Jaulin *et al*., 1998). These studies suggest that HDAC1 activity is essential for cell cycle regulation, and genes repressed by Rb are to some extent dependent on histone deacetylase activity. CBP/p300 is another co-regulator of E2F-dependent transcription factors and G1/S transition. The CBP/p300 complex possess intrinsic histone acetyltransferase activity and inactivation of this activity (mutation in the HAT domain) results in decreased E2Fs activity and abolishes G1/S transition (Ait-Si-Ali *et al*., 2000). The histone acetyltransferase Gcn5 has been shown to be required for proper G1/S transition and proper expression of E2F targets (Kikuchi *et al*., 2005).

The homologue of human HDAC1 in budding yeast, Rpd3, was also implicated in regulation of G1/S transcription. The studies on histone acetyltransferases and histone deacetylases global recruitment showed binding of Rpd3 to G1/S target promoters. ChIP-seq experiments showed that Rpd3 is recruited to SBF target promoters (Robert *et al*., 2004). Moreover, deletion of *RPD3* leads to upregulation of SBF targets *CLN2* and *SVS1* in asynchronous yeast culture (Fazzio *et al*., 2001).

Sin3 is a part of Rpd3 histone deacetylase complex and has also been shown to be involved in regulation of G1/S transcription (Stefan & Koch, 2009). Chromatin Immunoprecipitation showed that Sin3-Rpd3 complex is recruited to *CLN2* and *CLN1* promoters, which are both SBF targets. This recruitment is abolished in *swi4*Δ and *swi6*Δ mutants, indicating that the recruitment is SBF-dependent. In addition, the recruitment is cell cycle dependent, with maximum levels of Sin3-Rpd3 observed at G1 phase of the cell cycle, and not G2. Moreover, the pattern of binding correlated with transcriptional repression of SBF targets *CLN1*, *CLN2* and *PCL1*. Screening for additional SBF repressors

revealed Stb1 (Sin Three Binder 1) as a potential repressor of SBF target genes (Wang *et al*., 2009). Since Stb1 interacts with Sin3-Rpd3, these data further support involvement of Rpd3 in the regulation of SBF-dependent transcription. Furthermore, direct interaction between SBF repressor Whi5 and Rpd3 was established by affinity chromatography (Huang *et al*., 2009). ChIP results showed that both Whi5 and Sbt1 are required for Rpd3 recruitment, since Rpd3 did not bind G1 target promoters in *whi5*Δ*stb1*Δ mutant (Takahata *et al*., 2009). All these observations suggested a model, where Rpd3 is recruited to SBF target promoters by Swi4/Swi6/Whi5/Sbt1 and has a likely role in the repression of SBF targets in early G1 phase.

The histone acetyltransferase Gcn5 has been also implicated in the regulation of G1/S transcription in yeast. Gcn5 is a subunit of transcriptional co-activator SAGA. Chromatin immunoprecipitation showed that SAGA is recruited to the cell-cycle regulated promoter HO, which is regulated by SBF (Cosma *et al*., 1999). Direct binding of Gcn5 itself was confirmed with ChIP-seq studies, where Gcn5 was found at promoter regions of both SBF and MBF target genes (Robert *et al.*, 2004).

While these studies provide extensive evidence on Rpd3 and Gcn5 recruitment to SBF and MBF target promoters, the role of these histone modifying enzymes in the regulation of cell-cycle dependent transcription during G1 and S phases is not known. One of the objectives of my thesis is to establish what role HDAC Rdp3 and HAT Gcn5 play in regulation of G1/S cell cycle regulated transcription in *S. cerevisiae*.

1.2.4. The role of histone H3 lysine 36 methylation in genotoxic stress and DNA damage response.

Genotoxic stress, caused by internal or external factors, triggers the DNA damage response pathway to arrest the cell cycle and maintain genome integrity. The response includes the activation of specific groups of genes. While the role of histone methylation in the regulation of transcription in unperturbed conditions is established, little is known about the role of methyltransferases and demethylases in regulation of the transcriptional

response to genotoxic stress. In budding yeast, the histone demethylase Rph1, which is responsible of H3K36 de-methylation, is required for repression of DNA damage response (DDR) induced genes in normal conditions. These genes are significantly upregulated in *rhp1*Δ cells. After treatment with ultra violet or infrared light, Rph1 dissociates from promoters, which correlates with transcriptional induction of the DDR genes (Liang *et al*., 2013). However, deletion/depletion of the methyltransferase Set2 in both budding yeast and human cells does not affect transcription levels of DDR genes (Pfister *et al.*, 2014; Jha *et al.*, 2014).

A direct role, independent of transcription, of histone H3K36 methylation in response to genotoxic stress and DNA damage was established in both yeast and human cells. H3K36 methylation, which is carried out by Set2 and SETMAR and SETD2 in yeast and human cells respectively, creates a special microenvironment around double strand breaks (DBS) for recruitment of DNA repair machinery. DNA double strand breaks are the most severe type of DNA damage, and a significant source for genomic instability. DNA double strand breaks can be processed via two mechanisms: non-homologous end joining (NHEJ) and homologous recombination (HR). The choice of the repair mechanism depends on the cell cycle phase when the DNA damage occurs. The methylation state, as well as the cell cycle phase, determines the choice of the repair mechanism. In human cells H3K36Me2 is catalysed by methyltransferase SETMAR and favors NHEJ (Fnu *et al*., 2011). At the same time SETD2-dependent H3K36Me3 is involved in HR repair pathway. H3K36Me3 is required for the activation of the DNA damage response kinase ATM and recruitment of HR repair protein Rad51 to facilitate DNA resection and maintenance of genome stability (Carvalho *et al*., 2014; Pfister *et al*., 2014). In contrast, in both budding and fission yeast Set2-dependent H3K36Me3 is required for NHEJ repair, as in *set2*Δ cells DSB is exclusively repaired by HR (Pai *et al*., 2014; Jha *et al*., 2014). In my thesis, I am going to investigate the role of histone methyltransferase Set2 in regulation of G1/S transcription in response to replication stress.

1.3. Transcription regulatory network evolution.

1.3.1. Evolution of transcriptional networks via expansion.

Transcriptional control is a fundamental mechanism for optimizing cell survival prospects by allowing them to make the appropriate amount of proteins at the right time. Coordinated regulation of groups of genes in a transcriptional regulatory network facilitates synchronized expression of genes encoding for proteins with similar biological functions. Changes to these networks are thought to be important drivers of evolution. Changes can occur due to rewiring, expansion or contraction of transcriptional networks. Rewiring of transcriptional networks take place when old connections are broken and new connections are formed between regulators and target genes (Nocedal & Johnson, 2015). Transcriptional network expansion happens when new genes and regulators appear in the network.

A major driver of network expansion is gene duplication, which is thought to be the 'safest' way for driving changes in a transcriptional network without affecting pre-existed regulation and cell fitness. After the duplication one of the paralogs may acquire mutations in the coding sequence to gain a new role (*trans*-mutations). Often trans-mutations occur in the transcription regulatory protein gene (transcription factors) (Teichmann & Babu, 2004). Duplicated transcription factor may preserve the function and this creates redundancy when both paralogs respond to the same signal, recognise the same binding sites and regulate the same gene. Subsequently one or both duplicated genes may diverge and mutate in their DNA binding domain and therefore bind existing and new target genes with different affinity. Another scenario is when duplicated transcription regulator starts to respond to different signals or interact with different co-factors, still regulating the same target genes. Paralogos may divide functions and regulate a subset of genes within the network, previously regulated by the ancestral transcription factor (subfunctionalization). And finally, one of the paralogos may acquire a novel function, which ancestral regulator did not have (neofunctionalization) (Voordeckers *et al*., 2015).

Combinations of *cis*- (mutation in the upstream regulatory sequence) and *trans*-mutations also occur. Divergence of both target gene and transcription

factor allows new genes to be regulated by new transcription factor and old genes – by old regulators. These types of interactions contribute to a very small proportion of interactions in yeast (Teichmann & Babu, 2004). Most network interactions are though to result from gene duplications of target gene or transcription factor, followed by divergence and gain of new interaction.

Altogether gene duplication with subsequent *cis*- and *trans*-mutations allow expansion of transcriptional networks and evolution of cell complexity.

1.3.2. Expansion of G1/S transcriptional network in yeast.

The molecular mechanism controlling the G1/S transcriptional networks from yeast to human is conserved, while the regulatory proteins between yeast and human do not share sequence homology (Cross *et al*., 2011; Bertoli *et al*., 2013b). In human cells G1/S transcription is regulated by the E2F family of transcription factors, and in budding and fission yeast SBF/MBF and MBF are responsible for regulation, respectively. Evolution studies revealed that last eukaryotic common ancestor possessed activator and inhibitor E2Fs and pRb-family pocket proteins. SBF is homologous to DNA virus proteins, and most likely occurred via horizontal gene transfer, when viruses took over the cell cycle control. Ancestral fungi should have both E2Fs and SBF and subsequently lost E2F and Rb and indeed novel (SBF/Whi5) and ancestral (E2F/Rb) types of regulators still co-exist in some basal fungi (Medina *et al*., 2016).

Evolution of yeast species since the last common ancestor estimates millions of years. This led to dramatic differences between species, and yeast species are divided into clades based on genome similarity (Dujon, 2010).

However, the molecular mechanisms involved in the regulation of the G1/S transcriptional network is conserved and the G1/S regulators show considerable homology across distantly related clades. *S. cerevisiae* and *S. pombe*, which are the most commonly studied yeast species, are very distantly related. In *S. pombe* the G1/S transcriptional network encompasses around 80 genes and in regulated by single transcription factor MBF (Res1-Cdc10-Res2) (Lowndes *et al*., 1992; Tanaka *et al*., 1992; Miyamoto *et al*., 1994; Ayte *et al*.,

1995; Baum *et al*., 1997; Aligianni *et al*., 2009), while in *S. cerevisiae* two transcription factors, SBF and MBF, are involved in regulating more than 200 G1/S targets (Horak *et al*., 2002; Ferrezuelo *et al*., 2010). Both budding and fission yeast MBF recognise MCB sites ACGCGT in G1/S target promoters (Bähler, 2005), whilst budding yeast SBF recognizes SCBs (CGCGAAA) not found in fission yeast G1/S target promoters. At the same time, both Res1 and Res2 N-terminal domain are similar to Swi4 and Mbp1 N-terminal domains (Koch *et al*., 1993).

MCB DNA binding sites are also present in species, belonging to closer related to *S. cerevisiae* clades, while SCB elements and SBF are present only in species from clades very closely related to *Saccharomyces.* Transcriptional oscillation of genes during the G1-to-S transition in *Candida albicans* are involved in DNA replication and cell cycle processes as in budding and fission yeast and human cells. Sequence analysis of promoter regions of these genes revealed enrichment of MCB binding sites, suggesting regulation by MBF. However, transcription of *MBP1* is not cell cycle regulated, while *SWI4* and *SWI6* homologous are transcribed in cell cycle dependent manner. This, and other observations, suggest that it is likely that CaSwi4 and CaSwi6 bind to MCB elements in the cell cycle regulated genes to regulate transcription, since CaMbp1 is not present at G1 (Côte *et al*., 2009).

In G1 phase in *S. cerevisiae* SBF is inhibited by Whi5, which is then removed and SBF-dependent transcription is activated. In S phase SBF is phosphorylated and removed from promoters and transcription is inactivated. MBF-dependent transcription is active in G1 when MBF repressive function is inactivated. In S phase co-repressor Nrm1, which is an MBF target, binds MBF to co-repress transcription. *C. albicans* also possesses Nrm1. CaNrm1 is involved in repression in S phase, but in addition in *C. albicans* Nrm1 is also required for repression in G1 phase, performing ScWhi5 function (Ofir *et al*., 2012).

Proteins which sequences are similar to ScSwi4 and ScMbp1 were also identified in *Kluyveromyces lactis* (Koch *et al*., 1993) and *Neurospora crassa* (Galagan *et al*., 2003; Zámborszky *et al*., 2014). Thus, the G1/S regulatory network is conserved across yeast species with *S. cerevisiae* possessing the most complicated network.

1.4. The role of deregulated G1/S transcription in replication stress-induced DNA damage.

1.4.1. Replication stress-induced DNA damage.

Replication stress is defined as stalling or slowing down of replication fork progression. Replication stress is caused by various factors. Physical barriers, such as DNA secondary structures, unrepaired DNA lesions and RNA-DNA hybrids may cause stalling of replication machinery. The lack of building blocks for DNA replication (nucleotides) and compaction of newly synthesised DNA (chaperones, histones) may also lead to slowing down DNA replication. Replication fork stalling or slowing down results in the exposure of single strand DNA (ssDNA). This exposed ssDNA can be subjected to damage by external and internal factors. Accumulation of replication stress-induced DNA damage results in genomic instability, which contributes to many human diseases including cancer (Zeman & Cimprich, 2014).

1.4.2. Oncogene-induced replication stress.

Genomic instability, caused by replication stress and subsequent DNA damage is a common feature of most cancer cells. During the last decade, a central role for oncogene-induced replication stress and subsequent DNA damage in cancer initiation and development has been established (Halazonetis *et al*., 2008; Negrini *et al*., 2010; Hills & Diffley, 2014; Gaillard *et al*., 2015; Macheret & Halazonetis, 2015). Activation of oncogenes, such as Ras, Myc, and cyclin E, drives cell cycle entry by enhancing E2F activity. Uncontrolled proliferation leads to replication stress via altering the DNA replication pattern and timing. Increased CDK activity in G1 may result in origin under-usage, when not enough origins are licensed, whilst increased CDK activity in S phase may lead to more origins being fired or the same origins being reused (Hills & Diffley, 2014). These defects in replication control, under-,

over-, or re-replication can cause replication fork stalling and collapse and genomic instability.

1.4.3. The role of deregulated G1/S transcription in oncogene-induced replication stress.

In human cells the family of E2F transcription factors, together with the pocket proteins (pRb, p130 and p107), regulate G1/S transcription. Activation of G1/S transcription, leading to progression from G1 to S phase, is initiated by accumulation of G1 cyclin D-CDK4. Cyclin E-CDK2, an E2F target itself, creates a positive feedback loop by inactivating the transcriptional inhibitor Rb activating E2F-dependent transcription.

The G1/S transcriptional network is involved in cell cycle commitment and maintenance of genomic stability. Deregulation of G1/S transcription is found in many types of cancers. The proposed model of oncogene-induced replication stress is that activation of oncogenes, such as Ras and Myc, results in deregulation of G1/S transcription, which drives unscheduled S phase entry. Replication stress-induced DNA damage will activate the DNA damage checkpoint, which functions as a first barrier against oncogenesis. However, if the checkpoint is defective and allows proliferation these cells can become cancerous (Bartkova *et al*., 2006; Halazonetis *et al*., 2008).

In G1 phase repressor E2Fs (E2F4 and E2F5) together with pocket proteins from Rb family (p130 and p107) are bound to G1/S target promoters to repress transcription. Rb itself is bound to activator E2Fs (E2F1, E2F2 and E2F3) and inhibits their activity (Ivey-Hoyle *et al*., 1993; Helin *et al.*, 1993; Beijersbergen *et al*., 1994; DeGregori *et al*., 1995; Hijmans *et al*., 1995; Cartwright *et al*., 1998; Di Stefano *et al*., 2003). Cyclin E/CDK dependent hyper-phosphorylation of Rb removes this inhibition and allows transcriptional activation (Narashima *et al*., 2014). Other pocket proteins are also phosphorylated and repressor E2Fs are released from promoters. Rb is a tumour suppressor protein and found to be mutated in many cancer types. Rb inactivation results in uncontrolled E2F activity and premature S phase entry: depletion of Rb in mouse embryonic fibroblasts leads to inappropriate S phase entry (Almasan *et al.*, 1995; Chen *et al*., 2006). Moreover, cells with mutations

in all three proteins from Rb family (pRb, p130 and p107) do not arrest in G1 and enter S phase prematurely (Sage *et al.*, 2000). Increased Ras activity results in Rb inactivation and deregulation of G1/S transcription (Mittnacht *et al.*, 1997; Peeper *et al.*, 1997). The promoter of the activator E2F2 gene contains an E-box that is recognised by the oncogene Myc (Sears *et al.*, 1997). An increase in Myc activity also results in uncontrolled E2F activity and inappropriate S phase entry (Sheen & Dickson, 2002; Robinson *et al.*, 2009). Thus, deregulated G1/S transcription is a determinant factor in carcinogenesis. However, the mechanism, by which deregulation of G1/S transcription is causing replication stress is still elusive.

1.5. Exploiting cancer's addiction to deregulated G1/S transcription.

1.5.1. The role of G1/S transcription in tolerance to replication stress.

As discussed above, deregulation of G1/S transcription causes loss of cell cycle control resulting in replication stress and DNA damage. Many types of cancer exhibit an increase in G1/S transcription (Chen *et al*., 2006). Surprisingly, several studies have established that G1/S transcription is increased in response to replication stress. Studies in yeast showed that the replication checkpoint effector kinases Cds1 and Rad53 in fission and budding yeast, respectively, maintain G1/S transcription in response to replication stress via inactivation of the transcriptional G1/S co-repressor Nrm1 (de Bruin *et al*., 2008; Travesa *et al*., 2012; Bastos de Oliveira *et al*., 2012; Ivanova *et al*., 2013).

This maintenance of G1/S transcription upon replication stress is conserved from yeast to human. In human cells, in response to replication stress, the checkpoint effector kinase Chk1 phosphorylates and inactivates the transcriptional repressor E2F6 to maintain transcription in S phase (Bertoli *et al*., 2013a). Maintenance of E2F dependent transcription is important for replication fork stalling, stabilization and the resumption of replication. Thus, sustained E2F-dependent transcription provides tolerance to replication stress and is both required and sufficient to prevent replication stress-induced DNA damage (Bertoli *et al*., 2016).

1.5.2. Fission yeast with deregulated G1/S transcription as a model for cancer studies.

To recap some of the information discussed above, the mechanism of G1/S transcriptional regulation is conserved from yeast to human cells. Fission yeast MBF (Cdc10-Res1-Res2) is a functional analogue of mammalian E2F family of transcription factors. As in human cells repression of MBF-dependent transcription in S phase is regulated via negative feedback loop. Nrm1 and Yox1 are both MBF-targets and are expressed during the G1/S transition. In S

phase Nrm1 and Yox1 accumulate and bind to MBF to facilitate repression of G1/S transcription (de Bruin *et al*., 2006; Aligianni *et al*., 2009).

Deregulation of E2Fs by oncogene activation is observed in many types of cancer. Increased G1/S transcription drives cell cycle entry, which leads to replication stress. Oncogene-induced replication stress leads to DNA damage, which triggers DNA damage response. Cells with compromised DNA damage checkpoint will accumulate genomic instability and undergo transformation. Deletion of either *nrm1* or *yox1* results in constantly active G1/S transcription and cell cycle defects (elongated phenotype). Previous work in the de Bruin group has shown that deregulated G1/S transcription in *nrm1*Δ and *yox1*Δ cells results in accumulation of replication stress and DNA damage (Caetano *et al*., 2014). This resembles the accumulation of DNA damage resulting from oncogene-induced replication stress in human cells (Fig.2). Thus, fission yeast lacking co-repressor Nrm1 or Yox1 is a handy model system to study consequences of deregulated G1/S transcription in cancer.

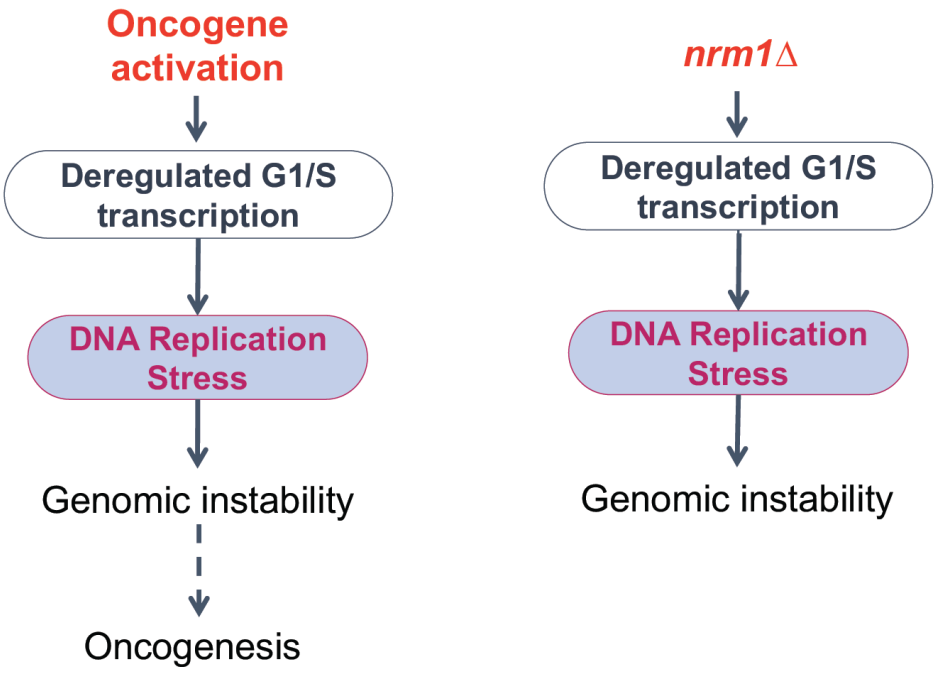


Figure 2. Comparison of the consequences of oncogene activation in human cells and deletion of *nrm1* in fission yeast. Description is in the text.

1.5.3. Exploiting cancer vulnerabilities via synthetic lethality screens.

Cancer cell transformation is a complex multistep process, during which cancer cells acquire multiple features via mutations. Mutations facilitate development of common features shared by cancer cells of different types. These features are characterised as hallmarks of cancer. Hallmarks include the ability to constantly proliferate without signalling and to resist growth suppressing and apoptosis signalling, to invade adjacent tissues to gain support from stromal cells and to facilitates angiogenesis to be supplied with nutrients and oxygen and eventually metastasise, disseminating throughout the body (Hannah & Weinberg, 2000; Hannah & Weinberg, 2011). Cancer cells experience high levels of genomic instability due to a number of cancer-associated stresses, which are recently added as novel hallmarks of cancer. These include: DNA damage/replication stress, proteotoxic stress, mitotic stress, metabolic stress, and oxidative stress. This genomic instability would result in senescence or apoptosis in healthy cells, but cancer cells bypass it by compromising the DNA damage checkpoint largely through inactivation of p53 (Gorgoulis *et al*., 2005; Halazonetis *et al*., 2008), which allows proliferation. Importantly, whilst genomic instability drives transformation in cancer cells their viability depends on cellular stress responses to prevent catastrophic levels of genomic instability. Cancer cells become extremely dependent on these support pathways and this is known as non-oncogene addiction. Genes, which are involved in these pathways are not oncogenes and are not critical for survival of normal cells (Luo *et al*., 2009). Therefore, agents, which specifically target proteins involved in these stress support pathways can selectively kill cancer cells, while normal cells will not be affected.

Synthetic lethality screens were proposed as a tool to identify dependencies/vulnerabilities of cancer cells (Kaelin, 2005). The idea behind synthetic lethality is that a mutation/deletion in either gene A or B is not essential for cell survival, but simultaneous mutation/deletion of both genes results in cell death. Now if mutations in gene A are associated with cancer, compounds that inactivate gene B would kill cancer cells without affecting healthy cells. Several synthetic lethality screens have been performed in several cancer cell lines using different approaches, such as RNA interference,

short hairpin RNA and barcoding (Luo *et al*., 2008; Luo *et al*., 2009; Cowley *et al*., 2014; Yu *et al*., 2016). These studies identified multiple pathways that are critical for the survival of the various cell lines used, but candidate genes largely depend on the specific cancer cell line and cell type and are therefore not applicable to a wide range of tumours. Ideally a synthetic lethality screen should focus on identifying the dependency on specific proteins/processes which deregulation is common to most if not all cancers. One such process is deregulation of G1/S transcription, which can be found in a wide range of cancers. Cancer associated mutations that activate oncogenes or inactivate tumour suppressors, deregulate a wide range of processes. This makes establishment of causal relationship very difficult. However, these mutations commonly deregulate G1/S transcription, which can be exploited for the identification of potential targets of anti-cancer therapy for a wide range of cancers.

2. MATERIALS AND METHODS.

2.1. Yeast strains.

The following *S. cerevisiae* (Table 1) and *S. pombe* (Table 2) strains were used in this thesis.

Table 1. *S. cerevisiae strains used in this thesis.*

Strain	***Strain genotype***	***Source***
RBY1 (wt)	*MATa, ade1, leu2-3, 112 his2, trp1-1, ura3Δns, bar1Δ*	de Bruin *et al*., 2004
RBY643 (*gcn5Δ*)	*RBY1 + gcn5::KAN*	This thesis
RBY280 (*rpd3Δ*)	*RBY1 + rpd3::URA3*	This thesis (created by Michael Harris)
RBY676 (*hda1Δ*)	*RBY1 + hda1::CloNat*	This thesis
RBY125 (*swi4Δ*)	*swi4:: KanMX*	de Bruin *et al*., 2006
mbp1Δ	*mbp1:: CloNat*	Hendler *et al*., 2017
*Kl*Mbp1BD-Swi4AD	SWI4*:: KlacMbp1BD-Swi4AD::*URA3	Hendler *et al*., 2017
*Kl*Swi4BD-Swi4AD	SWI4*:: KlacSwi4BD-Swi4AD::*URA3	Hendler *et al*., 2017
*Ca*Mbp1BD-Swi4AD	SWI4*:: CalbMbp1BD-Swi4AD::*URA3	Hendler *et al*., 2017
*Ca*Swi4BD-Swi4AD	SWI4*:: CalbSwi4BD-Swi4AD::*URA3	Hendler *et al*., 2017
*Yl*ResBD-Swi4AD	SWI4*:: YlipResBD-Swi4AD::*URA3	Hendler *et al*., 2017
*Nc*ResBD-Swi4AD	SWI4*:: NcraResBD-Swi4AD::*URA3	Hendler *et al*., 2017
*Sp*Res1BD-Swi4AD	SWI4*:: SpomRes1BD-Swi4AD::*URA3	Hendler *et al*., 2017
*Sp*Res2BD-Swi4AD	SWI4*:: SpomRes2BD-Swi4AD::*URA3	Hendler *et al*., 2017
*Kl*Mbp1BD-Swi4AD *mbp1Δ*	SWI4*:: KlacMbp1BD-Swi4AD::*URA3 *mbp1:: CloNat*	Hendler *et al*., 2017

Table 1. continued

Strain	*Strain genotype*	*Source*
*Ca*Mbp1BD-Swi4AD *mbp1Δ*	SWI4*:: CalbMbp1BD-Swi4AD::*URA3 *mbp1:: CloNat*	Hendler *et al*., 2017
*Yl*ResBD-Swi4AD *mbp1Δ*	SWI4*:: YlipResBD-Swi4AD::*LEU2 *mbp1:: CloNat*	Hendler *et al*., 2017
*Nc*ResBD-Swi4AD *mbp1Δ*	SWI4*:: NcraResBD-Swi4AD::*URA3 *mbp1:: CloNat*	Hendler *et al*., 2017
*Sp*Res1BD-Swi4AD *mbp1Δ*	SWI4*:: SpomRes1BD-Swi4AD::*LEU2 *mbp1:: CloNat*	Hendler *et al*., 2017
*Sp*Res2BD-Swi4AD *mbp1Δ*	SWI4*:: SpomRes2BD-Swi4AD::*URA3 *mbp1:: CloNat*	Hendler *et al*., 2017
*PRY2*promAA	P_{PRY2}::P_{PRY2} with AA mutation in the SCB motif	Hendler *et al*., 2017
*PRY2*promMCB	P_{NRM1}::P_{PRY2} with MCB mutation in the SCB motif	Hendler *et al*., 2017
swi4Δ *PRY2*promAA	P_{PRY2}::P_{PRY2} with AA mutation in the SCB motif *swi4:: KanMX*	Hendler *et al*., 2017
swi4Δ *PRY2*promMCB	P_{PRY2}::P_{PRY2} with MCB mutation in the SCB motif *swi4:: KanMX*	Hendler *et al*., 2017
*Kl*Swi4BD-Swi4AD *mbp1Δ*	SWI4*:: KlacSwi4BD-Swi4AD::*URA3 *mbp1:: CloNat*	Hendler *et al*., 2017

Table 2. *S. pombe* strains used in this thesis.

Strain	***Strain genotype***	***Source***
RBP11 (wt)	leu1-32, ura4-D18	de Bruin group collection
RBP571 (*set2*Δ)	h-, ade6-210, arg3-D4, his3D1, leu1-32, ura4-D18, *set2*::ura4	Timothy Humphrey's group
*set2*Δ*chk1*Δ	ade6-210, arg3-D4, his3D1, leu1-32, ura4-D18, *set2*::ura4, no marker for chk1	Timothy Humphrey's group
*chk1*Δ	RBP11+ *chk1*::ura	Timothy Humphrey's group
RBP1 (Res2-Myc)	h+, ade6-210, arg3-D4, his3D1, leu1-32, ura4-D18, res2-myc::KanMX4	de Bruin group collection
RBP44 (Cdc10-Myc)	h+, ade6-210, arg3-D4, his3D1, leu1-32, ura4-D18, cdc10-myc::KanMX4	Pai *et al*., 2017
*set2*Δ *Res1-Myc*	Cross RBP571xRBP1	Pai *et al*., 2017
*set2*Δ *Cdc10-Myc*	Cross RBP571xRBP44	Pai *et al*., 2017
*nrm1*Δ	h-, ade6-210, arg3-D4, his3D1, leu1-32, ura4-D18, nrm1::Hyg	de Bruin group collection
*yox1*Δ	h+, ade6-M210 ura4-D18 leu1-32 *yox1*::KanMX4	Bioneer Deletion Mutant library
*nrm1*Δ*yox1*Δ	Cross *nrm1*Δx*yox1*Δ	Louise Holland's undergrad report
*rad51*Δ	h+ ade6-M210 ura4-D18 leu1-32 *rad51*::KanMX4	Bioneer Deletion Mutant library
*nrm1*Δ*rad51*Δ	Cross *nrm1*Δx*rad51*Δ	Louise Holland's undergrad report
*hsp3105*Δ	h+ ade6-M210 ura4-D18 leu1-32 *hsp3105*::KanMX4	Bioneer Deletion Mutant library
*nrm1*Δ*hsp3105*Δ	Cross *nrm1*Δx*hsp31051*Δ	Louise Holland's undergrad report

Table 2. continued.

Strain	***Strain genotype***	***Source***
*meu17*Δ	h+ ade6-M210 ura4-D18 leu1-32 *meu17*::KanMX4	Bioneer Deletion Mutant library
*nrm1*Δ*meu17*Δ	Cross *nrm1*Δx*meu17*Δ	Louise Holland's undergrad report
*egt2*Δ	h+ ade6-M210 ura4-D18 leu1-32 *egt2*::KanMX4	Bioneer Deletion Mutant library
*nrm1*Δ*egt2*Δ	Cross *nrm1*Δx*egt217*Δ	Louise Holland's undergrad report
*wpl1*Δ	h+ ade6-M210 ura4-D18 leu1-32 *wpl1*::KanMX4	Bioneer Deletion Mutant library
P1nmt-nrm1	leu1-32, ura4-D18, P1nmt-*nrm1*::CloNat	de Bruin group collection
P41nmt-nrm1	leu1-32, ura4-D18, P41nmt-*nrm1*::CloNat	This thesis
P81nmt-nrm1	leu1-32, ura4-D18, P81nmt-*nrm1*::CloNat	This thesis

2.2. Media and growth conditions.

Complete media was purchased from Formedium: Yeast Peptone Dextrose (YPD; CCM0205), Yeast Peptone Dextrose Agar (YPD Agar; CCM0105), Yeast Extract Supplemented (YES; PCM0350), Yeast Extract Supplemented Agar (YES Agar; PCM0405). Selection complete media was supplemented with G418 200 µg/ml (Sigma G1279-1G) for Kan^R, Hygromycin 200 µg/ml (Formedium Hyg5000) for Hyg^R and with nourseothricin 100 µg/ml (Stratech AB-102L-JEN) for Nat^R. All strains were grown in liquid media at 30°C with aeration unless otherwise is stated.

Table 3. Media recipes used in this thesis.

Edinburgh Minimal Media for S. pombe*	
Ammonium chloride	5 g/l
Sodium hydrogen phosphate	2.2 g/l
Potassium hydrogen phthalate	3 g/l
Adenine	450 mg/l
Uracil, leucine, histidine**	225 mg/l each
Magnesium chloride hexahydrate	1.05 g/l
Calcium chloride dihydrate	0.0147 g/l
Potassium chloride	1 g/l
Sodium sulphate	0.04 g/l
Nicotinic acid	0.01 g/l
Inositol	0.01 g/l
Pantothenic acid	0.001 g/l
Biotin	0.00001 g/l
Citric acid	0.001 g/l
(Ortho)-boric acid	0.0005 g/l
Manganese sulphate	0.0004 g/l
Zinc sulphate heptahydrate	0.0004 g/l
Ferrous (3) chloride	0.0002 g/l
Molybdic acid	0.00004 g/l

Table 3. continued.

Potassium Iodide	0.0001 g/l
Copper sulphate pentahydrate	0.00004 g/l
Malt extract media (S. pombe mating media)	
Malt extract	30 g/l
Adenine, histidine, leucine, uracil	225 mg/l of each
Adjust pH to 5.5 with NaOH	
Agar	20 g/l
Drop-out media for S. cerevisiae	
Yeast nitrogen base w/o amino acids	6.7 g/l
Glucose	20 g/l
Agar	20 g/l
Drop-out amino acid mix CSM, –Leu, -Ura, + 40 Ade	700 mg/l
5-FOA, -Leu media for S. cerevisiae	
Yeast nitrogen base w/o amino acids	6.7 g/l
Glucose	20 g/l
Agar	20 g/l
Drop-out amino acid mix CSM, -Leu + 40 Ade	690 mg/l
5-Fluoroorotic acid	1 g/l

*separate glucose, salt, vitamin and minerals stocks were prepared and added after autoclaving; **-ura media was made to select *ura*+ clones.

2.3. Yeast strains generation: PCR-based method and LiAc transformation.

Gene deletions was carried out via PCR-based methods according to Longtine *et al*., 1998 and Bahler *et al*., 1998. Plasmid templates pFa6-NatMX4 and pFa6-KanMX4 were amplified with primers carrying homology arms to a target gene as described elsewhere (Longtine *et al*., 1998; Bahler *et al*., 1998). The size of the fragment was confirmed by DNA gel electrophoresis. Yeast culture were then transformed by LiAc method. 50 ml of yeast culture of OD=0.8-1.0 were spun, washed with water, transferred into micro centrifuge

tube and suspended in 1 ml of 100 mM LiAc/ 10 mM Tris-Cl pH7.5. Then 100 µl of the mixture were mixed with 1µg of PCR product and 2 µl of 10µg/ml ss-DNA and incubated for 5 min at room temperature. 280 µl of 40% PEG/100 mM LiAc/10 mM Tris-HCl pH 8 solution was added to the mixture and mixed by inversion. The tubes were incubated for 1 hour at 30°C shaking. After this 43 µl of DMSO were added and tubes were mixed gently. The mixture was subjected to 10 min heat shock at 42°C in a water bath and 5 min on ice. PEG and DMSO were removed, cells were washed in 1 ml of H_2O, suspended in 200 µl of H_2O and plated on complete media and incubated at 30°C overnight. The next day the plates were replica plated onto media containing G418 or Nat and incubated at 30°C until colonies appear. Colonies were re-streaked again on selective media. Deletion was confirmed by total DNA PCR with primer pairs 1) within open reading frame and 2) upstream ORF and within selection cassette (PCR mix for Q5 Polymerase NEB M0491S according to the manufacturer; 30 sec. at 98°C, followed by 25 cycles of 10 sec 98°C, 30 sec. 56°C, 72°C 1 min 20 sec, and 7 min extension at 72°C).

2.4. Yeast strains generation: *S. pombe* mating.

Double deletion and Myc-tagged *S. pombe* strains were generated by mating. The same amount of yeast cells was mixed on malt extract plate (ME) and let to grow for 3-4 days at room temperature. The efficiency of a cross was monitored by the presence of asci under a light microscope. The cells/asci mix was suspended in 400 µl of H_2O with 4 µl of glusulase (Perkin Elmer NEE154001EA). The mixture was incubated for 4 hours at 37°C to break asci walls. The spore mixture was then washed, diluted in water to 1:100 and 1:1000 and 100 µl aliquots were plated on selective media and incubated until colonies appear. The colonies were again re-streaked onto selective media and double selective media to identify double mutants.

2.5. Tetrad dissection.

Tetrad dissection was performed by Steffi Klier. *S. pombe* cells were mated for 2 days as described in 2.4. A loop of crossed cells was suspended in distilled water and a drop of mixture was spread onto YES plate. Individual asci

were placed in line using microdissection microscope (Singer Instruments). Plates were then incubated at 37°C for 4 hours and 4 individual spores were placed on a grid using microdissection microscope. The spores were then allowed to grow at 30°C for 48-72 hours and images of the plates were obtained with Epson Expression 1680 Pro scanner. Individual colonies were restricted onto plates containing G418 Kan and Hyg to confirm genotypes.

2.6. Cell cycle arrest and release.

Exponentially growing yeast cultures were synchronised with mating pheromone (8 µl of 2 mg/ml into 100 ml of culture; GenScript RP01002) for at least 90 min. The arrest was monitored by the absence of budding cells and the presence of “shmoos” under the light microscope. Cells cultures were then washed with fresh YPD media and suspended in warm YPD media. These cultures were incubated at 30°C with aeration and used for a time course.

2.7. RNA extraction and Reverse Transcriptase Quantitative PCR.

Transcript levels were analysed by RT-qPCR. Cells were harvested from 15 ml of yeast culture at different time points. Cell pellets were snap frozen in liquid nitrogen. Total RNA extraction was performed with RNeasy Plus Mini Kit (QIAGEN 74134). Cell pellets were suspended in 600 µl of RLT buffer supplemented with 1% of β-mercaptoethanol and disrupted with glass beads (Biospec 11079105) at 4°C for 20 minutes. The lysate was then separated from glass beads and spun for 2 min at 10,000 rpm. 350 µl of supernatant were used for RNA extraction according to the QIAGEN RNeasy Plus Mini Kit (74134) protocol. Total RNA was diluted to 20 ng/µl and analysed by RT-qPCR using primers from Table 4. RT-qPCR reaction was performed in 14 µl using One step qRT-PCR MasterMix for SYBR® assay No ROX (Eurogentec SYRT-032XNR) with Euroscipt/RNase inhibitor. Reactions were run on Chromo-4 Real-Time PCR detector (Bio-Rad). Obtained data were processed by Bio-Rad CFX Manager 3.0 software. The data was normalised against actin and analysed using Ct value method.

Table 4. Primers used for Reverse Transcriptase Quantitative PCR.

Gene name	***Primer sequence***
Sc*ACT1* FWD	*ATCGTCGGTAGACCAAGACACCAA*
Sc*ACT1* REV	*TCCCAGTTGGTGACAATACCGTGT*
Sc*CLN2* FWD	*TCCCAGGATAGTGATGCCACTGTA*
Sc*CLN2* REV	*GTACTGCCACGCGGATACATCAAT*
Sc*SVS1* FWD	*AGTTACAGCTGCTGCAGTTACCGA*
Sc*SVS1* REV	*TGGGTACCGTTGTTAGCAGAACCT*
Sc*TOS4* FWD	*GTTGGCAGAAACGTCACCCAAGTT*
Sc*TOS4* REV	*ATCACATTGCGAACTATTGCGCCC*
Sc*RNR1* FWD	*GCTCCATTCAAGGCTTACCAAACG*
Sc*RNR1* REV	*GAACGATCGGCTGCCATGTTAATG*
Sc*CDC21* FWD	*TGCTAAAGTTGTCGACATGGAGCC*
Sc*CDC21* REV	*CGGGAATGGTCTTGGATTTCTGGT*
Sc*PRY2* FWD	*ACCCAAGTCGTATGGAAGGGA*
Sc*PRY2* REV	*CCAGCGGCTTTGTAGGAACA*
Sp*cdc18* FWD	*GTAGGCATGCAATTGAACTTGCGG*
Sp*cdc18* REV	*TCATAGCAGATGTCGCTCGGACAA*
Sp*cdt1* FWD	*ACCGTATGGCCAGAGTCATTTGCT*
Sp*cdt1* REV	*AATTCAATGGAGCGGGAGAAGGCT*
Sp*cdc22* FWD	*TGCAACGTGTTGAACGTAACGAGC*
Sp*cdc22* REV	*AGGTAATGAACGACGACCACGGTT*
Sp*tos4* FWD	*TTCTGCAGTGAGAAGAGAGCCACT*
Sp*tos4* REV	*AACCGTGGATAGGACATGGTCACA*
Sp*nrm1* FWD	*GGGAAAGGCCAACAAACGAAGTGT*
Sp*nrm1* REV	*ATCGAACCGCAATCGGTGAAATCG*
Sp*act1* FWD	*CGCCGAACGTGAAATTGTTCGTGA*
Sp*act1* REV	*TCAAGGGAGGAAGATTGAGCAGCA*
Sp*rep2* FWD	*TCGCCGGAATGTCACTTATG*
Sp*rep2* REV	*TAAGCCCTTGTCTTGCTTTCT*

2.8. Chromatin Immunoprecipitation and quantitative PCR.

Exponentially growing yeast culture (around 45 ml) was collected for each time point and cross-linking was performed with 1% formaldehyde for 20 min at room temperature. The reaction was terminated with 2.5 M Glycine for 5 min. Then samples were washed 2 times with TBS (Tris-HCl 50 mM / NaCl 150 mM, pH 7.5). The pellet was suspened in 5 ml of TBS, 1 ml was aliquoted into screw cap tube and frozen in liquid nitrogen. The pellet was lysed in 500 µl ice cold ChIP lysis buffer (HEPES-KOH pH 7.5 50 mM / NaCl 140 mM / Triton X-100 1% / Sodium Deoxycholate 0.1% / EDTA 1mM) supplemented with Protease Inhibitors (Roche 04693124001) by shaking with glass beads (BioSpec Products 11079105) for 20 min. Lysate was separated from the beads and spun for 10 min at 14,000 rpm 4°C. Obtained pellet was suspended in 500 µl of ChIP lysis buffer supplemented with protease inhibitors. DNA was sheared by sonication (Qsonica sonicator, amplitude 100%, process time 5 min with pulse-ON 30 sec. and pulse-OFF 2 min). Sonicated mixture was separated (10 min 14,000 rpm 4°C) and 500 µl of chromatin lysate was collected. Whole cell extract (5 µl) were collected into separate tube and stored at -20°C until further use. Antibodies were added according to manufacture instruction and samples were incubated at 4°C overnight rotating. Next morning chromatin lysate with antibodies was mixed with 35 µl of ice cold 50% suspension of Protein A-Sepharose beads (Sigma P3391-1.5G) in lysis buffer and incubated for 3 hours rotating. Beads were then washed 6 times with 1 ml of freshly prepared cold wash buffer (Tris-HCl pH 7.5 / Triton X-100 1% / NaCl 150 mM / EDTA 5 mM / NP-40 0.5%). To de-cross link beads were mixed with 100 µl of 10% Chelex Resin (Bio-Rad 142-1253). Chelex suspension was also added to corresponding whole cell extract. The mixture was shaken for 10 sec., boiled for 10 min and then spun for 1 min at 12,000 rpm. Then 70 µl of the supernatant was moved to a new tube. 120 µl of water (Millipore H2OMB0501) was added to the remaining Chelex. The mixture was shaken and spun again. 100 µl of the supernatant were added to the previous 70 µl. DNA samples were used to run qPCR on Chromo-4 Real-Time PCR detector (Bio-Rad). The reactions were run in 14 µl with One step qRT-PCR MasterMix for SYBR® assay No ROX

(Eurogentec SYRT-032XNR) without Euroscript and with primers from the Table 5. Obtained data were processed by Bio-Rad CFX Manager 3.0 software. The data was analysed as % of the whole cell extract.

Table 5. Primers used for ChIP Quantitative PCR.

Gene name	***Primer sequence***
Sc*CLN2* FWD	*TGAGGATCTAACCTGCGAAATG*
Sc*CLN2* REV	*TGCGTGCGATACGCAAATA*
Sc*PCL1* FWD	*ACAGCGGCACGAACAAGAATTTCG*
Sc*PCL1* REV	*ATTTGGCTCCCGACATTTCGAGTC*
Sc*PRY2* FWD	*TGGCGATGTGCTTCGAG*
Sc*PRY2* REV	*GCCGGCTCGATTTCATTTG*
Sc*NRM1* FWD	*CAGCGCGGAGTTGAACGATTACAT*
Sc*NRM1* REV	*TCGGTCATTTACATTGGGAAGGGC*
Sc*MCD1* FWD	*GATTTCATTCCCGGCCTCTTA*
Sc*MCD1* REV	*CGTCCCTCCTCGAGTTATTTG*
Sc*ELO1* FWD	*ACGTGACGTGACGAAATATTAG*
Sc*ELO1* REV	*GGCTTCCTTTCTTTCCCTTATG*
Sp*cdc18* FWD	*GGCATTTCATATCTTTGAGGATGAGTCGT*
Sp*cdc18* REV	*ATGTCGCGTTCAACTCTACGTGTC*
Sp*cdt1* FWD	*TTTCAGAGAGCCTGAACTTGG*
Sp*cdt1* REV	*CTCCTTTGCTCTGCGAGATATTA*
Sp*cdc22* FWD	*ACTTAAAGTTCGGATGACGCGACG*
Sp*cdc22* REV	*GTTTGTAAGGTGGTAAATACCGGG*
Sp*tos4* FWD	*CACTGGGTTACTCTCGTTTCTT*
Sp*tos4* REV	*CCTGGGTATAAACACGCTATGA*
Sp*byr3* FWD	*TGGCAAGTTGTTGTGCTTCTTCCG*
Sp*byr3* REV	*TAACAAGCACATGGTGGCACTTGG*
Sp*rps17* FWD	*GCACCTGGTTTGTTGTTGGTTG*
Sp*rps17* REV	*TTCGTAACCTCCGTCGCTTCTGTT*

2.9. Western Blot Analysis.

Protein extraction for Western Blot analysis was performed as following. Exponentially growing yeast cultures were diluted to the same OD to have the same cell number. 10 ml of yeast cultures were spun and pellets were washed in ice cold distilled water. Obtained pellets were suspended in 300 µl of lysis buffer (Tris-HCl pH8 50 mM / NaCl 150 mM / EDTA 7 mM / DTT 5 mM) supplemented with protease inhibitors (Roche 04693124001) and broken with glass beads (BioSpec Products 11079105). The lysate was separated by centrifugation (5 min at 13000 rpm) and mixed with 1/6 volume of sample buffer (Tris-HCl pH 6.8 50 mM / Sodium Dodecyl Sulphate 2% / Bromophenol Blue 0.01% / Glycerol 10%). The samples were then boiled for 5 min and 20 µl were loaded onto NUPAGE™ 4-12% Bis-Tris gel (Invitrogen NP0322BOX) and run using MOPS SDS Running Buffer (Invitrogen NP0001). Then a wet transfer was performed using nitrocellulose blotting membrane (Amersham Protan 0.2 NC, 10600001, GE Healthcare Life Sciences). The membrane was blocked in 10% milk in PBS-tween (NaCl 8 g/l / KCl 0.2 g/l / Na_2HPO_4 1.44 g/l / KH_2PO_4 0.24 g/l / 1% Tween-20®; pH 7.4) at 60°C for 20 min. The membrane was then incubated with primary antibodies in 5% milk/PBS-tween overnight at 4°C rolling. The next day the membrane was washed 5 times for 10 min with PBS-tween and incubated with secondary antibodies (1:3000 dilution) in 5% milk/PBS-tween. The membrane was developed with Luminata™ Crescendo Western HRP substrate (Millipore, WBLUR0100) and XOGRAF Compact X4.

2.10. *S. cerevisiae* cell size and cell growth analysis.

Exponentially growing *S. cerevisiae* cultures were diluted 1:1000 in Isoton Diluent II (Beckman Coulter, 8448011) and cell size of 10,000 cells was analysed by Beckman Coulter Multisizer 4 Particle Counter using Beckman Software. Cell growth was analysed either by changes in OD_{600} or by accumulation of biomass. In the first case yeast cultures were diluted to OD_{600}=0.01 and an increase in optical density was monitored by spectrophotometer for 24 hours. Cell growth rate was calculated as a function of

optical density. Accumulation of biomass was measured by BioLector® (m2plabs). Yeast cultures were diluted to the same cell number and an increase in biomass was monitored for 24 hours at 30°C and 37°C.

2.11. Differential interference contrast microscopy.

Cell imaging was performed using Zeiss Axioplan 2 (63X magnification oil objective, optovar 1, DIC) with Qimaging QIclick Camera and Volocity Acquisition v.5.5.1 Software. Obtained images were processed with ImageJ software.

2.12. *S. pombe* cell length analysis.

Images obtained from DIC microscopy were processed with ImageJ software: cell length of 100 cells of each single and double mutant was measured using inbuilt ImageJ plugin. Cell length distribution was analysed with Graphpad Prism 6, outliers were manually calculated according to the Tukey's rule, where upper fence equals first quartile – 1.5* inner quartile range and lower fence equals third quartile + 1.5 * inner quartile range.

2.13. Synthetic genetic array and analysis of genetic interactions.

Plate-based synthetic genetic array was performed by Mimosa Hoti and is described elsewhere (Roguev et al., 2007). PEM-2 *nrm1*::CloNat query strain was mated to every single deletion strain from the Bioneer Library carrying G418 (KAN) resistance (3420 strains in total). After mating yeast cell underwent sporulation and were subjected to anti-diploid and mating type selection. Anti-diploid selection eliminated diploid cells which can overgrow slower growing double deletion strains. And elimination of one of the mating types was required to prevent re-mating. PEM-2 strategy allowed these two selections simultaneously: this strain contains cycloheximide sensitive allele in mating locus and cycloheximide resistant allele in endogenous ribosome gene. After mating and sporulation only cycloheximide resistant cells with single mating type remained. Then double deletion progeny was selected in quadruplicate on

media containing both CloNat and G418. The size of colonies was analysed with Spotsizer Software (Bischof *et al*., 2016) by Charalampos Rallis. The size of colonies of double deletion cells was compared to *nrm1*Δ colony size and scored from -2 to 2, with -2 poor growth/negative interaction and 2 better growth/positive interaction. Slim gene ontology term for a biological process for selected genes was established using PomBase database.

2.14. Promoter switch of *nrm1* and characterisation.

P1-nmt-nrm1 promoter was swapped with inducible promoters P41-nmt and P81-nmt according to Bähler *et al*., 1998. The fragments carrying P41-nmt and P81-nmt were amplified by PCR with forward primer *TGCCACAAGTACGCAACAATCGACGAGTCGCAAAAAAACTGTCTCTGATTA TTACTTTTCCTTCTGATTCTCTGCTACTAGAATTCGAGCTCGTTTAAAC* and reverse primer *CCATACTCATACACTTCGTTTGTTGGCCTTTCCCCCAAAGT GTTTAAACGAGATGGGGTCAATGGTTCCATTGACCTATCCATGATTTAACA AAGCGACTATA* from *P41nmt-pFa6a-natMX6* and *P81nmt-pFa6a-natMX6.* The resulting PCR product was transformed into wt and P1-nmt-*nrm1*::CloNat strain by LiAc transformation described in 2.3 and positive clones were selected on plates, containing CloNat. Transcript levels of *nrm1* and two MBF targets *cdc22* and *cdc18* were analysed before and after treatment with 5 μg/ml of thiamine for 18 hours by RT-qPCR.

2.15. Microarray analysis.

Microarray analysis was performed by Adi Hendler and Amir Aharoni and details can be found in Hendler *et al*., 2017.

2.15. Statistical analysis.

Unpaired two-tailed t-test was performed using Graphpad 6 to establish statistical significance in differences between transcript levels and *S. pombe* cell length.

3. RESULTS

3.1. The role of histone acetylation in G1/S cell cycle transcription in *S. cerevisiae*.

Histone posttranslational modifications provide an important level of transcriptional regulation via chromatin compaction by altering histone-DNA and histone-histone interactions. In addition, modified histones can serve as a platform for transcription factor binding and other histone modifying enzymes. Histone acetylation is a widely studied histone modification, which plays an important role in transcriptional regulation. Histone acetyltransferases catalyse addition of acetyl moieties to histone tails, which results in chromatin relaxation. DNA becomes accessible for transcriptional machinery and genes can be transcribed. Therefore, high acetylation levels are considered to be a mark of active transcription.

In budding yeast the transcription factor activator SBF (Swi4-Swi6) and repressor MBF (Mbp1-Swi6) complexes are both required for the regulation of the G1/S transcriptional regulon. Whilst SBF and MBF regulate transcription via completely different mechanisms (Fig.1A), the temporal patterns of expression of SBF and MBF-dependent genes are similar with peak transcription levels at the G1-to-S transition. Interestingly genome-wide studies show that histone deacetylase Rpd3 and histone acetyltransferase Gcn5 are recruited to SBF and SBF and MBF target genes respectively (Cosma *et al*., 1999; Robert *et al*., 2004; Stefan & Koch, 2009). The role of Rpd3 in G1/S transcription repression is supported by increase in transcription of such SBF targets as *CLN2* and *SVS1* in asynchronous culture in *rpd3*Δ cells (Fazzio *et al*., 2001). The current model of Rpd3-dependent repression of SBF targets suggests that Whi5/Stb1 (SBF inhibitors) recruit Rpd3 to SBF promoters (Takahata *et al*., 2009) to repress SBF targets in G1. However, the direct role of Rpd3 and Gcn5 in regulation of G1/S cell cycle regulated transcription is not established.

3.1.1. The increase in acetylation at SBF and MBF promoters corresponds with the increase in SBF and MBF-dependent transcription.

Based on previous studies, which implicate Rpd3 and Gcn5 in regulation of G1/S transcription in yeast the acetylation state of histones at the promoters of G1/S target genes changes during the cell cycle. The analysis of budding, transcription and acetylation by ChIP (Fig.3-5) were performed by Dr. Michael Harris. *CLN2* and *SVS1* were used as representative of SBF targets, since Rpd3 is recruited to *CLN2* promoter (Takahata *et al*., 2009), and *CLN2* and *SVS1* transcription increases in *rpd3*Δ mutants (Fazzio *et al*., 2001). *CDC21* and *RNR1* were picked as representative MBF targets. Exponentially growing *S. cerevisiae wt* culture was synchronised with mating pheromone to arrest cells in G1 and released into fresh medium. Samples for RNA and ChIP were collected at every 15 minutes for 75 minutes. Progression through the cell cycle was monitored by budding index (Fig.3). An increase in budding happened after 30 min, indicating that cells transit from G1 to S phase at 30 min after release. Transcript levels were established by RT-qPCR by Ct value method and levels of *ACT1* were used for normalization. Presence of histone acetylation marks was established by chromatin immunoprecipitation using antibodies to the following histone marks: H3K9ac (Millipore 07-352), H3K14ac (Millipore 07-353), H3K18ac (Millipore 07-354), H3K27ac (Millipore 07-360) and H4K5ac (Millipore 07-327).

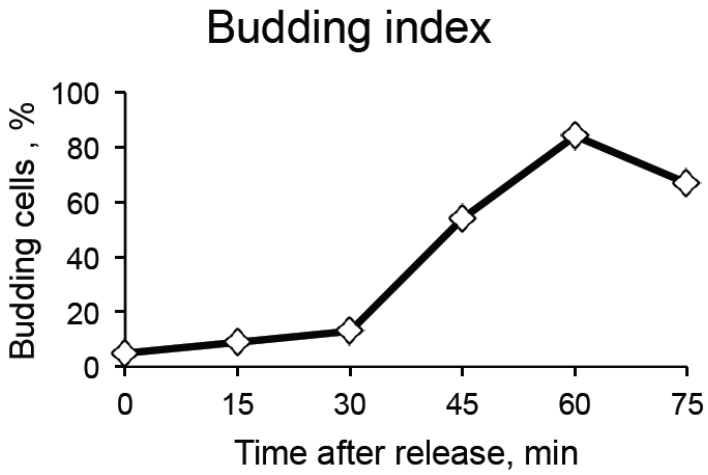


Figure 3. Budding index shows progression through the cell cycle. *S. cerevisiae* wt culture was synchronised with mating pheromone in G1 phase and released. Number of budding cells was counted under light microscope at each time point for 100 cells.

Transcription of all G1/S targets peaked at 30 min after release (G1/S transition) as expected (Fig.4A). We observed that acetylation at SBF and MBF target promoters followed the expression pattern of *CLN2* and *SVS1* (SBF targets) (Fig.4) and *RNR1* and *CDC21* (MBF targets) (Fig.5) with lower acetylation/expression levels in G1 phase and maximum levels upon G1-to-S phase transition. The enrichment in H3K9ac, H3K14ac and H3K27ac at G1/S promoters reached maximum levels at 30-45 minutes after release from G1 arrest, which coincides with a peak in transcription. At the same time levels of H4K5ac were already high in G1 with some fluctuation along the cell cycle. Our data shows that histone acetylation levels at SBF- and MBF-target gene promoters are cell cycle regulated and correlate with G1/S transcription levels. The cell cycle-dependent regulation of histone acetylation at G1/S promoters corresponds with the recruitment of both the histone acetyltransferases Gcn5 and the histone deacetylases Rpd3 to G1/S promoters, established by previous studies. These results suggest that histones at both SBF and MBF target genes undergo cell cycle dependent acetylation.

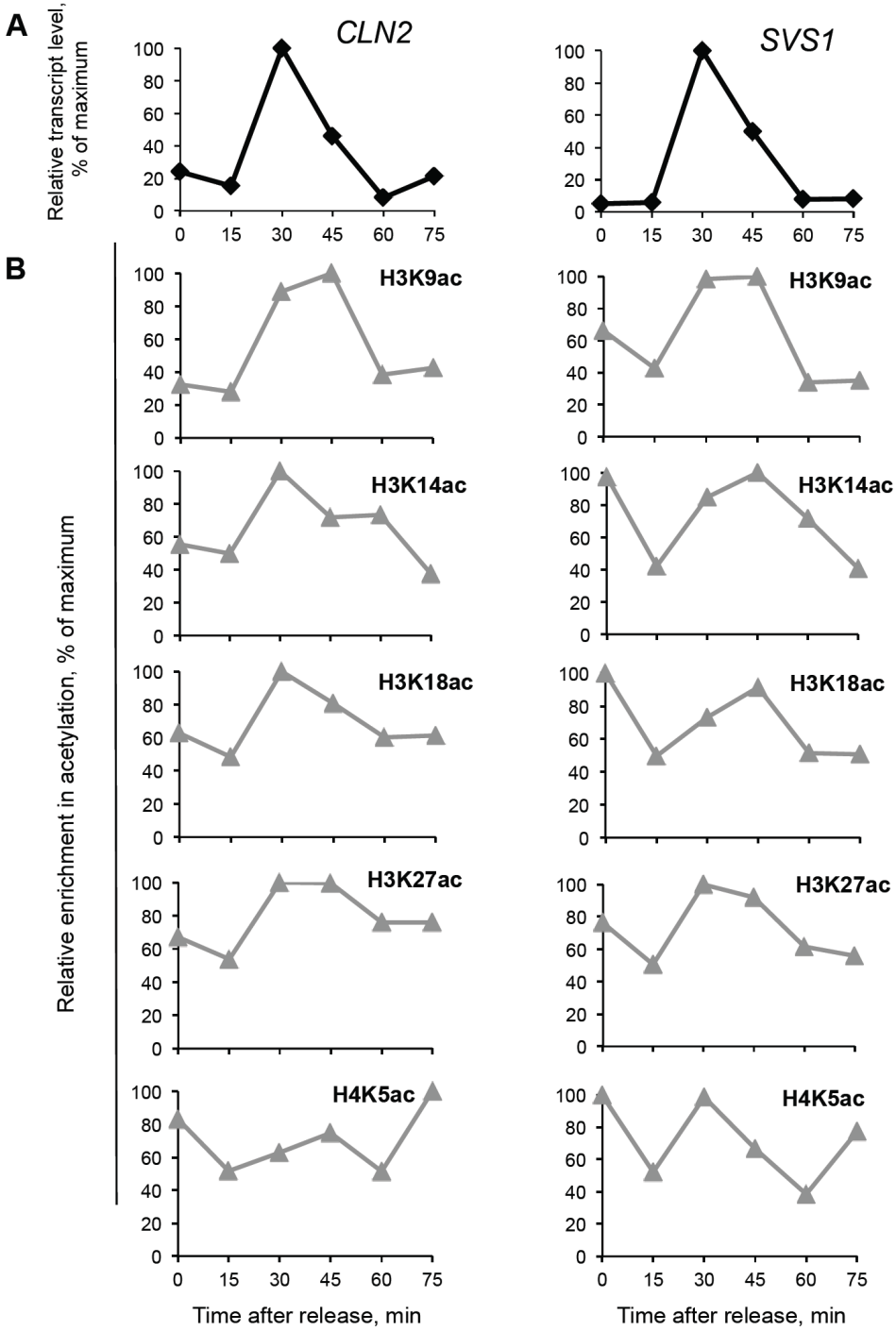


Figure 4. The correlation between levels of transcription of SBF targets *CLN2* and *SVS1* and acetylation at *CLN2* and *SVS1* promoters in wt *S. cerevisiae*. (A) Exponentially growing wt culture was synchronised in G1 and released. Transcript levels were established by RT-qPCR and normalised to *ACT1* (relative to the maximum level); (B) ChIP was performed according to the protocol with antibodies to H3K9ac, H3K14ac, H3K18ac, H3K27ac and H4K5ac and enrichment was established by qPCR with primers specific to *CLN2* and *SVS1* promoters. (Performed by Dr. Michael Harris)

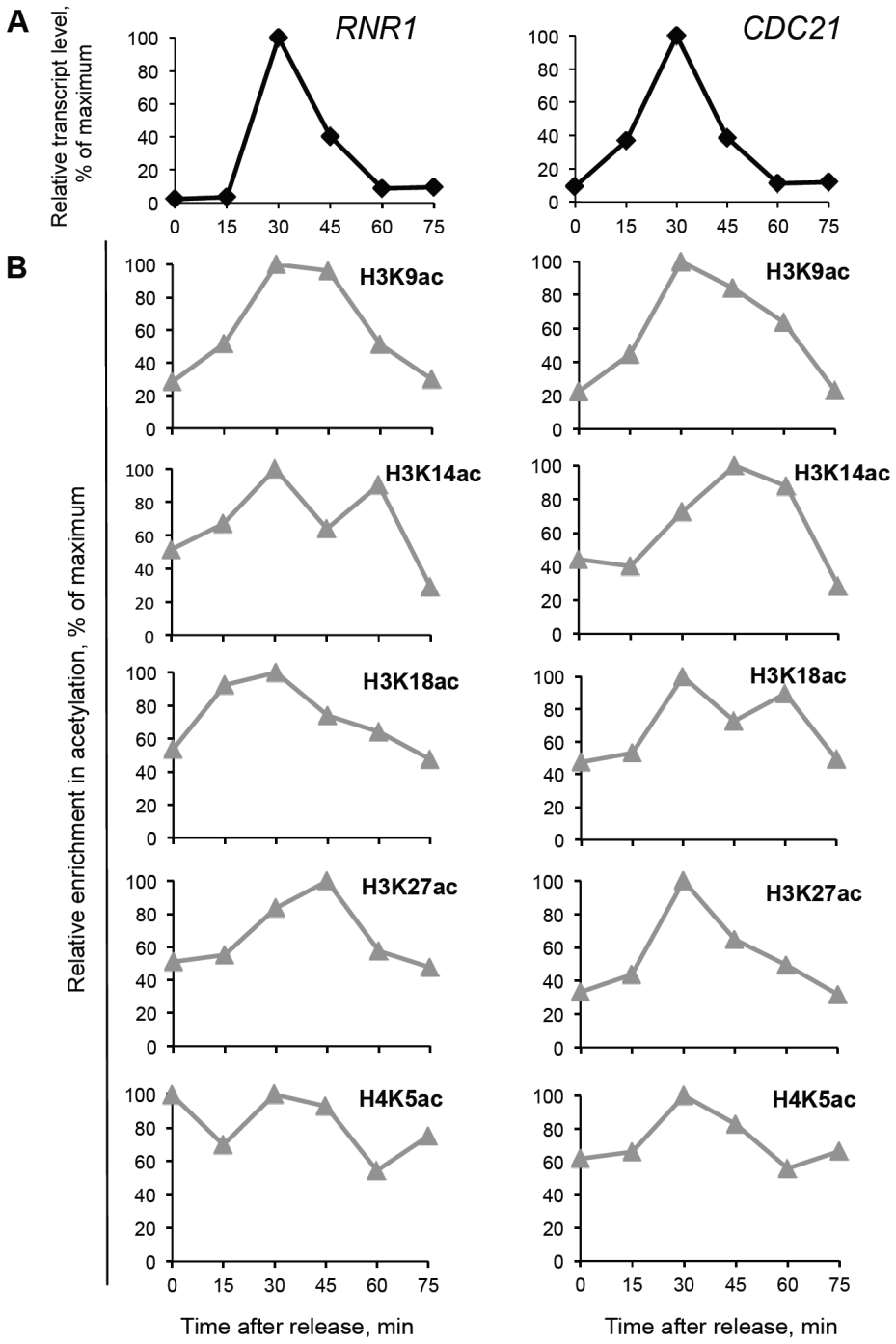


Figure 5. The correlation between levels of transcription of MBF targets *CDC21* and *RNR1* and acetylation at *CDC21* and *RNR1* promoters in wt *S. cerevisiae*. (A) Exponentially growing wt culture was synchronised in G1 and released. Transcript levels were established by RT-qPCR and normalised to *ACT1* (relative to the maximum level); (B) ChIP was performed according to the protocol with antibodies to H3K9ac, H3K14ac, H3K18ac, H3K27ac and histone H4K5ac and enrichment was established by qPCR with primers specific to *RNR1* and *CDC21* promoters. (Performed by Dr. Michael Harris)

3.1.2. HDAC Rpd3 is involved in repression of cell cycle transcription in G1 phase of the cell cycle.

Our data, showing that histone acetylation at G1/S promoters is low in G1, correlates with previous studies that showed that Rpd3 is recruited to *CLN1* and *CLN2* promoters in G1 (SBF targets). In addition, deletion of Rpd3 results in upregulation of the SBF targets *CLN2* and *SVS1* in asynchronous cultures. Overall this suggests that Rpd3 is required for repression of transcription in G1, however, to what extent Rpd3 contributes to repression in G1 has not been reported (Takahata *et al*., 2009). Based on the current model, deletion of Rpd3 should lead to de-repression of G1/S transcription in G1. To test this, I initially investigated if deletion of *RPD3* results in an increase of *CLN2* and *SVS1* transcription in G1 phase. Exponentially growing wt and *rpd3*Δ cultures were arrested in G1 with mating pheromone for 90 min, RNA was extracted and relative transcript levels were assessed by RT-qPCR using the Ct value method using *ACT1* levels for normalization. Whilst my data shows that *CLN2* and *SVS1* transcription is upregulated in G1 phase in *rpd3*Δ cells (*CLN2* not significantly, unpaired t-test P value = 0.0568 and *SVS1* significantly P value = 0.0037) transcription levels are still considerably lower than induced levels during the G1/S transition (see next section 3.1.3. and Fig.6). These results suggest that Rpd3 contributes to the repression of *CLN2* and *SVS1* in G1 phase, but is not required for preventing active transcription.

Next, I tested if Rpd3 is required for repression of MBF targets in G1. I compared transcript levels of representative MBF targets *CDC21* and *RNR1* in *wt* and *rpd3*Δ cells. I observed significant upregulation of both *CDC21* (P value = 0.006) and *RNR1* (P value = 0.0106) (Fig.6) suggesting that Rpd3 is required for repression of MBF targets in G1. Altogether these results indicate that Rpd3 is required for full repression of G1/S target in G1, but has a more prominent role in the repression of MBF vs SBF targets. To test this further I checked transcription levels of a G1/S switch gene *TOS4*, which is regulated by both SBF and MBF: by SBF in G1 phase and by MBF outside of G1. Transcription levels of *TOS4* were dramatically increased in G1 in some samples, but averaged not significantly (P value = 0.0716 based on unpaired t-test) in *rpd3*Δ cells, which corresponds with my results for the SBF target *CLN2*.

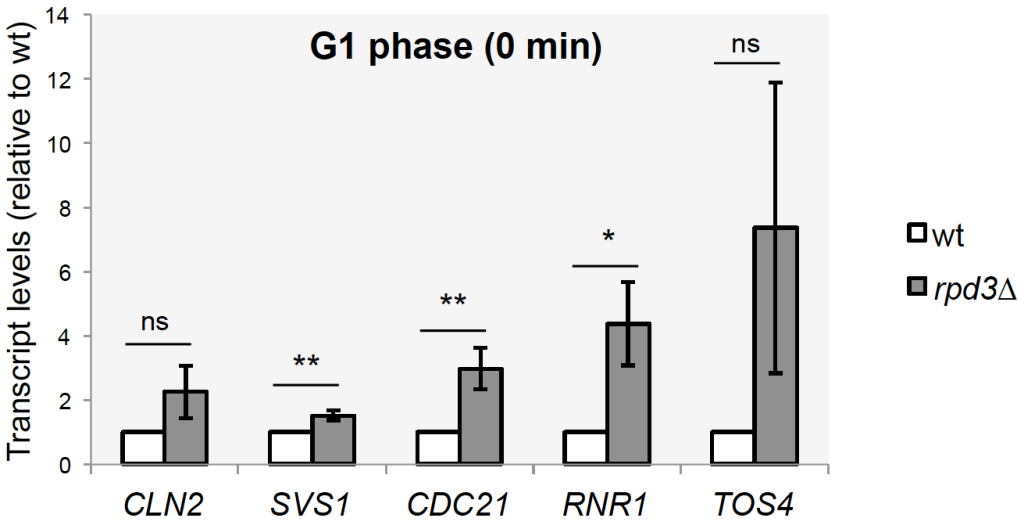


Figure 6. Transcription of SBF (*CLN2* and *SVS1*) and MBF (*CDC21* and *RNR1*) genes and switch gene *TOS4* is upregulated in *rpd3*Δ cells in G1 phase. Exponentially growing wt and *rpd3*Δ cultures were arrested in G1 phase. Transcript levels were established by RT-qPCR and normalised to *ACT1*. Transcript levels relative to wt are represented (3 biological repeats, error bars represent standard deviation; ** = P value ≤ 0.01; * = P value ≤ 0.05; ns = P value > 0.05).

3.1.3. HDAC Rpd3 is involved in modulation of G1/S cell cycle transcription.

The G1/S transcriptional wave is regulated by inhibition/repression in G1 phase, activation upon G1-to-S-phase transition and loss of activation/co-repression in S phase. Published data and my results suggest that Rpd3 is required for full repression of G1/S targets in G1, but how this compares to peak transcript levels during the G1/S transition and if Rpd3 has a role in S phase has not been studied. Based on this I decided to investigate whether deletion of *RPD3* affects the G1/S transcriptional wave. Exponentially growing wt and *rpd3*Δ cultures were arrested in G1 phase with mating pheromone and subsequently released. Samples for RNA extraction were collected every 15 min after release and transcript levels were quantified by RT-qPCR using Ct value were normalised to *ACT1*. Progression through the cell cycle was monitored by budding index. wt and *rpd3*Δ cells progressed through the cell cycle at the same rate and entered S phase at 45 min after release (Fig.7A). Therefore, the transition from G1 to S happened at 30 min after release.

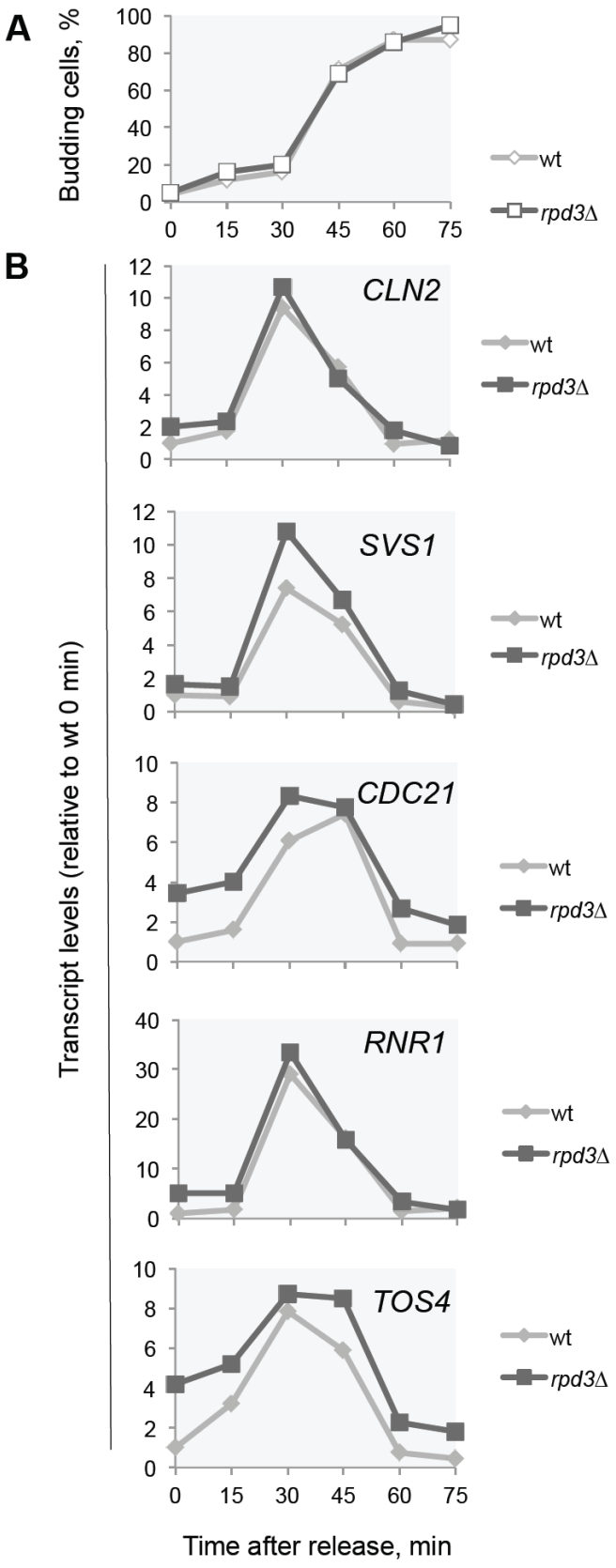


Figure 7. Progression through the cell cycle and G1/S transcriptional wave are not affected in the absence of Rpd3. (A) Budding indexes. Exponentially growing wt

(continued) and *rpd3*Δ cultures were arrested in G1 phase and released. Number of budding cells was counted under the light microscope at each time point, 100 cells were counted in total. (B) Transcript levels in wt and *rpd3*Δ cells during cell cycle. Exponentially growing wt and *rpd3*Δ cultures were arrested in G1 phase and released. Transcript levels were established by RT-qPCR and normalised to *ACT1*.

In agreement with previously published results I observed that in wt cells transcription of both SBF and MBF targets is low in G1 (0-15 min), reaches maximum levels at the G1/S transition (30 min) and is repressed once cells proceed into S phase (45-60 min) (Fig. 7B representative, Suppl.fig.1B). *rpd3*Δ cells show an increase in fold induction of transcription in G1 (0 min) and S phase (60 min) (Fig.7B representative, Suppl.fig.1B) suggesting that Rpd3 is required for full repression in G1 and during S phase. However, in the context of the level of transcriptional activation during the G1/S transition this de-repression seems largely insignificant, suggesting a limited role in the regulation of the G1/S transcriptional wave by Rpd3. To look at this in more detail I directly compared transcript levels at the G1-to-S phase transition (30 min) and late S phase (60 min) in wt and *rpd3*Δ cells (Fig.8). While at G1/S transition transcript levels of SBF and MBF targets were similar in wt and *rpd3*Δ, in S phase transcription is significantly upregulated in *rpd3*Δ cells in comparison to wt (*CLN2* P value = 0.02015; *SVS1* P value = 0.0317; *CDC21* P value = 0.0048; *RNR1* P value < 0.0001). Transcription of the switch gene *TOS4* was upregulated in both G1/S and S phase (P value = 0.0337; P value = 0.0325). Overall my results show, that whilst Rpd3 is required for full repression of G1/S transcription it is not essential for confining transcription to G1.

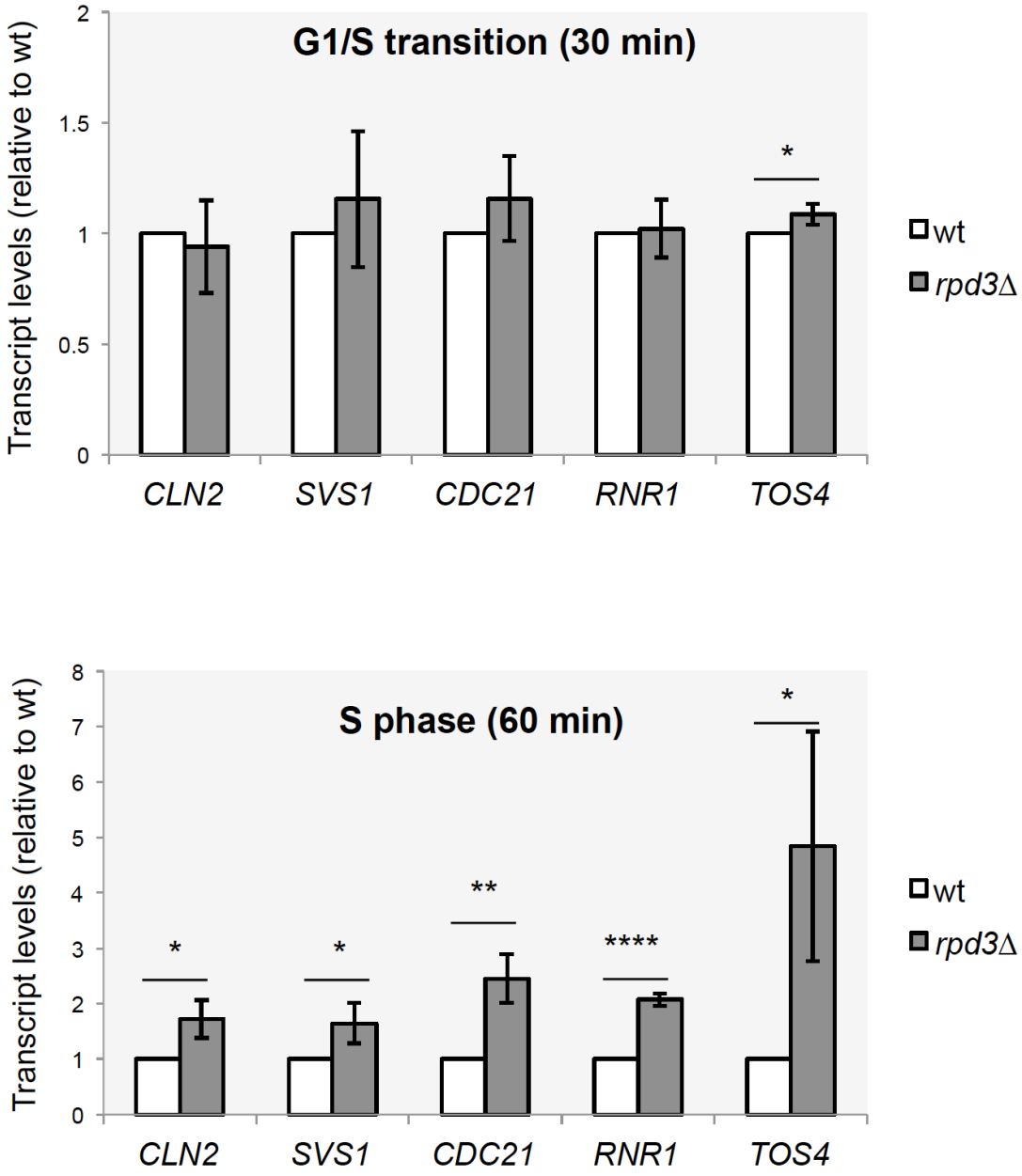


Figure 8. Transcription of SBF (*CLN2* and *SVS1*) and MBF (*CDC21* and *RNR1*) genes and switch gene *TOS4* is upregulated in S phase in *rpd3*Δ cells. Exponentially growing wt and *rpd3*Δ cultures were arrested in G1 phase. Transcript levels were analysed by RT-qPCR and normalised to *ACT1*. Transcript levels relative to wt are represented (3 biological repeats, error bars represent standard deviation; **** = P value < 0.0001; ** = P value ≤ 0.01; * = P value ≤ 0.05; ns = P value > 0.05).

3.1.4. Histone acetyltransferase Gcn5 is involved in peak G1/S cell cycle transcription.

We found that the increase in the levels of acetylation at SBF and MBF target genes coincides with increase in transcription (Fig.4 and 5) and that HDAC Rpd3 is involved in 'tuning' the repression of SBF and MBF targets in G1 and S phase. This suggests that histone deacetylation at G1/S promoters has a role in full repression of G1/S transcription. In turn histone acetylation at G1/S promoter is expected to have a role in transcriptional activation. The most likely candidate involved in histone acetylation at G1/S promoters is the HAT Gcn5, which together with SAGA, is recruited to SBF and MBF target genes (Cosma *et al*., 1999). Gcn5 is a part of SAGA transcriptional co-activator (Grant *et al*., 1998), and this complex is required for nucleosome displacement and recruitment of RNA Polymerase II and other co-activators. Therefore, I decided to assess to what extend Gcn5 is involved in regulation of G1/S transcription. As in previous experiments, exponentially growing cell cultures, wt and *gcn5*Δ*,* were arrested in G1 phase with the mating pheromone, released into the fresh media and samples were collected every 15 min after release. Transcript levels were established by RT-qPCR using Ct value method and normalised to *ACT1*. Progression through the cell cycle was monitored by percentage of budding cells (Fig.9A). Budding index revealed that deletion of *GCN5* leads to a cell cycle delay. In line with this transcription of all genes, except *CLN2*, reached peak levels later than in wt cells, which might be at the basis of transcription of G1/S targets not being induced to the same levels as in wt (Fig.9B representative; Suppl.fig.2B). Lower levels of G1/S transcription may be a consequence of the cell cycle delay, which causes de-synchronisation of the cell population and transcription. Based on this it is hard to conclude if Gcn5 is required for transcriptional induction. An interesting hypothesis is that the cell cycle delay in *gcn5*Δ may be caused by lower levels of G1-cyclin *CLN2,* which is required for the cell cycle progression, suggesting that Gcn5 is required for activation of G1/S transcription. The possible way to establish, whether Gcn5 is essential for activation of G1/S transcription, is discussed below (see Discussion).

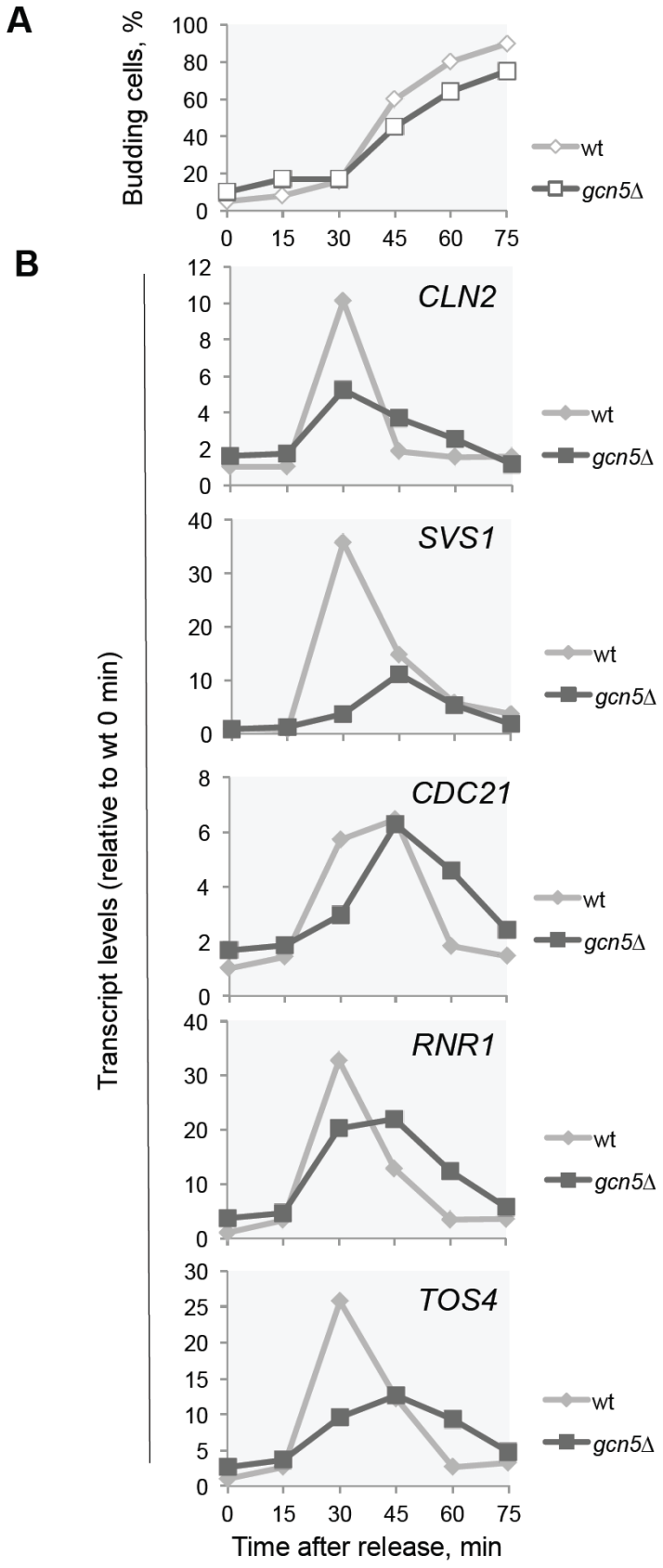


Figure 9. Deletion of *GCN5* results in the cell cycle delay and lower levels of G1/S transcription. (A) Budding indexes. Exponentially growing wt and *gcn5*Δ cultures were

(continued) arrested in G1 phase and released. Number of budding cells was counted under the light microscope at each time point, 100 cells were counted in total. (B) Transcript levels in wt and *gcn5*Δ cells during cell cycle. Exponentially growing wt and gcn5Δ cultures were arrested in G1 phase and released. Transcript levels were established by RT-qPCR and normalised to *ATC1.*

I then compared transcript levels in wt and *gcn5*Δ cells at different time points separately (Fig.10). In G1 phase (0 min after release), apart from *RNR1*, transcription was not significantly affected (P value = 0.0289). This is in line with acetylation levels at G1/S promoters being low in G1 phase. Upon G1-to-S phase transition deletion of *GCN5* leads to significant down-regulation of genes regulated by SBF: *CLN2* (P value = 0.0206); *SVS1* (P value < 0.0001) and *TOS4* (P value = 0.0006), while transcript levels of MBF targets are not significantly affected. As mentioned before the lower peak levels might result from the cell cycle delay observed in *gcn5*Δ cells. In addition, the up-regulation of all G1/S targets except *SVS1*, in *gcn5*Δ cells in S phase, could be the consequence of the delay in cell cycle progression. Overall, my data suggest that Gcn5 is likely to be required for full induction of G1/S transcription.

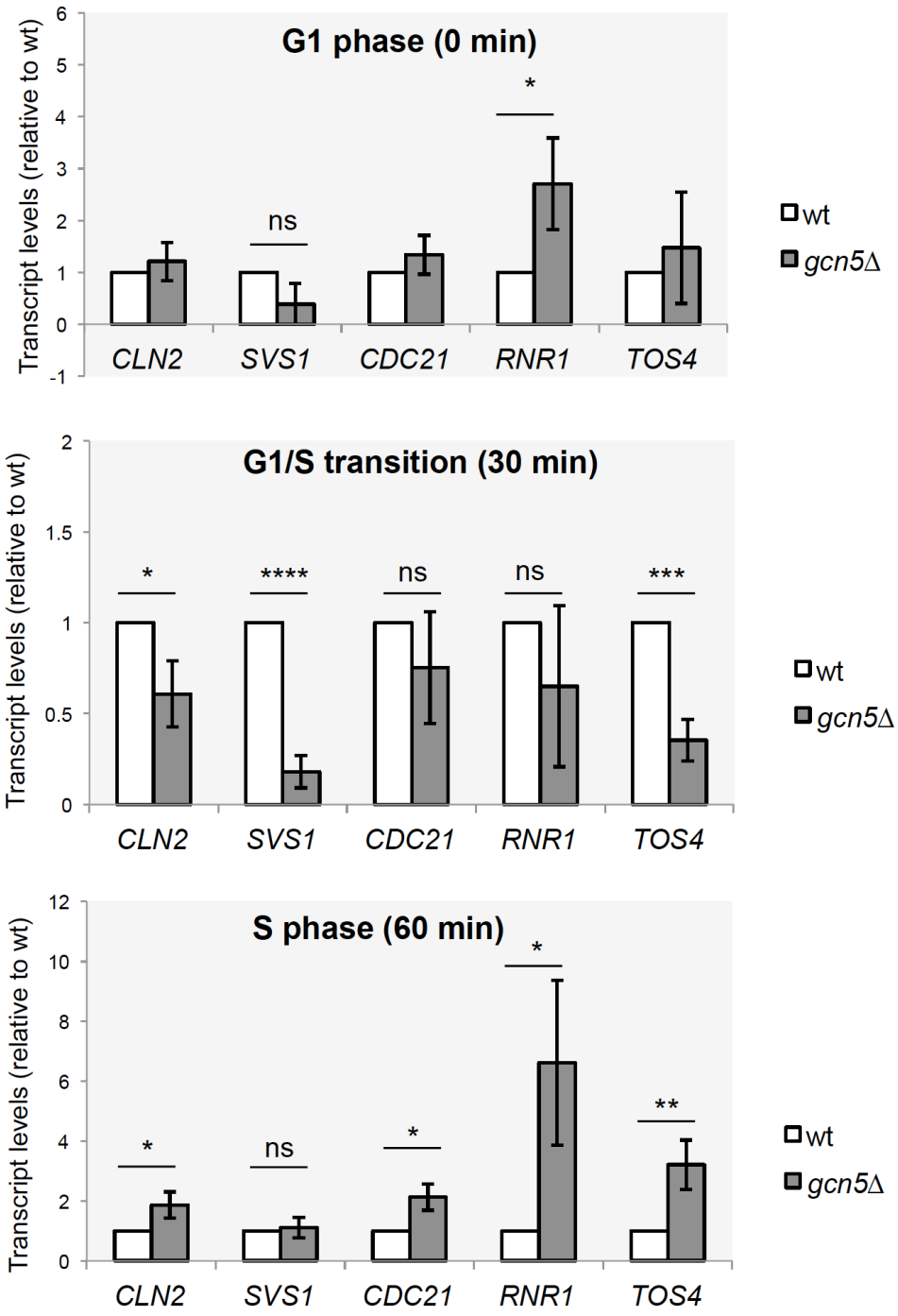


Figure 10. Transcription of SBF-dependent genes *CLN2* and *SVS1* and switch gene *TOS4* is down regulated at G1-to-S phase transition in *gcn5*Δ cells. Exponentially growing wt and *gcn5*Δ cultures were arrested in G1 phase. Transcript levels were analysed by RT-qPCR and normalised to *ACT1.* Transcript levels relative to wt are represented (3 biological repeats, error bars represent standard deviation; **** = P value < 0.0001; *** = P value ≤ 0.001; ** = P value ≤ 0.01; * = P value ≤ 0.05; ns = P value > 0.05).

3.1.5. Gcn5 counteracting partner HDAC Hda1 is not required for repression of G1/S transcription.

My data shows that deletion of *rpd3*Δ only slightly affects repression of G1/S transcription, which can be due to a possible redundancy in HDACs. Acetylation introduced by Gcn5 has been shown to be removed by the histone deacetylase Hda1 (Vogelauer *et al*., 2000). Based on this I decided to test whether Hda1 is involved in repression of G1/S transcription in G1 and S phases. Exponentially growing wt and *hda1*Δ cells were synchronised in G1 with mating pheromone and released. Samples for RNA extraction were collected every 15 min after release, transcript levels were established by RT-qPCR using Ct value method and *ACT1* for normalisation. Progression through the cell cycle was monitored by budding indexes: wt and *hda1*Δ cultures progressed through the cell cycle at the same rate and entered S phase at 45 min after release (Fig.11A). Transcription of all analysed G1/S targets reached maximum levels at 30 min after release (G1/S transition) in both wt and *hda1*Δ cells, but levels of induction were lower of *CLN2* and *RNR1* in *hda1*Δ mutant (Fig.11B representative). At the same time, peak level of *SVS1* was higher in *hda1*Δ, but no difference was observed between wt and *hda1*Δ cells in transcript levels of *CDC21* and *TOS4* in this representative experiment. In this representative experiment transcript levels in wt cells were much higher than expected, however, transcript levels in *hda1*Δ were as high as in wt (except *RNR1*). At this point I can only conclude that the difference in *RNR1* transcript levels are probably due to the stochastic nature of transcriptional wave. Results from subsequent experiments were inconsistent with large variations in relative transcript levels in *hda1*Δ cells (Suppl.fig. 3B), which made it difficult to reach a solid conclusion.

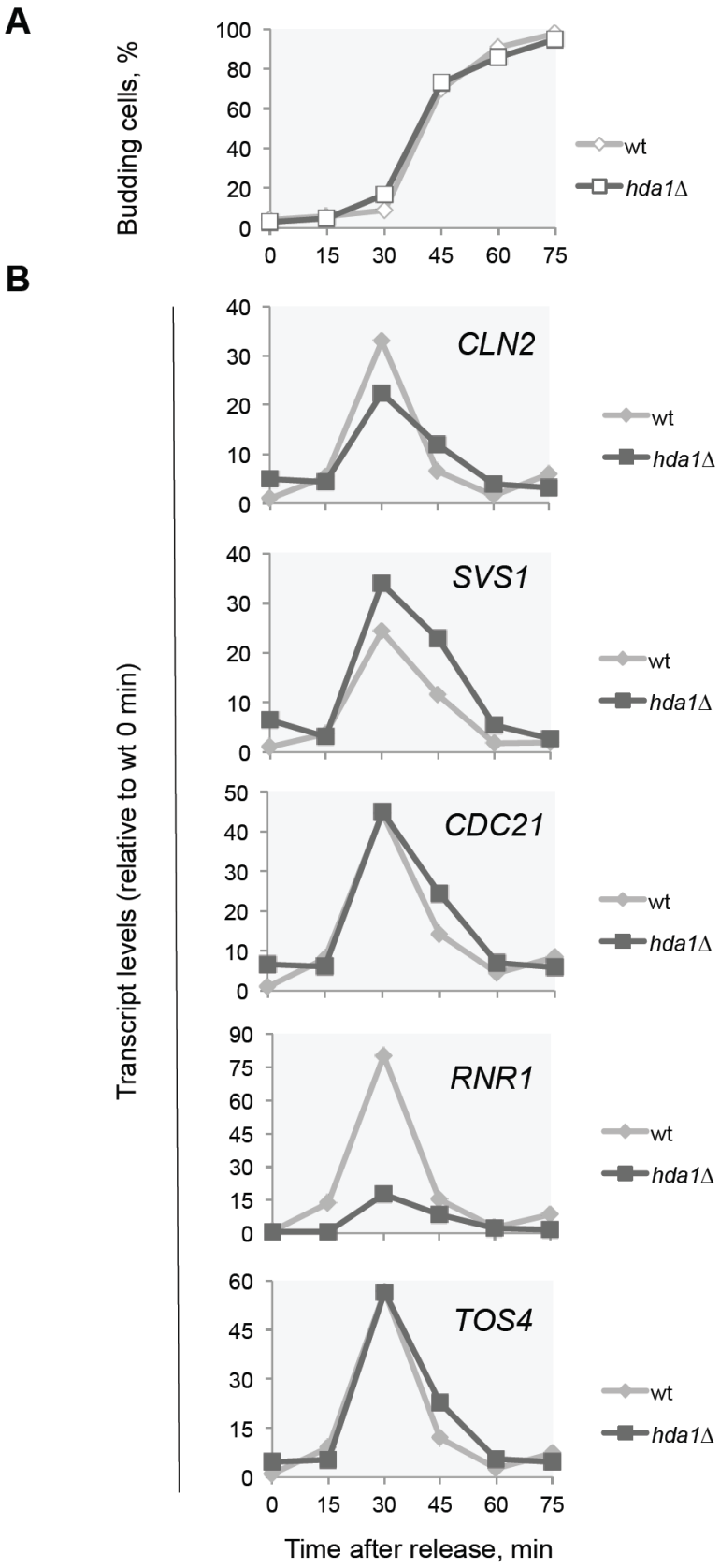


Figure 11. HDAC Hda1 is not involved in repression of G1/S transcription in S phase. (A) Budding indexes. Exponentially growing wt and *hda1*Δ cultures were

(continued) arrested in G1 phase and released. Number of budding cells was counted under the light microscope at each time point, 100 cells were counted in total. (B) Transcript levels in wt and *hda1*Δ cells during cell cycle. Exponentially growing wt and *hda1*Δ cultures were arrested in G1 phase and released. Transcript levels were established by RT-qPCR using *ACT1* as a reference gene.

However, when I compared absolute levels from different biological repeats at G1 (0 min), G1-to-S phase transition (30 min) and S phase (60 min) (Fig.12), no significant difference in relative transcript levels in wt and *hda1*Δ cells was observed in G1 and G1-to-S phase transition except for *RNR1* for which transcript levels were slightly lower (P value = 0.0257). In S phase only *CLN2* and *TOS4* transcription levels were significantly upregulated (P value = 0.0053 and P value = 0.0161 respectively). A large range of variation in transcription levels in the repeats does not allow me to draw any solid conclusions about the role of Hda1 is G1/S transcription regulation. However, most likely Hda1 is not required for repression of G1/S transcription in G1 or S phase, but might have a role in tuning the transcript levels in S phase.

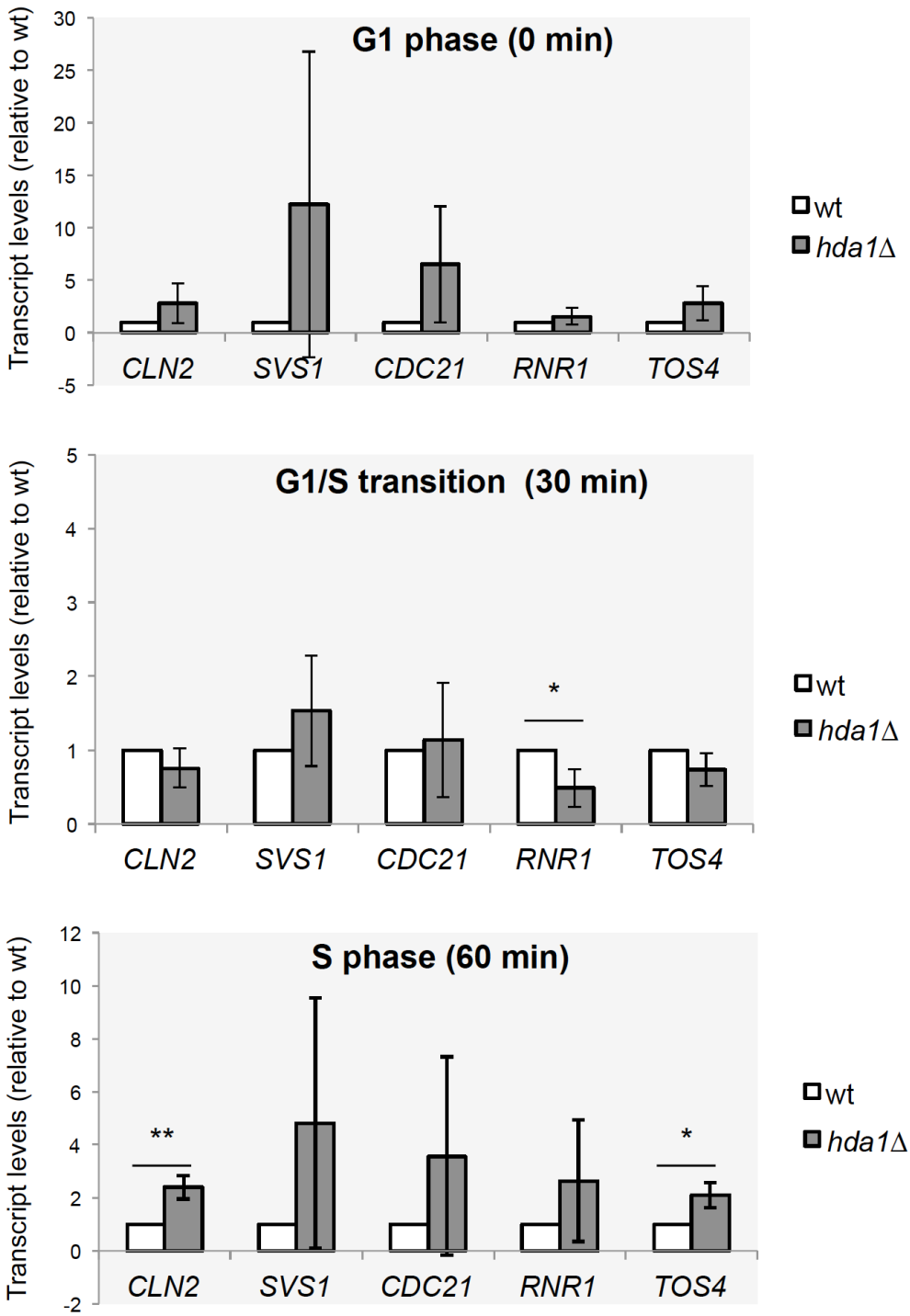


Figure 12. Transcription of SBF-dependent gene *CLN2* and switch gene *TOS4* is up regulated in S phase in *hda1*Δ cells. Exponentially growing wt and *hda1*Δ cultures were arrested in G1 phase and released. Transcript levels were analysed by RT-qPCR and normalised to *ACT1*. Transcript levels relative to wt are represented (3 biological repeats; error bars represent standard deviation; ** = P value ≤ 0.01; * = P value ≤ 0.05).

3.1.6. Summary.

G1/S transcription is crucial for driving cell cycle entry in both yeast and human cells. Previous studies suggest that histone acetylation plays an important role in the regulation of G1/S transcription in yeast and human cells. Our data shows, that acetylation at G1/S target promoters is cell cycle regulated (Fig.4 and Fig.5). This suggests cell cycle dependent recruitment of HDACs and HATs to G1/S promoters. Rpd3 is recruited to G1/S promoters (*CLN2* and *CLN1*) in G1 phase (Takahata *et al*., 2009). Based on this it was suggested that Rdp3 is required for repression of G1/S transcription in G1. My work shows that whilst Rpd3 is required for full repression of several G1/S targets in G1, deletion of *RPD3* only de-represses transcription slightly and does not lead to a significant increase of *CLN2* transcription in G1 phase arrested cells (Fig. 6). In addition, all G1/S targets tested are de-repressed in S phase (Fig.8). However, peak transcription levels during the G1-to-S phase transition, unaffected in *rpd3*Δ, are significantly higher than de-repressed levels observed in G1 and S phase in *rpd3*Δ cells (Fig.7). These data suggest that Rpd3 is not essential for regulation of G1/S transcription, but might be required for full repression in G1 and S phase. The HAT Gcn5 has also been shown to be recruited to G1/S target promoters. My work shows that deletion of *GCN5* results in lower levels of peak transcription of G1/S targets at the G1/S transition (Fig.9B), with specifically SBF, but not MBF, significantly down-regulated at the G1/S transition in *gcn5*Δ cells (Fig.10). However, the reduced G1/S peak transcript levels in *gcn5*Δ cells might be due to a cell cycle delay observed in *gcn5*Δ cells and/or loss of synchrony (Fig. 9A). The delay could be a consequence of lower levels of G1 cyclin *CLN2*. These possibilities will be discussed below. Surprisingly, G1/S transcription was up-regulated in *gcn5*Δ cells in S phase (Fig.10), however this may also be due to the cell cycle delay/loss of synchrony. Lack of a prominent effect of *RPD3* deletion may be due to redundancy in HDACs. Another HDAC Hda1 is a counteracting partner of Gcn5. Relative G1/S target transcript levels varied in *hda1*Δ cells from experiment to experiment, but overall the G1/S transcriptional wave was similar to that observed in wt cells (Fig.11). Comparison of transcript levels in G1, G1/S and S phases did not reveal significant difference in transcription in wt and *hda1*Δ, except down-

regulation of *RNR1* at G1/S transition and *CLN2* and *TOS4* up-regulation in S phase (Fig.12). All these data lead to the conclusion that Rpd3, Gcn5 and Hda1 are not required for the regulation of G1/S transcription but might be required to modulate full repression and activation of G1/S transcription and regulation is carried out by transcription factors SBF and MBF.

3.2. The role of histone methyltransferase Set2 in G1/S transcription in *S. pombe* upon genotoxic stress.

Genotoxic stress leads to DNA damage and failures in the DNA repair can lead to genomic instability. DNA double stand breaks (DSBs) are one of the most dangerous forms of DNA damage and are processed by two different mechanisms: homologous recombination and non-homologous end joining. In human cells, the histone methyltransferase SETD2 is involved in homologous recombination, while in yeast cells Set2-dependent methylation is required for non-homologous end joining. Set2 creates special microenvironment around DSBs. This in turn allows activation of DNA damage signalling pathway, recruitment of DNA damage repair proteins and DNA resection (Fnu *et al.,* 2011; Carvalho *et al*., 2014; Jha *et al*., 2014; Pai *et al*., 2014; Pfister *et al*., 2014). However, deletion/depletion of Set2/SETD2 does not affect transcription of DNA damage repair genes in response to DNA damage stress (Jha *et al*., 2014; Pfister *et al*., 2014),

Previously RNA-seq analysis in fission yeast identified a number of genes, which were up- and down-regulated upon bleomycin treatment in *set2*Δ cells (Pai *et al*., 2014). Bleomycin is an antibiotic, which interacts with ions of Fe^{2+} and binds DNA. Then Fe^{2+} undergoes oxidation into Fe^{3+}, which can in turn react with oxygen to form superoxide radicals. These free radicals attack bonds in DNA molecule and induce double strand breaks (Hecht, 2000). Short exposure to bleomycin causes replication fork slowing in fission yeast (Iyer & Rhind, 2017) and replication slowing and arrest in G1 phase in budding yeast (D'Amours & Jackson, 2001). Slowing down or stalling of replication forks or replication stress (RS), activates replication stress response (RS response), which prevents replication stress-induced DNA damage, and induces cell cycle arrest. Previous studies showed that during replication stress response G1/S transcription is maintained active in both yeast and human cells (de Bruin *et al*., 2008; Travesa *et al*., 2012; Bastos de Oliveira *et al*., 2012; Ivanova *et al*., 2013; Bertoli *et al*., 2013a). Moreover, G1/S transcription is required for tolerance of replication stress (Bertoli *et al*., 2016). Among genes identified in microarray analysis as down-regulated in *set2*Δ, was a cluster of MBF target genes

(regulator of G1/S transcription in fission yeast): replication origin licensing factors encoding genes *cdt1* and *cdc18*, subunit of ribonucleotide reductase *cdc22* and putative transcription factor *tos4.* These data suggest that Set2 may be involved in regulation of G1/S transcription in response to bleomycin treatment. We decided to further investigate the role Set2 in regulation MBF-dependent transcription in response to genotoxic stress caused by bleomycin in fission yeast.

3.2.1. Histone methyltransferase Set2 is required for MBF-dependent transcription activation in response to Bleomycin treatment.

To assess the role of Set2 in MBF-dependent transcription I have compared G1/S transcript levels by RT-qPCR in untreated or bleomycin treated wt and *set2*Δ fission yeast cells. Cultures were grown to exponential phase in YES media and samples for RNA extraction were collected before and after 30 min treatment with 5 μg/ml of bleomycin (as was used for RNA-seq analysis). RNA levels were analysed by RT-qPCR using Ct value method and normalised to *act1*. Whilst all MBF targets were upregulated in wt cells in response to 30 minutes bleomycin treatment, only *cdc22* was significantly up-regulated (P value=0.0158) (Fig. 13). These results are in line with the RNA-seq results using the same conditions. The up-regulation of the MBF targets *cdc18*, *cdt1*, *nrm1*, *tos4* and *rep2* was not significant, which might be due to the short time of the treatment. Interestingly the expression level of these genes in treated and untreated *set2*Δ cells was lower than those found in untreated wt cells. Furthermore, transcription of MBF targets was not induced in *set2*Δ upon treatment with bleomycin (Fig.13). Comparing transcription levels in treated wt and *set2*Δ cells, *cdc18*, *cdt1* and *tos4* are significantly down-regulated (P values are 0.0042, 0.0469 and 0.0166), while no significant difference was observed for *cdc22*, *nrm1* and *rep2*. These data suggest that Set2 has a role in transcriptional activation of MBF targets in response to short bleomycin treatment.

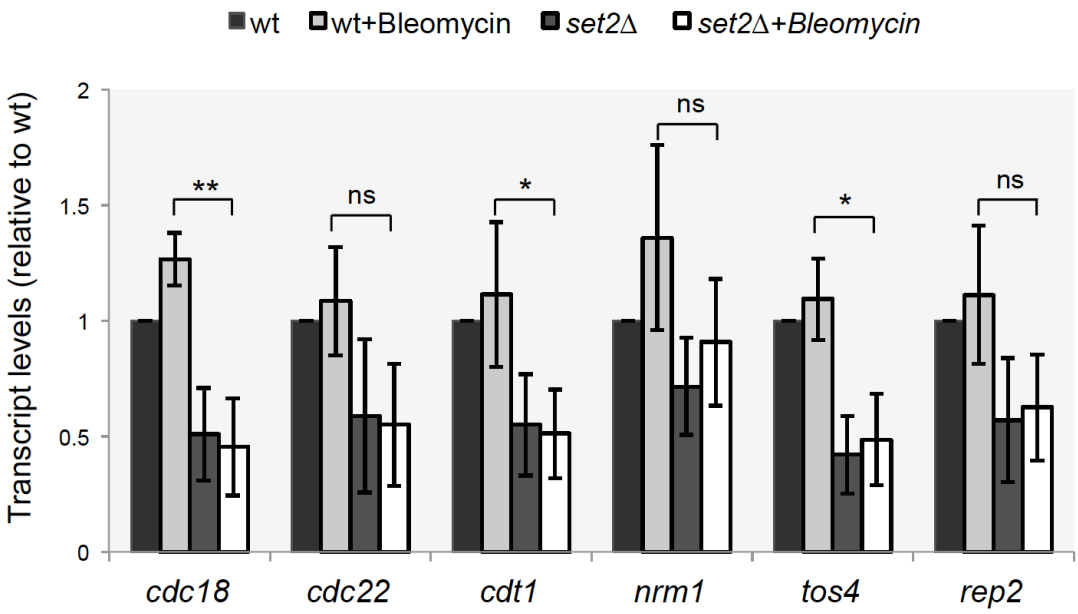


Figure 13. Set2 is required for activation of MBF targets in fission yeast in response to bleomycin treatment. Exponentially growing cultures of wt and *set2*Δ were collected for RNA extraction before and after 30 min of treatment with 5 μg/ml of bleomycin. Transcript levels were quantified by RT-qPCR. Transcript levels relative to wt are represented (3 biological repeats; error bars represent standard deviation; ** = P value ≤ 0.01; * = P value ≤ 0.05; ns = P value > 0.05).

3.2.2. Set2 is required for induction and maintenance of MBF-dependent transcription in response to Hydroxyurea.

Induction of MBF-dependent transcription after short bleomycin treatment is most likely caused by activation of RS response. To investigate whether Set2 is indeed required for activation and maintenance of MBF-dependent transcription in response to replication stress I have assessed transcription levels in cells treated with hydroxyurea (HU). HU is a ribonucleotide reductase inhibitor which causes a reduction of deoxyribonucleotides pool and thus inhibits DNA synthesis inducing replication stress and S phase arrest. Activation of replication stress response leads to the maintenance of G1/S transcription. In fission yeast Cds1 is a checkpoint kinase activated upon replication stress. Cds1 inactivates MBF transcriptional co-repressors Nrm1 and Yox1 to maintain

MBF-dependent transcription, as MBF targets are involved in replication stress response (de Bruin et al., 2006; Dutta *et al*., 2008; Dutta *et al*., 2009; Ivanova *et al*., 2013). Yeast cultures were grown in EMM to OD_{595}=0.1 and samples for RNA extraction were collected before and after 1, 3 and 5 hours of treatment with 12 mM HU. RNA was extracted with Qiagen RNeasy Kit and transcript levels were established by RT-qPCR using Ct value method and normalised to *act1*. I observed that in *set2*Δ cells, *cdc22* and *cdc18* transcription is not induced or maintained to the same level as in wt cells (Fig.14 representative; Suppl.3.3). These data suggest a role for Set2 in both the induction and maintenance of replication stress induced transcription. However, it is unclear if this is a direct or indirect role.

Replication stress, if not dealt with properly, can lead to DNA damage, which triggers the activation of the DNA damage checkpoint response through Rad3-dependent activation of Chk1 (Walworth *et al.*, 1993; Walworth & Bernards, 1996). Previous work has shown that Chk1 phosphorylates Cdc10, the MBF regulatory subunit, to inactivate MBF-dependent transcription (Ivanova *et al.*, 2013). If Set2 has a role in the tolerance to replication stress the reduced levels of MBF-dependent transcription in response to HU treatment in *set2*Δ cells could result from an increase in the levels of replication stress-induced DNA damage. To investigate this possibility, I performed the HU experiment in *chk1*Δ background. Inactivation of the DNA damage checkpoint protein kinase Chk1 allows me to establish if the reduced levels of MBF-dependent transcription in response to HU in *set2*Δ cells are due to activation of DNA damage response or loss of Set2 activity. While in *chk1*Δ cells MBF-dependent transcription was activated and maintained along the time course as in wt cells (HU treatment did not induce DNA damage response), transcription in cells lacking both *set2* and *chk1* was down-regulated as in *set2*Δ (Fig.14 representative; Suppl.fig.4). These findings indicated that Set2 has a direct role in the activation and maintenance of MBF transcription upon HU treatment.

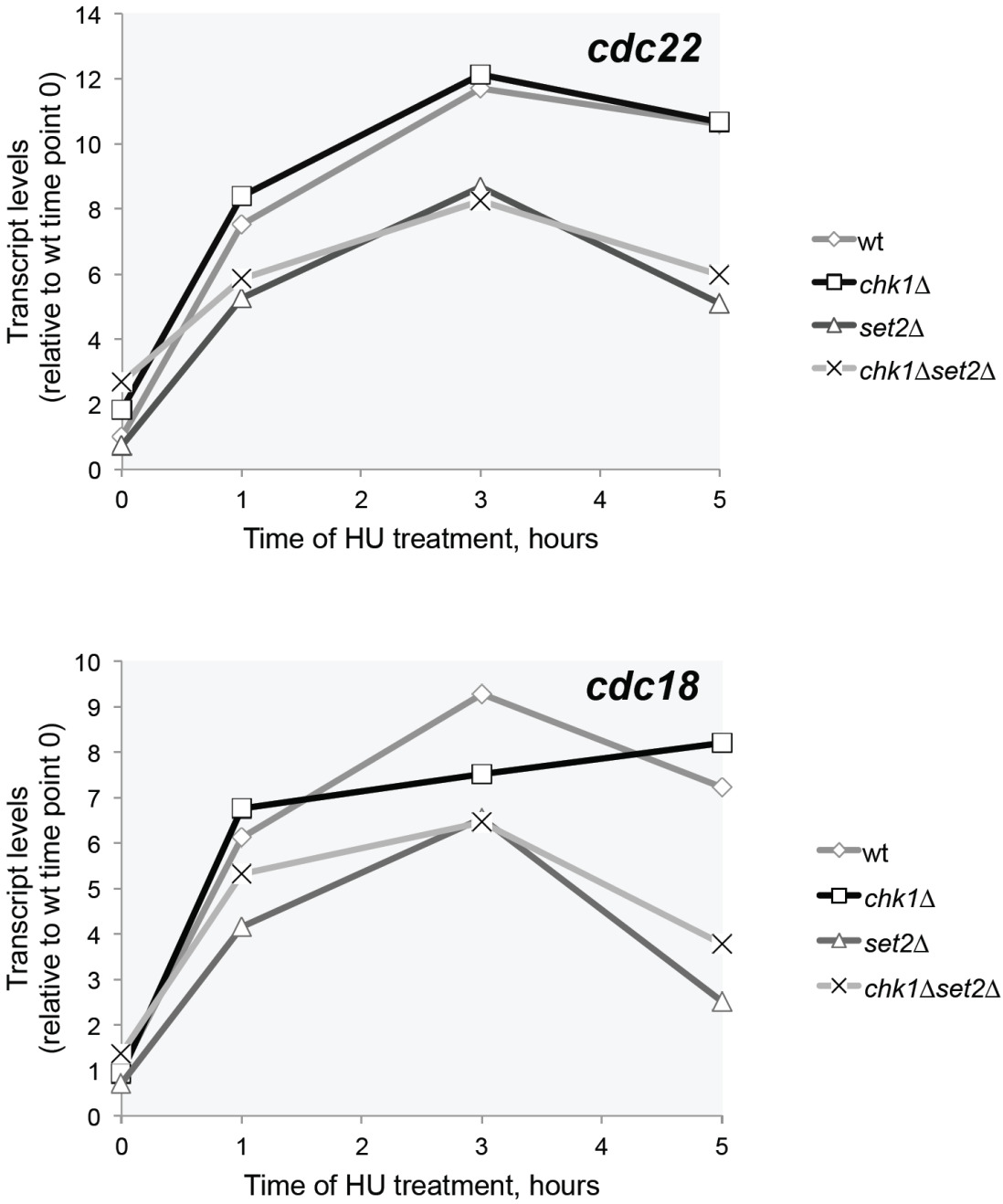


Figure 14. MBF transcription is not fully activated and maintained in *set2*Δ cells in response to HU. Fission yeast wt, *chk1*Δ, *set2*Δ and *chk1*Δ*set2*Δ cells were grown to early exponential phase and treated with 12 mM HU for 5 hours. Samples were collected every hour, and transcript levels were quantified by RT-qPCR with primers, specific to *cdc22* and *cdc18* coding regions. Transcript levels are relative to wt time point 0.

3.2.3. Licensing factors Cdt1 and Cdc18 and ribonucleotide reductase Cdc22 are misregulated in *set2*Δ cells.

Deletion of Set2 results in down-regulation of replication licensing factors *cdc18* and *cdt1* and ribonucleotide reductase subunit *cdc22* in asynchronous cells (Fig.13). This down-regulation is accompanied by replication delay (data not shown, Timothy Humphrey's group, Oxford). To investigate whether in *set2*Δ cells MBF target genes are not activated to the extent sufficient for normal cell cycle progression, or transcriptional activation is delayed, we performed a G1 phase block and release experiment, where wt and *set2*Δ cells were synchronised by nitrogen starvation. Samples for RNA extraction and FACS were collected every hour after release (Timothy Humphrey's group, Oxford). I extracted RNA using the Qiagen RNeasy Kit and analysed transcript levels by RT-qPCR using Ct value method and normalised to *act1.* As expected I observed peak transcription levels of the *cdc18*, *cdt1* and *cdc22* genes at 2 hours after release, which in our experiment coincides with G1-to-S phase transition (Fig.15) (based on Baum *et al*., 1997). In contrast, in *set2*Δ cells the highest transcription levels were at 3 hours after release showing a clear shift in activation timing. Moreover, peak levels of *cdc18*, *cdt1* and *cdc18* transcripts were lower than in wt (Fig.15). Progression through the cell cycle was analysed by FACS (performed by Dr. Chen-Chun Pai), which revealed a cell cycle delay in *set2*Δ cells (data not shown). These data support the role of Set2 in the activation of MBF-dependent transcription.

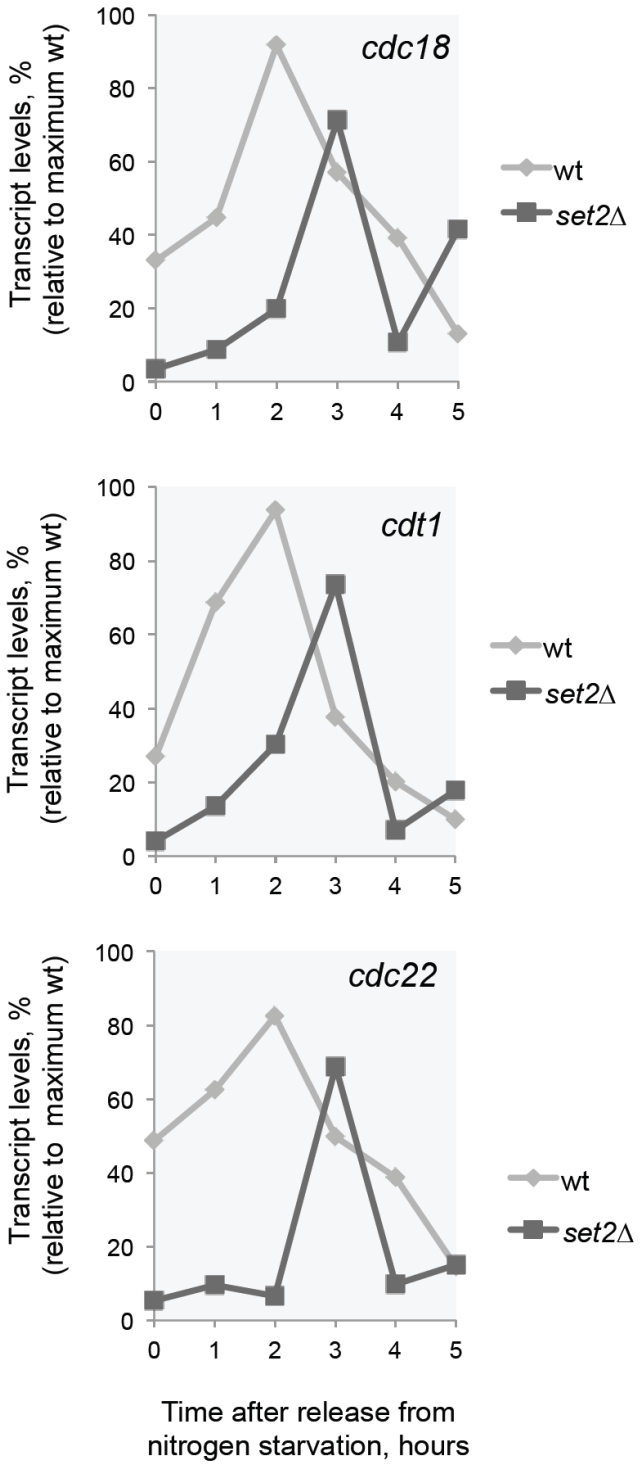


Figure 15. Activation of transcription of MBF targets *cdc18*, *cdt1* and *cdc22* is delayed in *set2*Δ cells. wt and *set2*Δ cultures were synchronised in G1 by nitrogen starvation. Samples for RNA extraction were collected every hour at 0-5 hours after release. 2 hours correspond to G1/S transition, 3 hours – S phase. Transcript levels were analysed by RT-qPCR and normalised to *act1*. Transcript levels are relative to maximum wt value within 3 technical repeats.

3.2.4. Set2-dependent di- and tri-methylation at *cdc22*, *cdc18* and *cdt1* promoters is induced in response to genotoxic stress.

Set2 is the sole histone methyltransferase is responsible of all mono-, di- and tri-methylation of H3K36 in fission yeast. Previous study with phleomycin, which belongs to the bleomycin family of antibiotics, showed that Set2-dependent H3K36me3 globally increased after 1 hour treatment and then returns to the basal levels (western blot analysis of total H3K36me3) (Jha and Strahl, 2014). In contrast, H3K36me2 reached maximum levels at the end of a time course (5 hours treatment). I decided to investigate, by Chromatin Immunoprecipitation, whether promoter regions of MBF target genes are methylated in Set2-dependent manner in response to bleomycin treatment. Yeast cultures were grown in YES media to exponential phase and ChIP samples were collected before and 30 min after treatment with 5 μg/ml bleomycin. ChIP was performed according to the protocol with specific antibodies (anti-H3K36me2 Active Motif 39255; anti-H3K36me3 Active Motif 61101, UK). Immunoprecipitated *cdc22*, *cdc18* and *cdt1* promoter DNA was quantified by qPCR as % of input (whole cell extract). ChIP revealed that in response to the 30 minutes bleomycin treatment di-methylation, H3K36me2, is induced at the MBF-dependent *cdc22*, *cdc18* and *cdt1* promoters (P values 0.0165, 0.0467 and 0.0033 showed significant increase within analysed sample), while di-methylation levels did not significantly change at promoters of non-MBF target genes *byr3* and *rps17* (Fig.16A). Deletion of *set2* led to complete loss of di-methylation at promoters of all analysed genes in both treated and untreated conditions. Levels of tri-methylation, H3K36me3, also increase at *cdc22* and *cdt1* promoters (P values 0.004 and 0.0378) in response to bleomycin, but not significantly at the *cdc18* promoter (P value 0.1054; Fig.16B). Tri-methylation of H3K36 at control promoters *byr3* and *rps17* did not change significantly after treatment with bleomycin. As in the case of di-methylation, tri-methylation was also abolished in *set2*Δ cells in both treated and untreated conditions confirming that Set2 is responsible of both di- and tri-methylation in fission yeast.

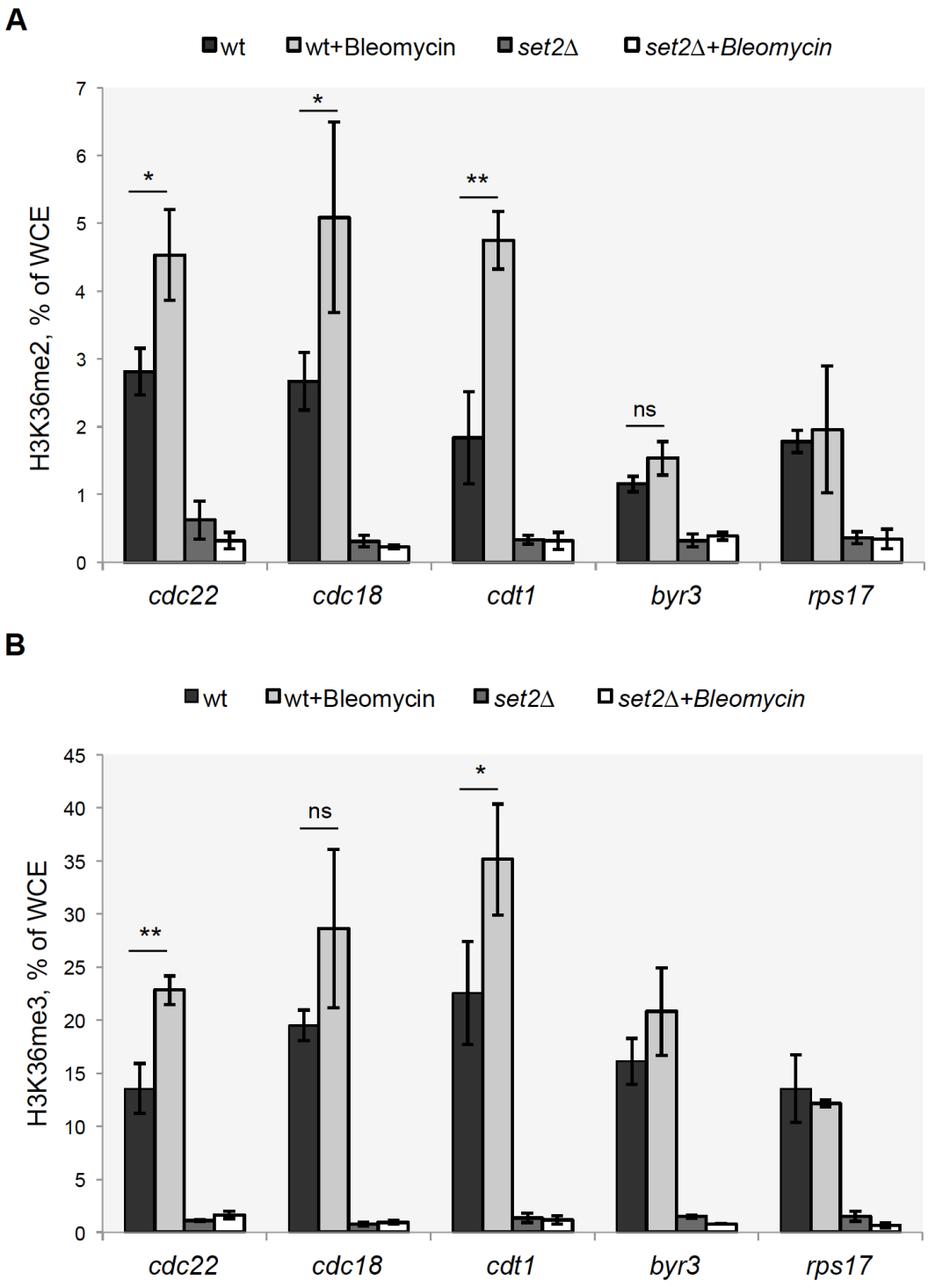


Figure 16. MBF target promoters are di- and tri-methylated at H3K36 in response to bleomycin treatment in Set2-dependent manner. ChIP was performed according to the protocol with anti-bodies against H3K36me2 and H3K36me3 and DNA fragments were analysed by qPCR with primers, specific to *cdc22*, *cdc18*, *cdt1*, *byr3* and *rps17* promoter regions (3 technical repeats, error bars represent standard deviations within one sample, ** = P value ≤ 0.01; * = P value ≤ 0.05).

My results indicate, that both H3K36me2 and H3K36me3 are induced at MBF target promoters in response to bleomycin treatment in a Set2-dependent manner.

3.2.5. Set2 facilitates binding efficiency of the MBF DNA binding subunit Res1 and activation subunit Cdc10.

In fission yeast G1/S transcription depends on the MBF TF complex. MBF is composed of two DNA binding subunits Res1 and Res2 and an activation subunit Cdc10 (Lowndes *et al*., 1992; Tanaka *et al*., 1992; Miyamoto *et al*., 1994; Ayte *et al*., 1995; Baum *et al*., 1997). I decided to investigate if Set2-dependent methylation is involved in recruitment of MBF components to MBF-dependent *cdc22*, *cdc18* and *cdt1* promoters in response to bleomycin. Exponentially growing *Res1-Myc*, *Cdc10-Myc*, *set2*Δ *Res1-Myc* and *set2*Δ *Cdc10-Myc* cultures were treated with 5 µg/ml bleomycin for 30 min and samples for ChIP were collected before and after the treatment. ChIP was performed according to the protocol with c-Myc antibodies (Santa Cruz (9E10): sc-40). *set2*Δ cells were used as negative control, to establish background signal, and the *byr3* promoter was used as a control of a non-MBF target genes. Anti-Myc IP DNA fragments were analysed by qPCR and values were normalized to % of WCE (Fig.17A). Signal for the negative control *byr3*, in all strains, was comparable to those observed in the untagged control *set2*Δ cells indicating low levels of background. In contrast, I found enrichment of the MBF-dependent *cdc22*, *cdc18* and *cdt1* promoters in the Res1-Myc pull down. Signals in untreated wt and *set2*Δ cells are comparable, but while in response to bleomycin treatment signals in the wt background tend to be higher, in *set2*Δ cells they tend to be lower. This indicates that binding of MBF to promoters in response to bleomycin treatment is compromised in *set2*Δ cells. For Cdc10 binding I observed clear signals in wt cells in both untreated and bleomycin treated conditions, with a reduction for the *cdc18* promoter in response to bleomycin (Fig 17B). Surprisingly, while binding tends to be lower in untreated *set2*Δ cells treatment with bleomycin reduced Cdc10-Myc recruitment to MBF promoters (Fig.17B). This result may be explained by lower levels of DNA binding subunit Res1 which is required for Cdc10 binding. Overall my results suggest that Set2 facilitates Res1 and Cdc10 binding to target promoters in response to bleomycin treatment.

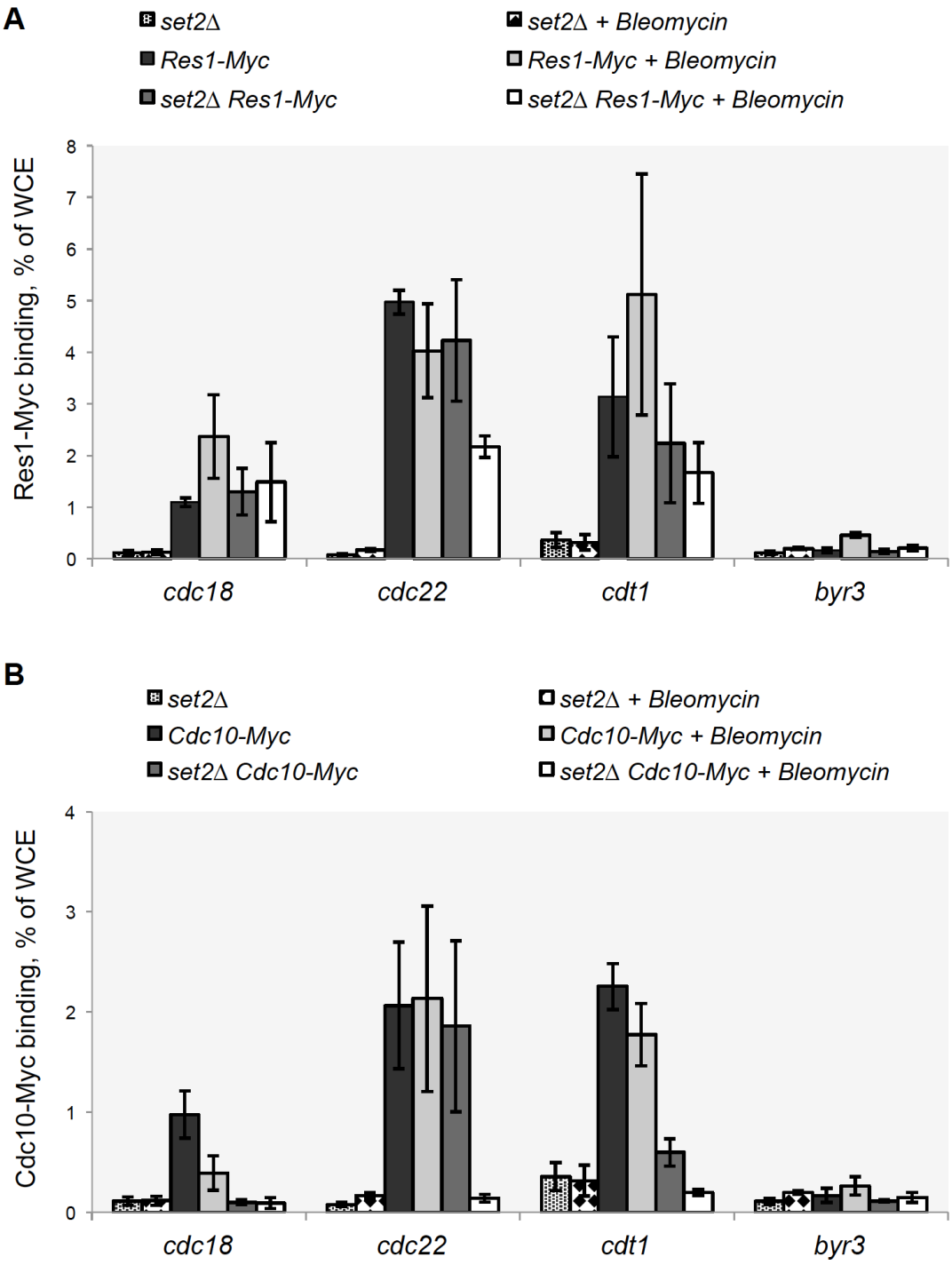


Figure 17. Recruitment of Res1-Myc and Cdc10-Myc to MBF target promoters is affected in *set2*Δ cells in bleomycin treatment conditions. ChIP was performed with exponentially growing cells according to the protocol with c-Myc antibodies. DNA fragments were analysed with qPCR with primers, specific to *cdc22*, *cdc18*, *cdt1* and *byr3* promoter regions (3 technical repeats, error bars represent standard deviations within one sample). Res1-Myc (A) and Cdc10-Myc (B) binding to MBF target (*cdc18*, *cdc22* and *cdt1*) and non-MBF target promoters.

3.2.6. Summary.

Set2-dependent methylation is directly involved in DNA repair pathways in both yeast and human cells (Fnu *et al.,* 2011; Carvalho *et al*., 2014; Jha *et al*., 2014; Pai *et al*., 2014; Pfister *et al*., 2014), however is not required for activation of transcription of genes involved in DNA damage response (Jha *et al*., 2014; Pfister *et al*., 2014). I have shown that Set2 is necessary for activation of MBF-dependent transcription in response to genotoxic stress, caused by bleomycin (Fig. 13). Activation and maintenance of G1/S transcription in response to HU treatment is also dependent on Set2 (Fig.14), which suggests that Set2 is indeed required for the replication stress transcriptional response. Moreover, deletion of Set2 also leads to cell cycle delay in unperturbed conditions (data not shown, Tim Humphrey's group) due to delayed transcriptional activation of replication licensing factors Cdt1 and Cdc18 (Fig.15). Set2-dependent methylation facilitates transcription elongation, and promoters of MBF targets *cdc18*, *cdc22* and *cdt1* become highly methylated at H3K36 upon treatment with bleomycin, and this methylation is Set2-dependent (Fig.16). The binding of MBF DNA binding subunit Res1 was reduced in *set2*Δ cells treated with bleomycin, while binding the activation subunit Cdc10 was reduced even more than Res1 binding (Fig.17). This indicates that Set2-dependent methylation facilitates efficient MBF binding.

3.3. Expansion of G1/S transcriptional network in yeast.

The number of genes bound by the G1/S TFs varies from around 80 in fission yeast to more than 200 in budding yeast (Iyer *et al*., 2001; Horak *et al*., 2002; Rustici *et al*., 2004). The complexity of the network is different as well. In *S. cerevisiae* and species from the same clade, both SBF (Swi4/Swi6) and MBF (Mbp1/Swi6) exist. Despite that the specific SCB and MCB DNA binding sites *in vivo*, *in vitro* SBF and MBF can bind both sequences (Bean *et al*., 2005). SBF biding sites SCB are found only in *S. cerevisiae* and closely related species. Ancestral Res (progenitor of Swi4 and Mbp1) bound MCB sites and probably underwent duplication to evolve into more specialised SBF, which bind the distinct binding sequence SCB. In other yeast species G1/S transcriptional wave is regulated by MBF only and gene promoters are enriched for only the MCB motifs (Koch *et al*., 1993; Galagan *et al*., 2003; Bähler, 2005; Côte *et al*., 2009; Ofir *et al*., 2012; Zámborszky *et al*., 2014). At the same time, MBF components from these species share similarity with SBF components in *S. cerevisiae* (Koch *et al*., 1993; Côte *et al*., 2009). Overall, this makes the yeast G1/S transcription network a great model to study how transcriptional networks expanded during evolution and the role of co-evolution of DNA binding sites (SCB and MCB) and DNA binding domains (Swi4 and Mbp1).

3.3.1. The Swi4 DNA binding domain is functionally conserved and is sufficient to drive G1/S transcription in *S. cerevisiae.*

The presence of MCB binding sites, but not SCB, in most yeast species suggests that MBF is likely to be an ancestral transcription factor. However, regulators of G1/S transcription in other yeast species share sequence homology with both Swi4 and Mbp1 (Fig.18). Therefore, either Swi4 or Mbp1 could be the ancestral TF DNA binding component.

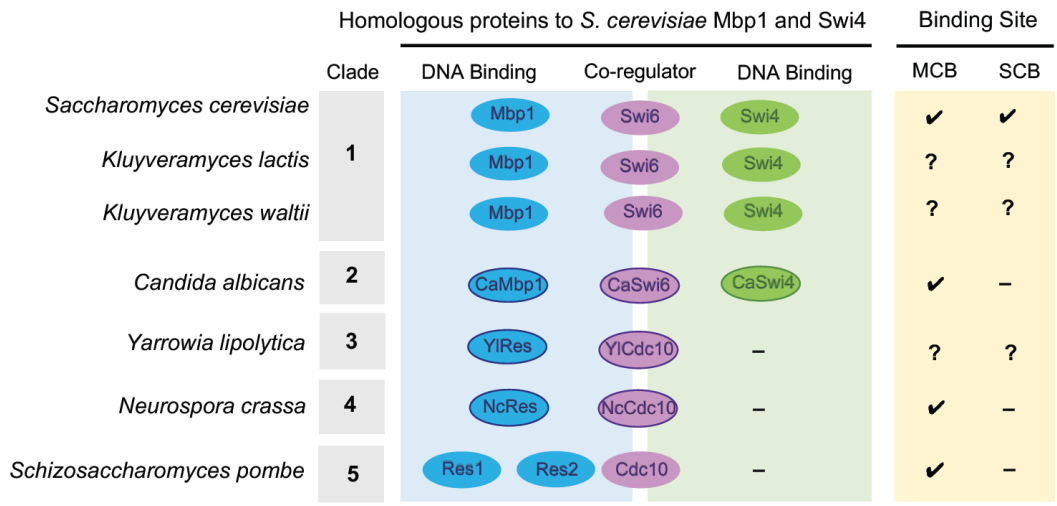


Figure 18. Regulators of G1/S transcriptional network are conserved across yeast species. The summary table is based on previous studies. Homologs of *S. cerevisiae* Swi4 and Mbp1 are found across the species. Most of the species possess MCB binding site and not SCB. CaMbp1 is not cell cycle regulated and CaSwi4 and CaSwi6 most likely bind MCB to regulate G1/S transcription. Question mark means that the analysis of the binding sites has not been performed before.

To test whether MBF or SBF is the likely ancestral TF, DNA binding domains of *S. cerevisiae* Swi4 and Mbp1 were swapped with DNA binding domains from Swi4 or Mbp1 from distant yeast clades (Fig.19 and Fig.20). This would establish if the DNA binding domains from different clades were able to bind MCB and/or SCB elements in *S. cerevisiae* and function as native Mbp1 or Swi4 and drive G1/S transcription. While single deletion mutants of Swi4 or Mbp1 in budding yeast are viable, deletion of both is lethal, but can be rescued by ectopic expression of ScSwi4 or ScMbp1 (Koch *et al*., 1993). Adi Hendler (Ben-Gurion University of Negev) created strains with hybrid transcription factors, where native Mbp1 or Swi4 DNA binding domains in *S. cerevisiae* (ScMbp1BD or ScSwi4BD) were replaced with Mbp1BD (Fig.19 top panel) and Swi4BD (Fig.20 top panel) from yeast species from different clades and hybrid proteins were expressed from plasmids (clade 1: *K. lactis*, *K. waltii;* clade 2: *Debaryomyces hansenii*, *C. albicans*; clade 3: *Y. lypolytica*; clade 4: *N. crassa*; clade 5: *S. pombe*). Based on previous structural analysis (Taylor *et al*., 2000) 125 amino acids of Mbp1 and 166 amino acids of Swi4 at the end of DNA

binding domain were chosen to be replaced, while C-terminal activation domain was preserved. Endogenous Swi4 and Mbp1 were deleted, and native Swi4 was expressed from plasmid with URA marker (pRS316-Swi4-URA3), while hybrid transcription factors were expressed from centromeric plasmid with LEU marker (pRS315-LEU2), which can be selected against on media lacking uracil or leucine respectively. Thus, *swi4*Δ*mbp1*Δ *S. cerevisiae* cells carried hybrid Swi4 or Mbp1 expressed from *pRS315* plasmid (functional *LEU2* gene) and native Swi4 expressed from *pRS316* plasmid (functional *URA3* gene). Cells were first plated on media lacking both uracil and leucine (Sc-leu/-ura) to select those carrying both plasmids and expressing native Swi4 to drive G1/S transcription. Then colonies were replica plated on media without leucine, but containing 5-Fluoroorotic Acid (Sc–leu/+5FOA). 5-FOA is toxic for cells, carrying functional *URA3* gene, and cells do not survive on these plates. The 5-FOA treatment therefore selects against the plasmid with native Swi4 (plasmid with *URA* marker), leaving the hybrid TF (plasmid with *LEU* marker) as a sole source of G1/S transcription regulation: only strains where hybrid TF could bind target genes and activate transcription would survive. Empty *pRS315-LEU2* plasmid was also transformed as a negative control, since these cells would not have G1/S transcription and survive on Sc–leu/+5FOA media. Adi Hendler observed, that hybrid Mbp1 from clades 2-5 could not rescue lethality, while hybrids from closer species from clade 1 (KlMbp1BD-Mbp1AD and KwMbp1BD-Mbp1AD) could (Fig.19 bottom panels). This indicates, that Mbp1 DNA binding domain from distant species (clade 2-5: DhMbp1BD-Mbp1AD, CaMbp1BD-Mbp1AD, YlResBD-Mbp1AD, NcResBD-Mbp1AD, SpRes1BD-Mbp1AD and SpRes2BD-Mbp1AD) cannot bind MCB in *S. cerevisiae* and drive G1/S transcription. However, hybrids of ScSwi4, where the DNA binding domain was replaced with Swi4BD from yeast from clades 1-5, could rescue the *swi4*Δ*mbp1*Δ double mutant lethality, but to different extent based on the growth rate (Fig.20 bottom panels). To test if the inability of the Mbp1 hybrids to rescue the *swi4*Δ*mbp1*Δ double mutant lethality was due to low protein levels I analysed protein levels by Western blot. Western blot analysis confirmed that the hybrid proteins were expressed to a comparable level to wt ScMbp1 (Fig.21) indicating cells did not survive because hybrid Mbp1 could not bind target genes and drive transcription.

These results suggest, that Swi4 hybrids, carrying either Swi4BD or Mbp1BD/ResBD from other species and native Swi4AD, are able to drive a critical subset of genes, required for G1/S transition and that Swi4 is likely to be an ancestral TF.

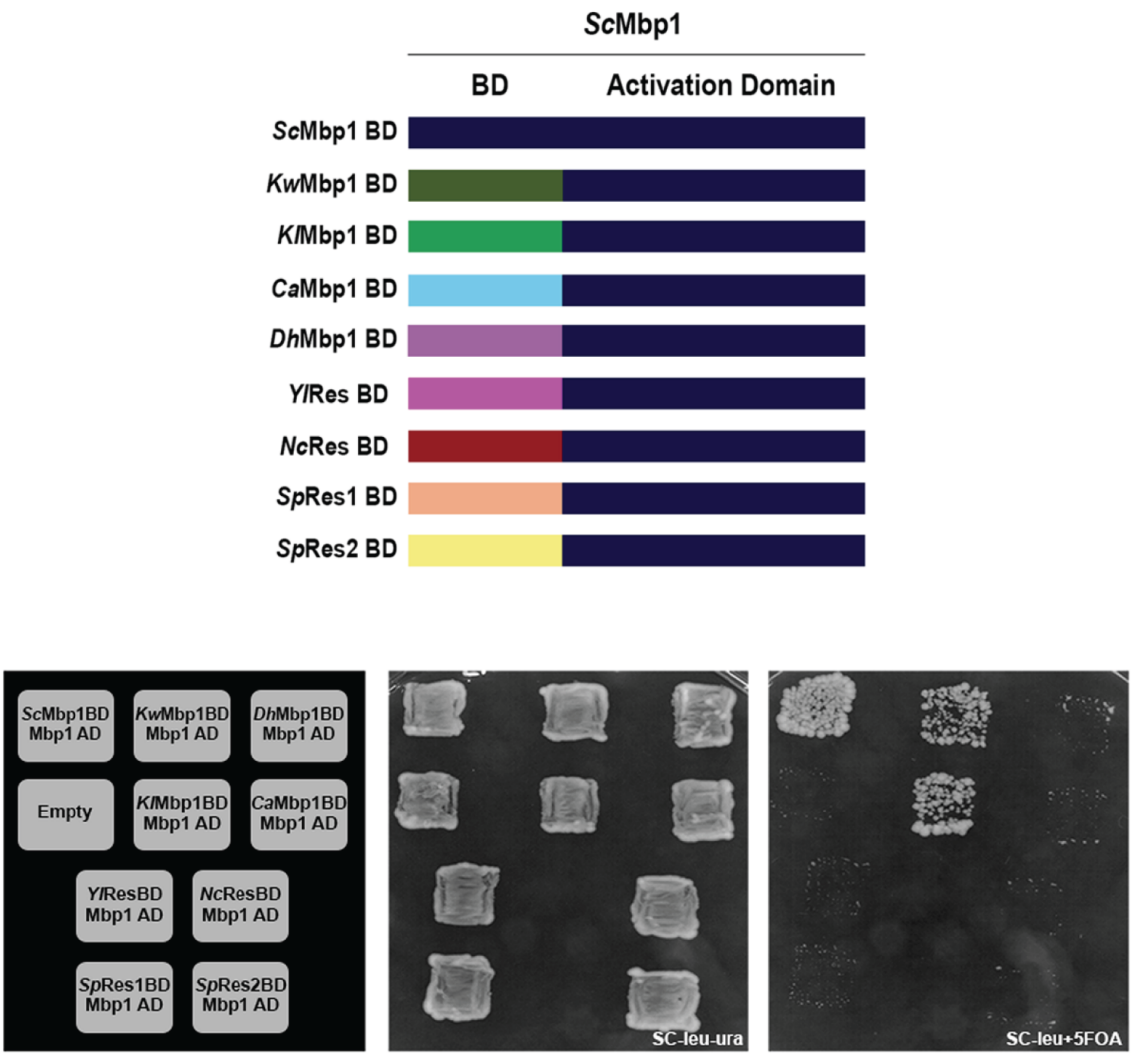


Figure 19. Hybrid Mbp1 cannot rescue lethality of *swi4*Δ*mbp1*Δ *S. cerevisiae*. (Top panel) Schematic representation of replacement of *S. cerevisiae* Mbp1 DNA binding domain with Mbp1/Res DNA binding domains from other yeast species. (Bottom panel) Complementation assay with 5FOA. Cells carrying hybrid Mbp1 (LEU marker) and native Mbp1 (URA marker) grow on SC-leu-ura plates. Only cells, where hybrid Mbp1 drives transcription of critical G1/S targets, are viable on SC-leu+5FOA.

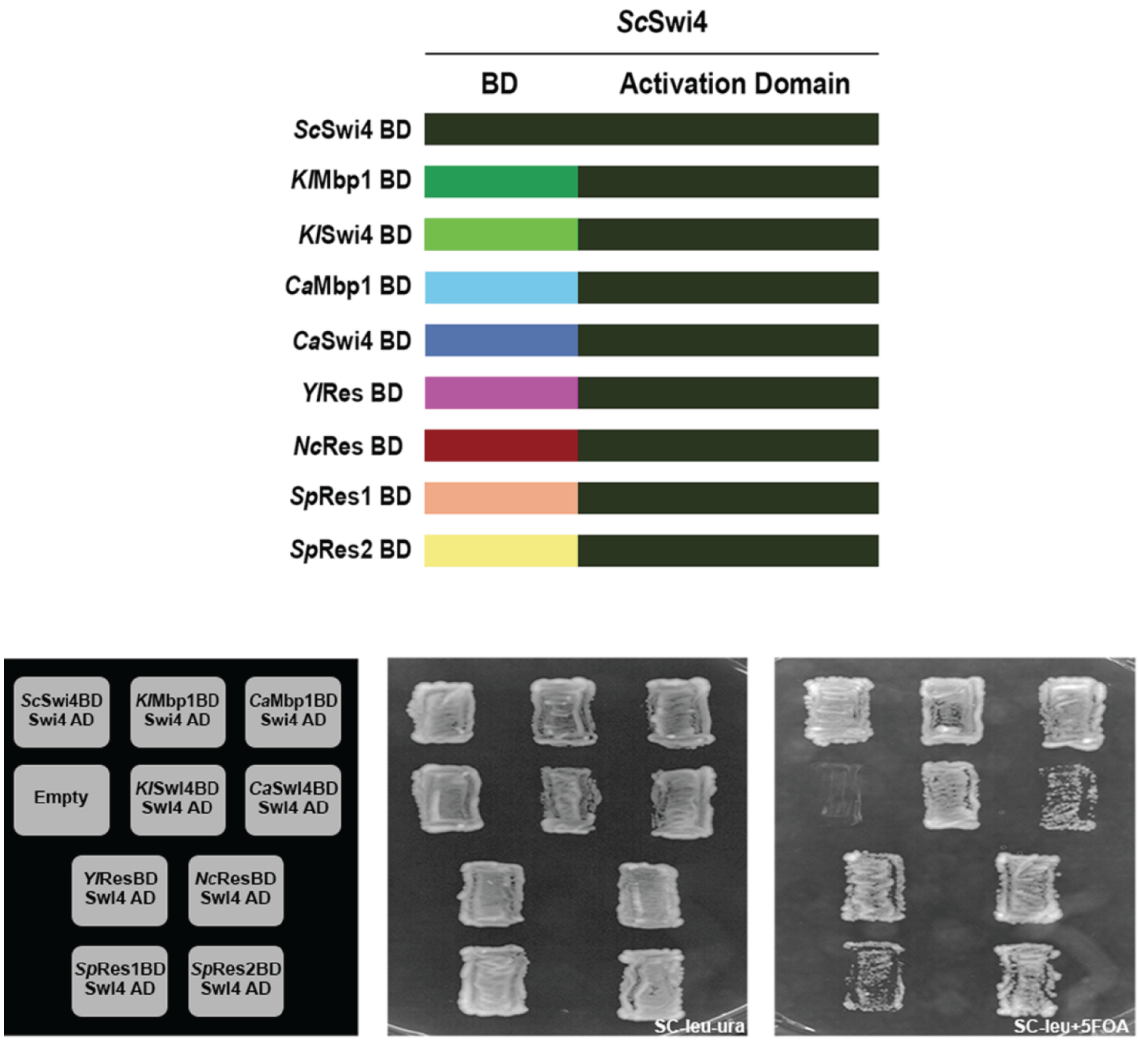


Figure 20. Hybrid Swi4 is sufficient to rescue lethality of *swi4*Δ*mbp1*Δ *S. cerevisiae*. (Top panel) Schematic representation of replacement of *S. cerevisiae* Swi4 DNA binding domain with Swi4/Mbp1/Res DNA binding domains from other yeast species. (Bottom panel) Complementation assay with 5FOA. Cells carrying hybrid Swi4 (LEU marker) and native Swi4 (URA marker) grow on SC-leu-ura plates. Only cells, where hybrid Swi4 drives transcription of critical G1/S targets, are viable on SC-leu+5FOA.

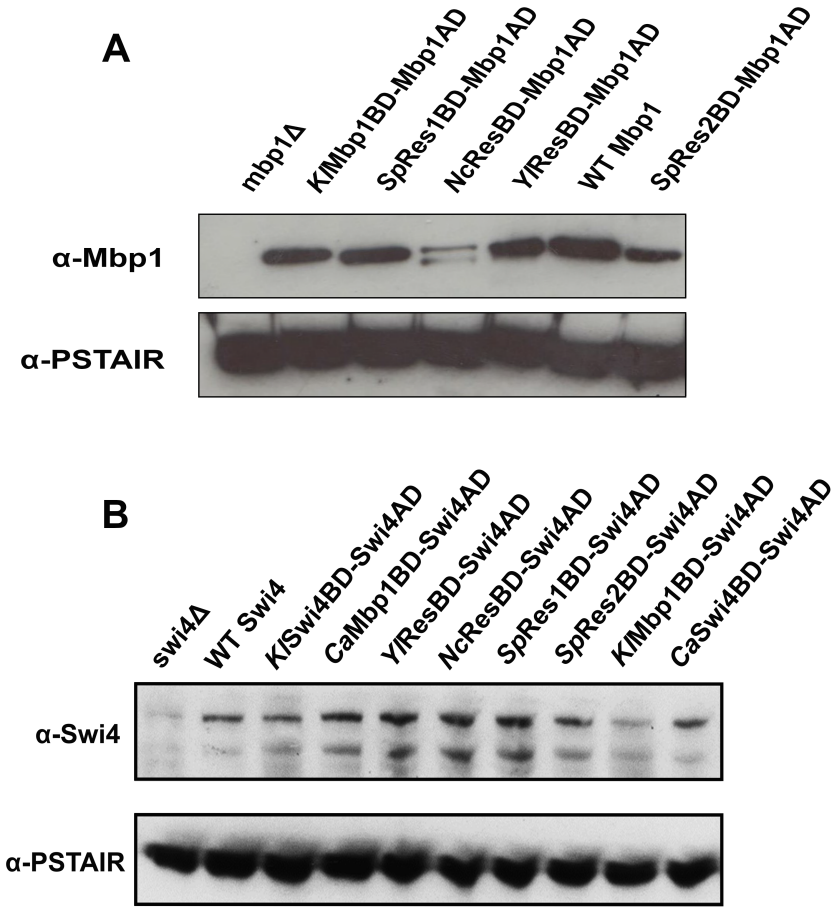


Figure 21. Hybrid Swi4 and Mbp1 are expressed to the same level as ScSwi4 and ScMbp1. Exponentially growing cells were diluted to the same OD_{600}. Protein extraction and Western Blot with specific antibodies to Mbp1 (Panel A) and Swi4 (Panel B) C-terminal domains were performed according to the protocol. PSTAIR levels were used as loading control.

3.3.2. Regulation of G1/S transcription by hybrid Swi4 causes phenotypic defects.

Swi4, but not Mbp1, hybrid can rescue the lethality of *swi4*Δ*mbp1*Δ cells. Interestingly, the hybrid Swi4 can rescue lethality to different extent based on the growth rate. However, these experiments were carried out using plasmid born copies of the hybrids, which could affect the expression levels. To investigate this in more detail Adi Hendler constructed strains were the endogenous ScSwi4BD was replaced, via knock-in/knock-out transformation, with the DNA binding domains from different clades, in *mbp1*Δ cells. Adi Hendler established growth rates as a function of increase in optical density measured by OD_{600} with automatic plate-reader (Fig.22). Cells with Swi4 DNA binding domain from clades 3-5 (YlResBD-Swi4AD, NcResBD-Swi4AD, SpRes2BD-Swi4AD) show impaired growth and doubled generation times, while cells with DNA binding domains from clades 1-2 (KlMbp1BD-Swi4AD and CaMbp1BD-Swi4AD) showed the same growth rate as *mbp1*Δ cells.

The decrease in growth, likely the result of cell cycle defects, correlates with abnormal cell morphology (Fig.23). Cell morphology was examined under light microscope and the number of cells with defects were counted by Steffi Klier. Cells with hybrid Swi4 showed elongated phenotype with cell bundles. Moreover, the number of cells with morphological defects was higher in clades 3-5 (YlResBD-Swi4AD, NcResBD-Swi4AD and SpRes2BD-Swi4AD) with the highest in clade 5, showing an increase in severity that correlates with the increase in phylogenetic distance. These results suggest that the hybrid Swi4 proteins can regulate an increasingly limited number of G1/S targets or regulateG1/S targets to an increasingly limited extend.

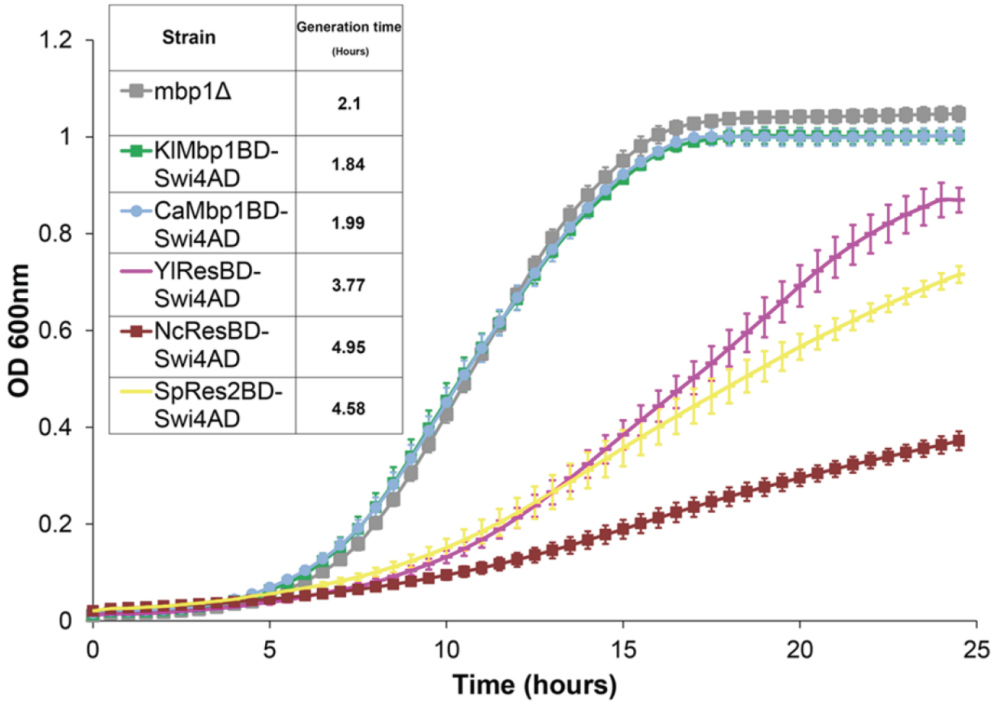


Figure 22. Cells with G1/S transcription driven by hybrid Swi4 from clades 3-5 have growth defects in *mbp1*Δ background. Exponentially growing cell cultures were diluted to the same OD in complete media. Growth rate was analysed by measuring OD_{600} during 24 hours at 30°C.

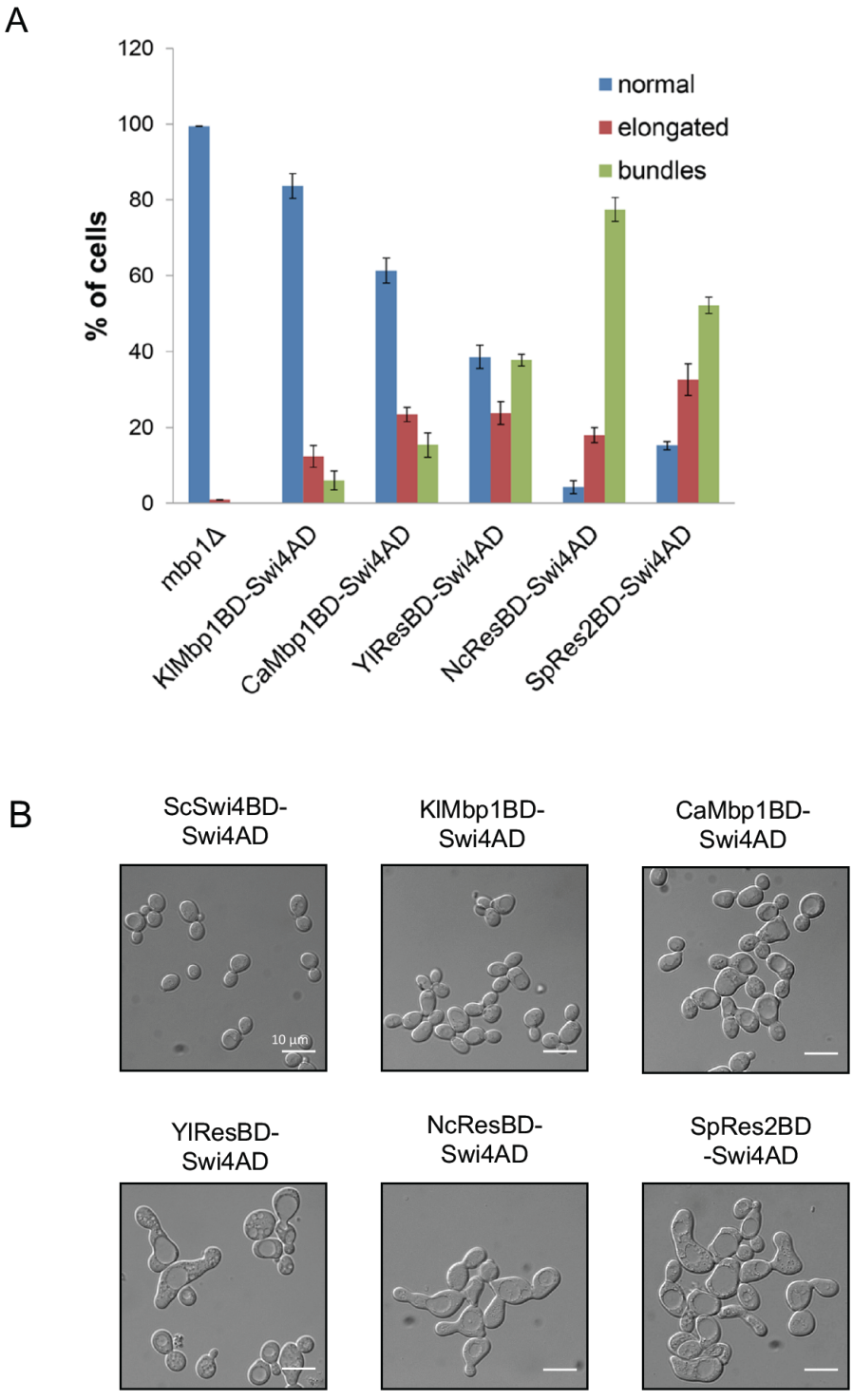


Figure 23. Cells with G1/S transcription driven by hybrid Swi4 exhibit cell morphology defects in *mpb1*Δ background. (A) Quantification of cell with morphology defects: elongation and cell bundles. Exponentially growing yeast cells were analysed by light microscopy (Zeiss AxioPlan, 63X magnification, oil objective). Each strain was analysed in triplicate and at least 100 cells were counted. (B) Representative DIC images, showing cells elongation and bundling.

Next, I investigated the phenotype, when G1/S transcription is driven by hybrid Swi4, in *MBP1* wt background. Exponentially growing cells were diluted to the same cell number and accumulation of biomass was measured during 24 hours by BioLector (m2p-Labs) at 30°C and 37°C degrees in triplicate. I found, that, in this case, replacement of DNA binding domain did not affect growth rates significantly in both 30°C and 37°C (Fig.24A and B). These results were largely expected since growth in *swi4*Δ cells is not significantly affected. A clear phenotype of losing SBF function is the increase in cell size in *swi4*Δ cells. I therefore established cell size in cells depending on hybrid Swi4 to test if SBF function is compromised. In my experiment *swi4*Δ mean cell diameter is 8.51 µm versus wt with mean diameter 5.33 µm. Cell expressing Swi4 hybrids with the DNA binding domain from clade 1 (KlSwi4BD-Swi4AD) had the closest to wt cell diameter 5.73 µm. All other strains with hybrid Swi4 from the clades 2-5 had larger cell diameters than wt, but smaller than *swi4*Δ (∅ for CaSwi4BD-Swi4AD = 6.95 µm; YlResBD-Swi4AD = 7.20 µm; NcResBD-Swi4AD = 7.45 µm; SpRes1BD-Swi4AD = 7.22 µm, SpRes2BD-Swi4AD = 6.96 µm) (Fig.25).

Our work establishes that *mbp1*Δ cells depending on distantly related hybrid Swi4 display severe phenotypic defects, such as impaired growth rate and abnormal cell morphology. This indicates that whilst the hybrid TFs can regulate G1/S transcription to maintain viability it cannot regulate critical G1/S targets required for proper cell cycle progression. Interestingly, the fitness of *MBP1* wt cells with hybrid TF is not affected. This suggests that Swi4 DNA binding domain from distantly related clades cannot compensate for *MBP1* loss, but are sufficient to regulate critical SBF targets. However, cell size is increased in *MBP1* wt cells with hybrid TF, which indicates SBF function is compromised.

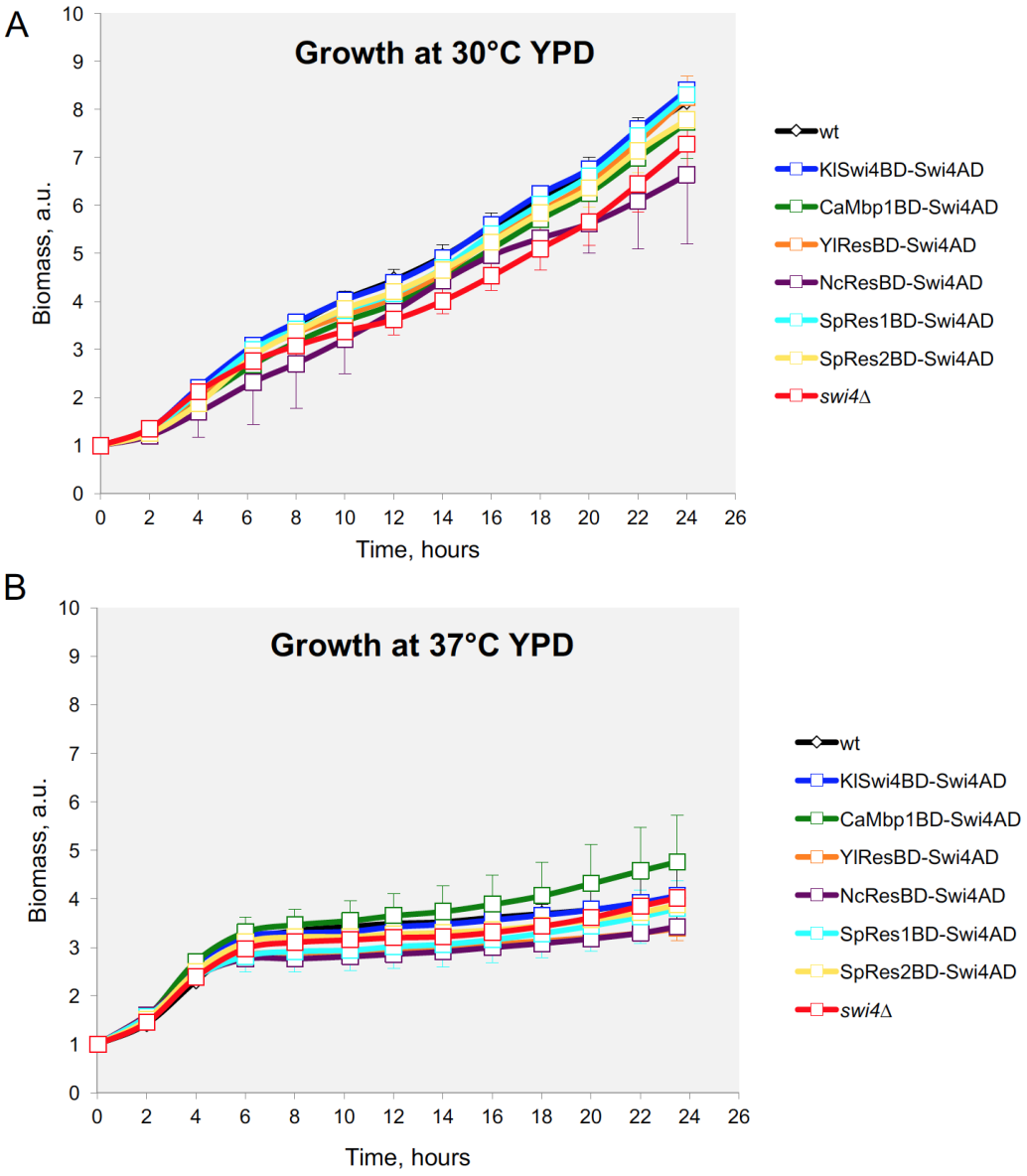


Figure 24. Replacement of ScSwi4 DNA binding domain with Swi4BD from other clades did not affect growth rates in *swi4*Δ background. Exponentially growing yeast cultures were diluted to the same cell number and grown for 24 hours at 30°C (A) and 37°C (B). Growth in biomass was monitored by BioLector (all strains were analysed in triplicate; error bars represent standard deviation).

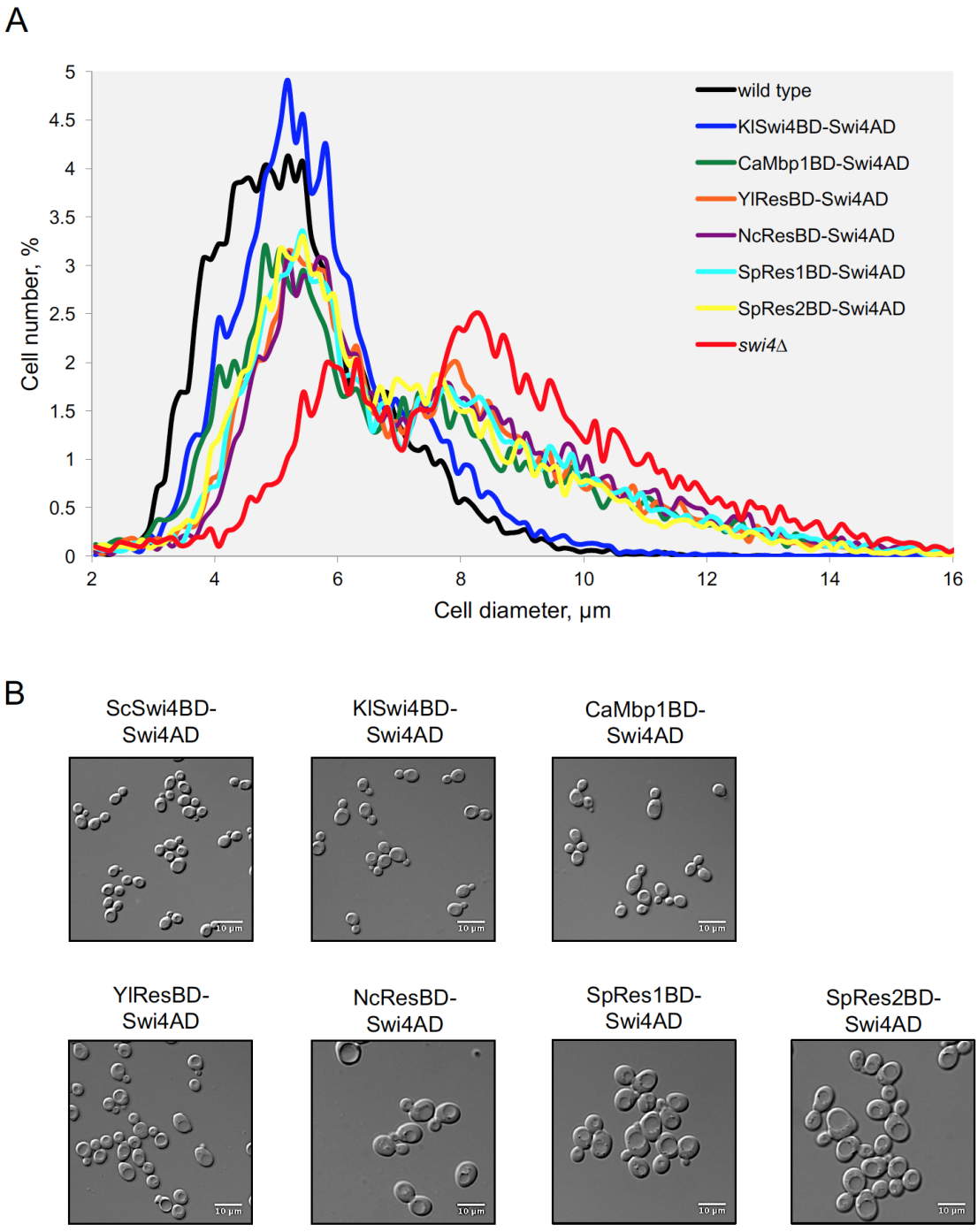


Figure 25. Replacement of ScSwi4 DNA binding domain with Swi4BD from other clades resulted in the increased cell size in *swi4*Δ background. (A) Cell size distribution measured by Multisizer 4 (Beckman Cell Coulter Counter). 10,000 cells were analysed in total. (B) Representative DIC images of wt ScSwi4BD-Swi4AD and strains with hybrid Swi4 (Zeiss AxioPlan, 63x magnification, oil objective).

3.3.3. G1/S transcriptional network expansion via inclusion of SBF specific and MBF specific targets.

Decreased fitness of strains with hybrid Swi4 can be a result of different modes of action of the hybrid Swi4. DNA binding domains from distant clades might bind all SBF targets with lower affinity or only a subset. It would be predicted that this would either lead to lower levels of expression of SBF targets or no activation of some. Therefore, we investigated the transcription levels of an SBF (*CLN2*) and an MBF (*RNR1*) target in wt, *swi4*Δ and cell expressing a hybrid Swi4 from clade 1 (KlMbp1BD-Swi4AD), clade 2 (CaMbp1BD-Swi4AD), clade 4 (NcResBD-Swi4AD) and clade 5 (SpRes2BD-Swi4AD). Cells expressing a hybrid in an *MBP1* background were used for expression analysis, since these strains don't display a growth defect. Wt and *swi4*Δ strains were used as positive and negative controls, respectively. Exponentially growing cultures were arrested in G1 phase with mating pheromone and released. Samples for RNA extractions were collected every 15 min after release and transcript levels were established by RT-qPCR by Ct value method and normalised to *ACT1*. Progression through the cell cycle was monitored by budding indexes (Fig. 26A). Replacement of Swi4 DNA binding domain did not affect cell cycle progression. Transcription of another cell cycle regulated gene *RNR1*, which is not regulated by SBF, served as a control for proper transcription timing and was repressed in G1 phase (0 min), activated upon G1-to-S phase transition (30 min) and inactivated in S phase (60 min) (Fig.26). This indicated, that transcription timing was not affected in hybrid strains. At the same time, transcription of SBF-dependent gene *CLN2* was affected. In *swi4*Δ cells activation of the SBF target *CLN2* was largely lost, but still with a slight peak at 30 min. *CLN2* transcript levels were increasingly reduced in the cells with hybrid Swi4 correlating with evolutional distance between species. These results suggest that the ability to regulate *CLN2* was decreased with the phylogenetic distance of the yeast species.

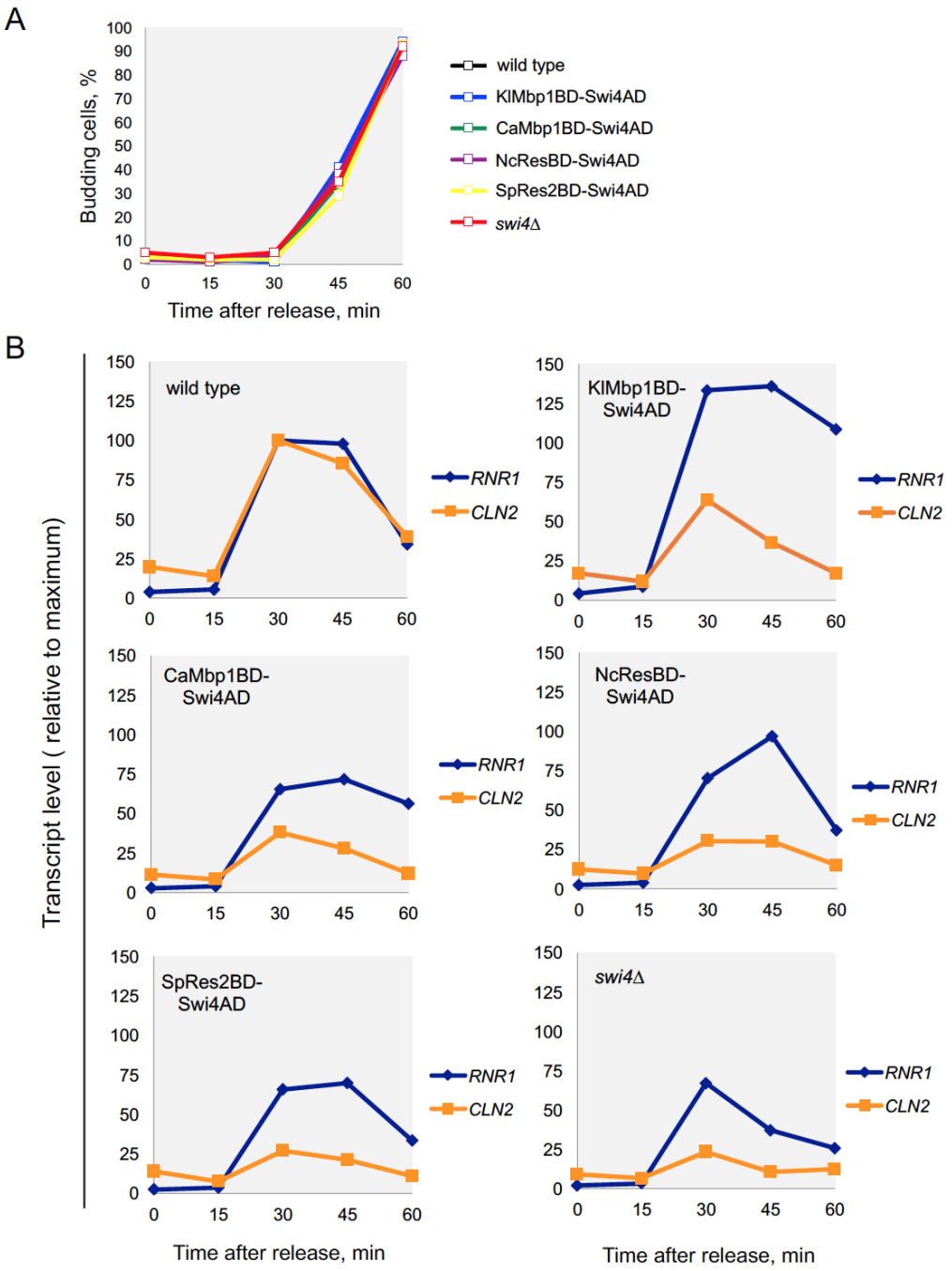


Figure 26. Transcription of SBF target gene *CLN2* cannot be fully activated by hybrid Swi4. Yeast cultures were arrested in G1 phase and released. Samples for budding counting and RNA extraction were collected every 15 minutes after release. (A) Budding indexes. Number of budding cells was counted under the light microscope. At least 100 cells were analysed in total. (B) Relative transcript levels SBF (CLN2) and MBF (RNR1) target genes in wt and stains with Mbp1/Res DNA biding domains from species of clad 1 (KlMbp1BD-Swi4AD), clade 2 (CaMbp1BD-Swi4AD), clade 4 (NcResBD-Swi4AD) and clade 5 (SpRes2BD-Swi4AD). Transcript levels were established by RT-qPCR and normalised to *ACT1*.

To investigate transcriptional regulation by the hybrid Swi4 TF of the entire G1/S regulon our collaborators, Adi Hendler and Amir Aharoni, performed RNA-seq analysis. Strains, used for RT-qPCR analysis, were arrested in G1 phase with mating pheromone and released into fresh media. Samples were collected at 0 (G1) and 30 min (G1/S), since G1/S transcription is inhibited in G1 and reaches its maximum levels at G1-to-S transition.

They first established SBF-dependent genes by selecting those genes whose expression levels were increased in wt cells at 30 minutes but not in *swi4*Δ cells (complete description of the analysis in Hendler *et al.,* 2017) (Fig.27). 68 genes were identified to be down-regulated in *swi4*Δ cells in comparison to wt (SBF regulon), 30 of which shared with SBF target genes identified in previous study (Ferrezuelo *et al*., 2010).

They then compared the expression level of these SBF-dependent genes in the strains with hybrid Swi4 to wt. This analysis established that hybrid Swi4 from different clades can only regulate a smaller subset of SBF target genes. The highest number of SBF targets can be regulated by the hybrid TFs closet related to *S. cerevisiae* (clade 1 *K. lactis* 26 genes and clade 2 *C. albicans* 27 genes). At the same time, DNA binding domain from more distant species *N. crassa* and *S. pombe* can only activate 10 and 8 genes respectively. And only 3 genes from SBF-dependent regulon are activated by all hybrid TFs (*DSE2, CIT2, TRF5*). The analysis also revealed a set of SBF target genes (33 genes), which are specific to *S. cerevisiae* and can be regulated only by wt Swi4. Altogether, our results suggest that the Swi4 hybrids can activate only a limited number of SBF targets, and some SBF-targets to lower extent. Importantly, the DNA binding domains from more distantly related clades can only regulate an increasingly limited set of SBF-dependent targets to a lower extend.

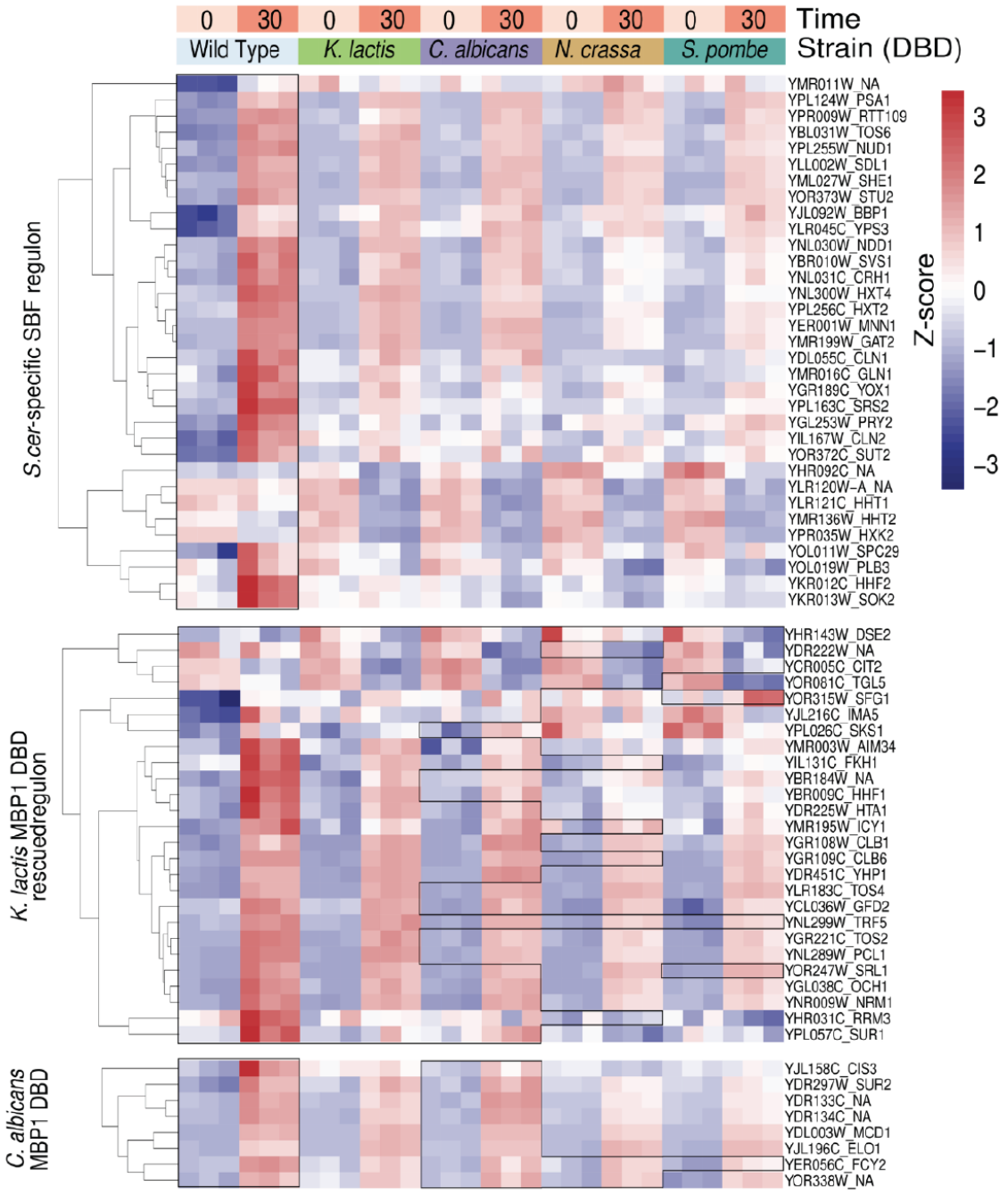


Figure 27. Genome-wide expression analysis revealed subsets of SBF-activated genes, which can be regulated by hybrid Swi4 with DNA binding domain from other species. Detailed description can be found in Hendler *et al.*, 2017.

3.3.4. *S. cerevisiae* Swi4 binds SCB motifs with higher affinity than MCB-like motifs.

Promoter enrichment analysis was performed by Dr. Edgar Medina to investigate DNA motifs in the subset of SBF targets that can be regulated by the various TF hybrids (Duke University, USA) (data not shown). In the analysis promoter regions (1000 bp upstream gene coding region) of SBF target genes, which can be regulated by hybrid TF from different clades, were analysed to identify differences in motifs for wild type and hybrid Swi4 binding. These differences are most likely to determine the extent to which SBF-regulon can be activated by hybrid TFs. As expected, ScSwi4-regulated genes were enriched in SCB motifs, while genes, which were activated by CaSwi4BD had more MCB-like motifs. Surprisingly, MCB-like motif was second enriched in genes activated by ScSwi4. This observation suggested that the MCB-like motif is likely to be an ancestral DNA motif with target genes containing the more specific SCB motif, which cannot be regulated by the Swi4 hybrids, likely to be the result of the network expansion. To investigate this differential activation of SBF-regulon I compared the binding affinity of ScSwi4 to promoters containing SCB and MCB-like motifs by chromatin immunoprecipitation (ChIP). Yeast cultures were grown to the same OD_{600} to have the same cell number and arrested in G1 phase with mating pheromone. Samples for ChIP were collected at 0, 30 and 60 min after release. ChIP was performed according to the protocol described using specific Swi4 antibodies recognising the C-terminal domain, which doesn't include the DNA binding domain (Harris *et al*., 2013). DNA fragments were analysed by qPCR with primers specific to SBF target promoter regions containing SCBs (*CLN2, PCL1, PRY2*) and MCB-like (*NRM1, MCD1, ELO1*) motifs. qPCR values were determined as % of WCE, and Swi4 binding was established as enrichment of values detected in *swi4*Δ cells, used as negative control. Comparing binding affinity in G1 phase (0 min), the time when Swi4 binds strongly to the target promoters, revealed that *S. cerevisiae* Swi4 binds to SCB motifs of *CLN2*, *PCL1* and *PRY2* with significantly higher affinity than to the promoters containing MCB-like motifs of *NRM1*, *MCD1* and *ELO1* (Fig.28A). Swi4 leaves target promoter in S phase when transcription is inactivated. Comparing binding affinity during the cell cycle, at time points 0, 30 and 60 min,

shows that binding is lost at 60 minutes, when cells are in S phase (all budded) establishing the specificity of Swi4 binding in my ChIP analysis (Fig.28B).

To confirm that Swi4 preferentially binds to promoters containing SCB, I compared Swi4 binding to a wt *PRY2* promoter and *PRY2* promoters with mutated SCB motifs at their endogenous locus. The SCB in the *PRY2* promoter was either disrupted by mutations in the core-binding motif (CGCG to CAAG: wt *PRY2* AA) or mutated to resemble a MCB-like motif (ATCGCGA to AACGCGT: wt *PRY2* MCB). Cell cultures were treated as above, and G1 phase arrested cells were analysed by ChIP according to the protocol. I observed, that disruption of the core-binding motif (wt *PRY2* AA) led to loss of Swi4 binding (Fig.29A left panel). Binding to an SCB mutated to resemble a MCB-like motif (wt *PRY2* MCB) was highly reduced in comparison to wt SCB. At the same time binding to the SCB containing promoter *CLN2* was not affected in any of the strains (Fig.29A right panel), confirming that this reduced binding is specific to mutations in the *PRY2* promoter. Moreover, analysis of *PRY2* transcription by RT-qPCR revealed that transcript levels correlate with the ability of SBF to bind to the *PRY2* promoter showing a significant reduction when SCB was mutated (Fig.29B) (performed by Adi Hendler).

Our analysis revealed that SBF-dependent genes containing MCB-like motifs are enriched in the set of genes that can be regulated by hybrid Swi4 TFs, but those with SCBs are not. My results show that Swi4 has binds more to promoters containing SCBs than those with MCB-like sequence, indicating that the SCB sequence is an optimized DNA binding sequence, and that this is important for proper regulation of transcription.

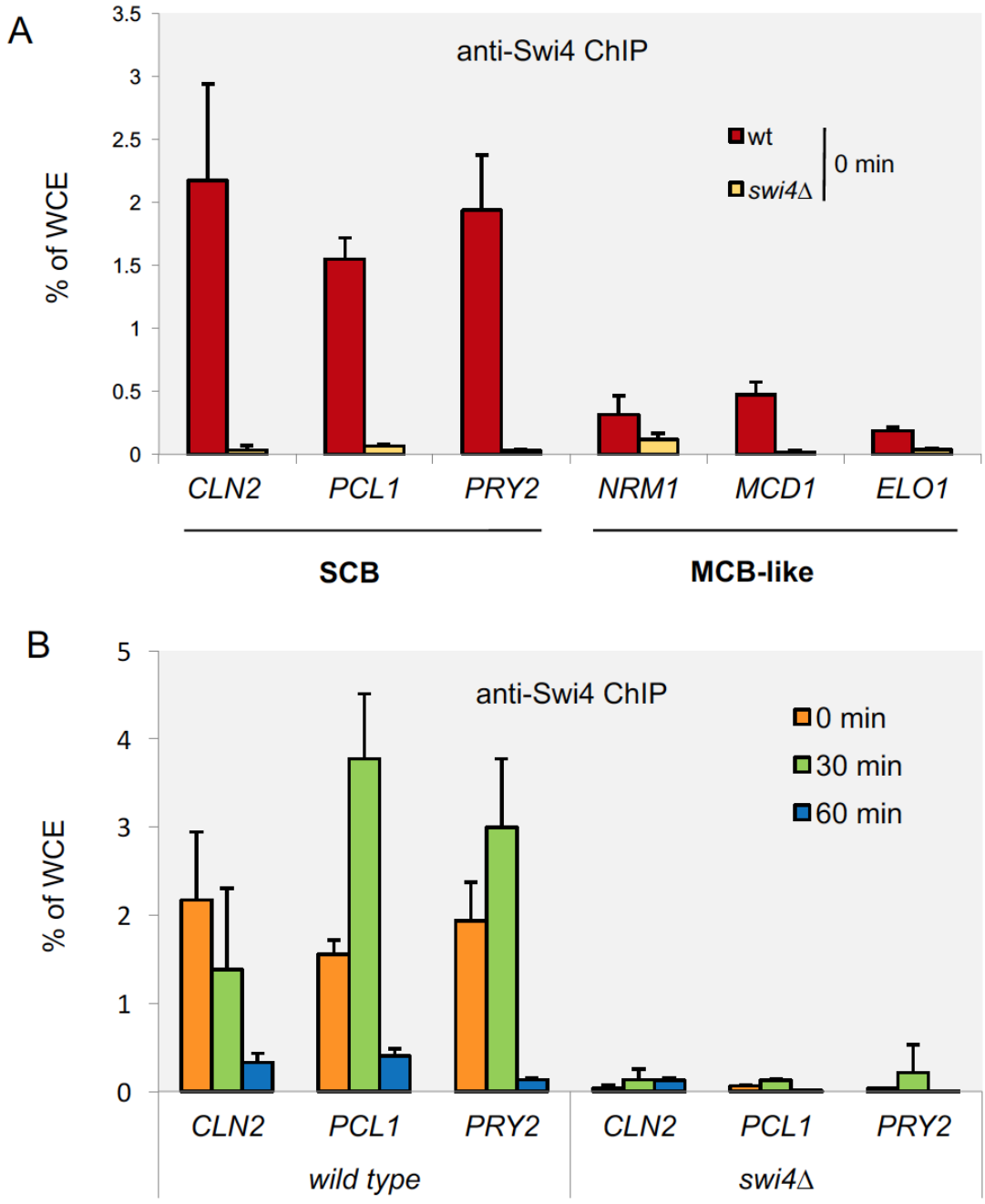


Figure 28. Swi4 binds to SCB motifs with higher efficiency, than to MCB-like motifs. Yeast cultures were diluted to the same OD_{600}, arrested in G1 phase with mating pheromone and samples were collected at 0, 30 and 60 min after release. ChIP was performed with anti-Swi4 antibodies. DNA fragments were analysed by qPCR and % of WCE was calculated. (A) Enrichment in Swi4 binding at SCB (*CLN2*, *PCL1* and *PRY2*) and MCB-like (*NRM1*, *MCD1* and *ELO1*) containing promoters in G1 phase. (B). Enrichment in Swi4 binding at SCB containing promoters in G1 phase (0 min), G1/S transition (30 min) and S phase (60 min). (3 technical repeats; error bars represent standard deviation within one sample).

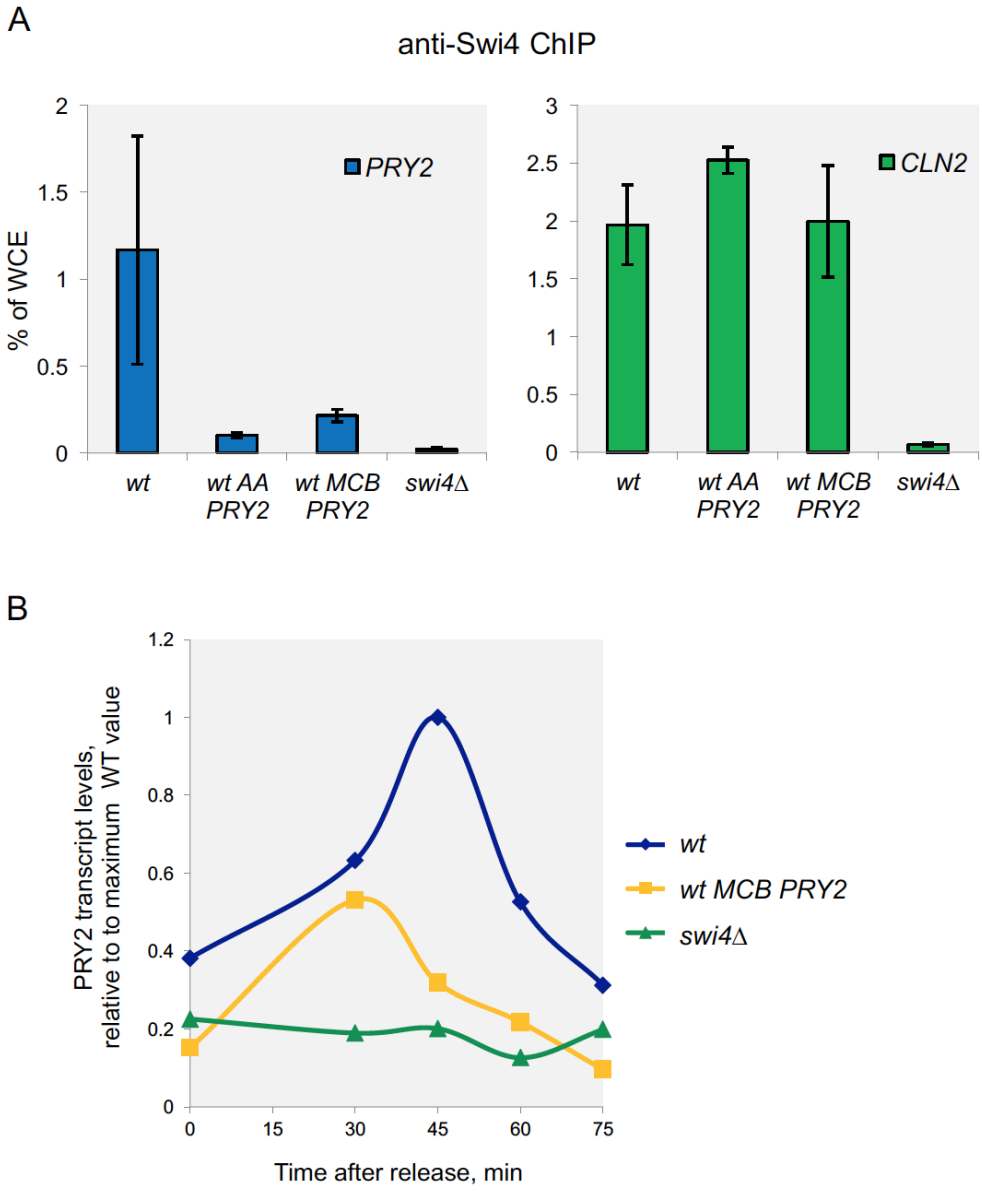


Figure 29. SCB motif determines high Swi4 affinity and transcription activation of SBF target *PRY2*. Yeast cultures were diluted to the same OD_{600}, arrested in G1 phase with mating pheromone and G1 arrested cells were analysed by ChIP with anti-Swi4 antibodies. DNA fragments were analysed by qPCR and % of WCE was calculated. (A) Enrichment in Swi4 binding at *PRY2* and *CLN2* promoters in cells carrying wt *PRY2* SCB and mutated *PRY2* SCB (wt AA with disrupted core binding motif and wt MCB with SCB mutated into MCB-like motif) (3 technical repeats; error bars represent standard deviation with one sample). (B). Transcript levels of *PRY2* in wt, wt MCB and *swi4*Δ cells, analysed by RT-qPCR using Ct value method and *ACT1* as a reference gene. Yeast cultures were synchronised in G1 phase with mating pheromone and samples for RNA extraction were collected at 0 min and every 15 minutes after release.

3.3.5. Hybrid Swi4 can bind MCB-like motifs in SBF target promoters.

We have established that Swi4 binds SBF-dependent target promoters with SCB motifs with higher affinity than those with MCB-like motif, which is important for proper regulation of G1/S targets by Swi4. Promoter analysis, of SBF-dependent targets that can be regulated by Swi4 hybrids (Fig.27; data for analysis is not shown, Edgar Medina), revealed enrichment for the MCB-like motifs. SCB motifs can only be found in G1/S target promoters in yeasts from clade 1 species (for example *S. cerevisiae* and *K. lactis*).

This would suggest that Swi4 hybrids should be able to bind to promoters containing MCB-like motif, but not those with SCB motifs. To test this, I performed ChIP analysis. Exponentially growing cell cultures were diluted to the same OD_{600} and arrested in G1 phase with mating pheromone. ChIP was performed according to the protocol with antibodies against C-terminal domain of Swi4 activation domain. I observed that binding affinity of hybrid Swi4 to SBF target promoters with MCB-like motifs (*NRM1*, *MCD1*, *ELO1*) is comparable to binding efficiency of wt Swi4. However, Swi4 hybrids have much lower affinity to SBF target promoters with SCB motifs (*CLN2, PCL1, PRY2*), than wt Swi4 (Fig.30). My data indicate that Swi4 hybrids can bind MCB-like motifs, but not SCB, which correlates with their ability to regulate only this subset of SBF genes.

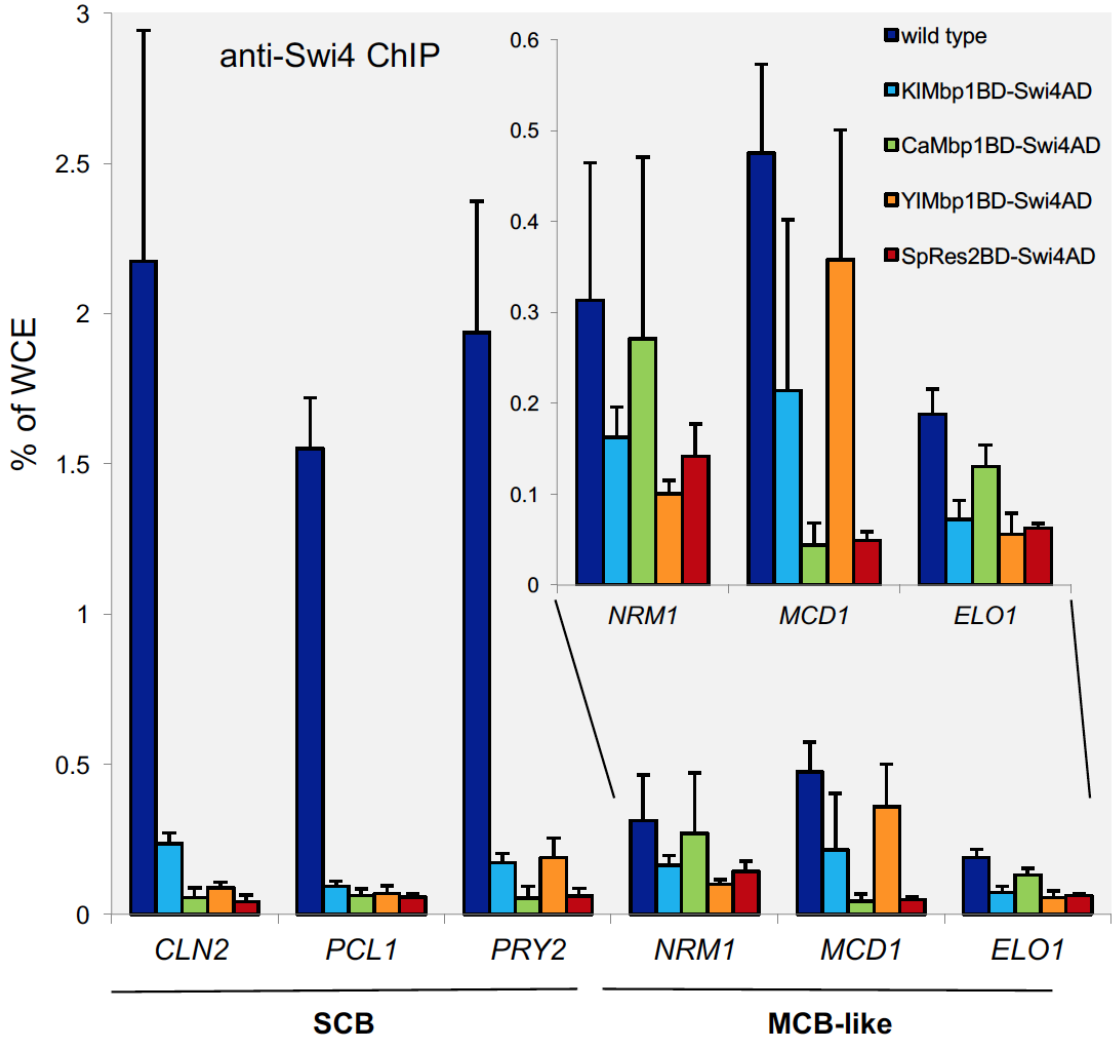


Figure 30. Hybrid Swi4 recognise and bind MCB-like motifs in SBF target promoters. ChIP was performed with antibodies against ScSwi4AD. DNA fragments were analysed by qPCR with primers, specific to promoter regions containing SCB (*CLN2, PCL1, PRY2*) and MCB-like motifs (*NRM1, MCD1, ELO1*). Enrichment was calculated as % of WCE. (3 technical repeats, error bars represent standard deviation within one sample).

3.3.6 Summary.

G1/S transcriptional network size varies in different yeast species from 80 in fission yeast to more than 200 in budding yeast. To address the question of evolution of G1/S transcriptional network and investigate if Swi4 or Mbp1 is an ancestral transcription factor, we performed a functional analysis, where DNA binding domains of Swi4 and Mbp1 were replaced with DBD from distantly related yeast species. The aim of the analysis was to establish if DBD from distant species could functionally complement native *S. cerevisiae* Mbp1 or Swi4. Replacement of ScSwi4 DNA binding domain with DNA binding domains from distantly related yeast clades allowed regulation of critical G1/S transcription regulon and rescue lethality of *swi4*Δ*mbp1*Δ cells (Fig.19), but not Mbp1BD replacement (Fig.18). Cells relying on Swi4 hybrid as a regulator of G1/S transcription exhibited severe phenotypic defects in *mbp1*Δ background (impaired growth rate and abnormal cell shape) (Fig.21 and Fig.22), while in a wt background the phenotype was less severe (Fig.23 and Fig.24). Our data shows that the severity of the phenotypes of cells with replaced Swi4BD from distantly related yeasts is the result of some SBF-dependent target not being activated to the full extend and subsets of SBF targets not being activated at all. Analysis of gene promoters of SBF targets that can be regulated by Swi4 hybrids revealed enrichment of a MCB-like, not SCB, motif in the promoter region. My work shows that wt Swi4 can bind to SCB motifs with higher affinity than to MCB-like motifs (Fig.27A), which is required for activation of SBF targets such as *PRY2* (Fig.28B). Hybrid Swi4 cannot recognise and bind SCB motifs, but can bind to MCB-like motifs comparable to wt Swi4 (Fig.29). Overall our work suggests that the MCB-like motif represent the ancestral binding motif and the SCB motif is an optimised Swi4 binding sequence more likely added to the regulon during expansion of the network.

3.4. Fission yeast with deregulated G1/S transcription as a model for cancer development.

Oncogene activation leads to improper cyclin-CDK activity and deregulation of G1/S transcription. Deregulation of G1/S transcription is observed in many types of sporadic cancer. Such deregulation results in uncontrolled proliferation, replication stress and subsequent DNA damage and genomic instability. The DNA damage checkpoint provides the first tumorigenic barrier (Bartkova *et al*., 2006), but when this is compromised allowing proliferation cancer can be initiated. Cancer cells accumulate high levels of replication stress and DNA damage, which are detrimental for normally proliferating cells. The mechanism allowing cancer cells to cope with high levels of genomic instability is still elusive. The ability of cancer cells to survive and proliferate successfully can be explained by the concept of stress support pathways. The concept suggests, that cancer cells become more dependent on certain regulation pathways than normal cells: DNA damage stress, mitotic stress, proteotoxic stress, metabolic and oxidative stress. Thus, cancer cells become addicted to non-oncogenes – genes, which are crucial for cancer cells survival, but not in normally proliferating cells (Luo et al., 2009).

Identifying and targeting proteins involved in stress support pathway represent a new strategy for development of anti-cancer therapy, since these dependencies are specific to cancer cells and not surrounding healthy cells. One of the approaches to identify these genes is performing synthetic lethality screens. This approach is borrowed from yeast genetics, where two strains with single deletion are crossed to each other and viability of double deletion strain is assessed.

The mechanism of G1/S transcription is conserved from yeast to human. Moreover, deletion of co-repressor Nrm1 leads to deregulated G1/S transcription and accumulation of replication stress and DNA damage in fission yeast (Caetano *et al*., 2014). Based on this *nrm1*Δ fission yeast represents a great model system to study dependencies of cells with deregulated G1/S transcription, as observed in many cancer cells. Therefore, we decided to take

an approach of synthetic lethality to identify genes, which become essential for survival of *nrm1*Δ fission yeast cells.

3.4.1. Deregulation of G1/S transcription makes fission yeast dependent on stress support pathways – novel hallmarks of cancer.

To identify synthetic lethal and sick interactions of *nrm1* a Synthetic Genetic Array (SGA) was performed according to Roguev *et al*., 2007. Plate-based synthetic genetic array approach is based on the analysis of growth rates and colony sizes of different strains. Colony size of each double deletion strain is compared to the colony size of query strain: smaller size of double deletion colony indicates worse growth and synthetic sick/lethal interaction, while bigger size suggests positive interaction. The crosses and plating was performed by Mimosa Hoti (Bahler lab, UCL). The *nrm1*::CloNat PEM-2 query strain was crossed to each of 3420 of Bioneer viable deletion collection strain, carrying G418 (KAN) resistance. After mating, meiosis and sporulation and rounds of selection for double deletion strains, by selecting for the NAT and KAN resistant markers, double mutants were grown in quadruplicate colonies on plates, containing both Nat and G418, until colonies are visible. Analysis of colony sizes was performed by Charalampos Rallis (UEL) with Spotsizer Software (Bischof *et al*., 2016). The size of colonies of double deletion cells was compared to *nrm1*Δ colony size and scored from -2 to 2, with -2 poor growth/negative interaction and 2 better growth/positive interaction. I have applied a cut off < -0.2 and identified 250 strong negative interactions. Gene ontology analysis with AnGeli GO tool revealed enrichment in various biological processes from regulation of cellular metabolism to histone methylation. I have chosen 100 genes with the lowest score (strongest negative interaction) and manually established slim gene ontology term for a biological process they are involved in, using PomBase database. This analysis revealed, that half of these genes encode for proteins involved in the cancer associated stress support pathways (Table 6). Moreover, the function of these genes covers all the types of cancer associated stresses, suggesting that fission yeast cells with

deregulated G1/S transcription become dependent on these pathways. It also proves, that our *nrm1*Δ model is robust enough for our purposes.

Table 6. Genes showed the strongest negative interaction with *nrm1* according to Synthetic Genetic Array.

Stress type	Gene names	GO Biological process
DNA damage stress	*abp2, cmb1, def1, pnk1, rhp14*	*GO:0006281* - DNA repair
Mitotic stress	*atb2, cdr1, hip1, mal3, mto1, mug134, nud3, pyp3, slm9, ssu72*	*GO:0000226* - microtubule cytoskeleton organization *GO:1901990* - regulation of mitotic cell cycle phase transition *GO:0000070* - mitotic sister chromatid segregation
Proteotoxic stress	*SPBC839.03c, fub2, int6, pin1, pop2, rex2, ubr1, ufd2*	*GO:0070647* - protein modification by small protein conjugation or removal *GO:0030163* - protein catabolic process *GO:0006461* - protein complex assembly *GO:0006457* - protein folding *GO:0042254* - ribosome biogenesis

Table 6. continued.

Stress type	Gene names	GO Biological process
Metabolic stress	*SPAC1F12.10c, SPAC23H3.11c, SPAC27E2.01, SPBC725.03, SPBC902.03, SPBPB2B2.05, SPBPB2B2.11, arg6, exo5, fap1, lys2, met3, met6, mug180, nem1, scp1*	*GO:0051186* - cofactor metabolic process *GO:0005975* - carbohydrate metabolic process *GO:0006629* - lipid metabolic process *GO:0006520* - cellular amino acid metabolic process *GO:0007005* - mitochondrion organization
Oxidative stress	*mcs4*	*GO:0071588* - hydrogen peroxide mediated signalling pathway

3.4.2. The approach to validate potential hits from SGA.

Currently, hits from SGA are being validated by other members of the de Bruin group. For this a negative genetic interaction is investigated by analysing the double mutants initially by random spore analysis to select double deletion cells and subsequently by tetrad dissection analysis. The phenotype of the double mutant cells is assessed by microscopy and cell length is measured. An increase in cell length is an indicator of cell cycle defects. *nrm1*Δ cells have elongated phenotype due to the increased levels of RS and DD and an additional increase in cell length suggests that double deletion further negatively affects cell fitness.

Previous work in our group has established that G1/S transcription is necessary for the tolerance of oncogene-induced replication stress in human cells (Bertoli *et al*., 2013a; Bertoli *et al*., 2016). A small-scale synthetic lethality screen with only G1/S (MBF) target genes was also carried out by Louise Holland (now Immunocore) and Steffi Klier (now Kings College London). They have crossed *nrm1*Δ strain to 35 non-essential MBF targets deletion and assessed their phenotype and viability. They established, that an astonishing 24 non-essential MBF target genes become important and even essential in *nrm1*Δ cells (data not shown). Moreover, 50% of these genes belonged to the cancer stress support pathways: DNA damage stress, mitotic stress, proteotoxic stress, metabolic stress and oxidative stress. I have analysed a selection of these interactions via cell length of double deletion strains, which should have defects in each of the support pathways: *yox1* (proteotoxic stress), *rad51* (DNA damage stress), *hsp3105* (proteotoxic stress), *meu17* (metabolic stress) and *egt2* (oxidative stress) (Fig.31 and Fig.32). I have observed, that deletion of heat shock protein *hsp3105* and ergothioneine biosynthesis protein *egt2* in *nrm1*Δ background, proteotoxic and oxidative stress respectively, did not lead to significant increase in cell length in comparison to *nrm1*Δ (Fig.32). But when viability of double deletion strains was assessed by tetrad dissection analysis (Fig.33) (when each colony represents a progeny of one spore, which genotype can be established on selective media) by Louise Holland and Steffi Klier, they found that *nrm1*Δ*hsp3105*Δ cells are not viable. Moreover, they established, that deletion of *wpl1*, which encodes for cohesin loading/unloading factor, is

lethal in *nrm1*Δ background (Fig.33). Thus, combining cell length analysis and viability assay of double mutants we can validate potential hits from SGA, which belong to different cancer stress support pathways.

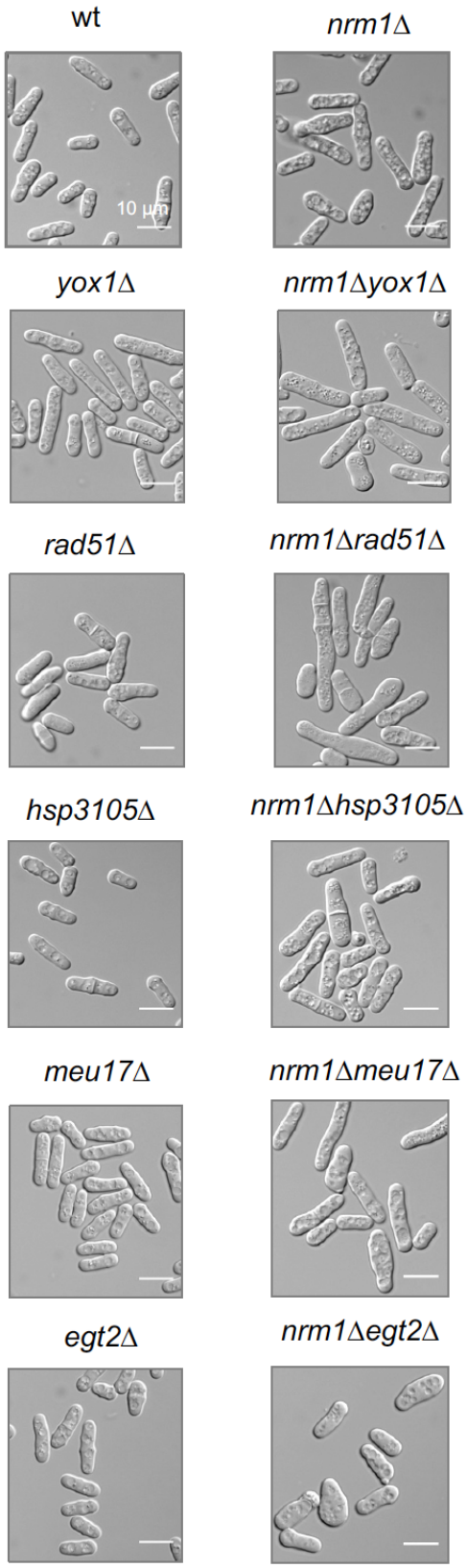


Figure 31. Double deletion cells have increased cell length in comparison to single deletion. Representative DIC images of wt and single and double deletion strains (Zeiss AxioPlan2, 63x magnification, oil objective).

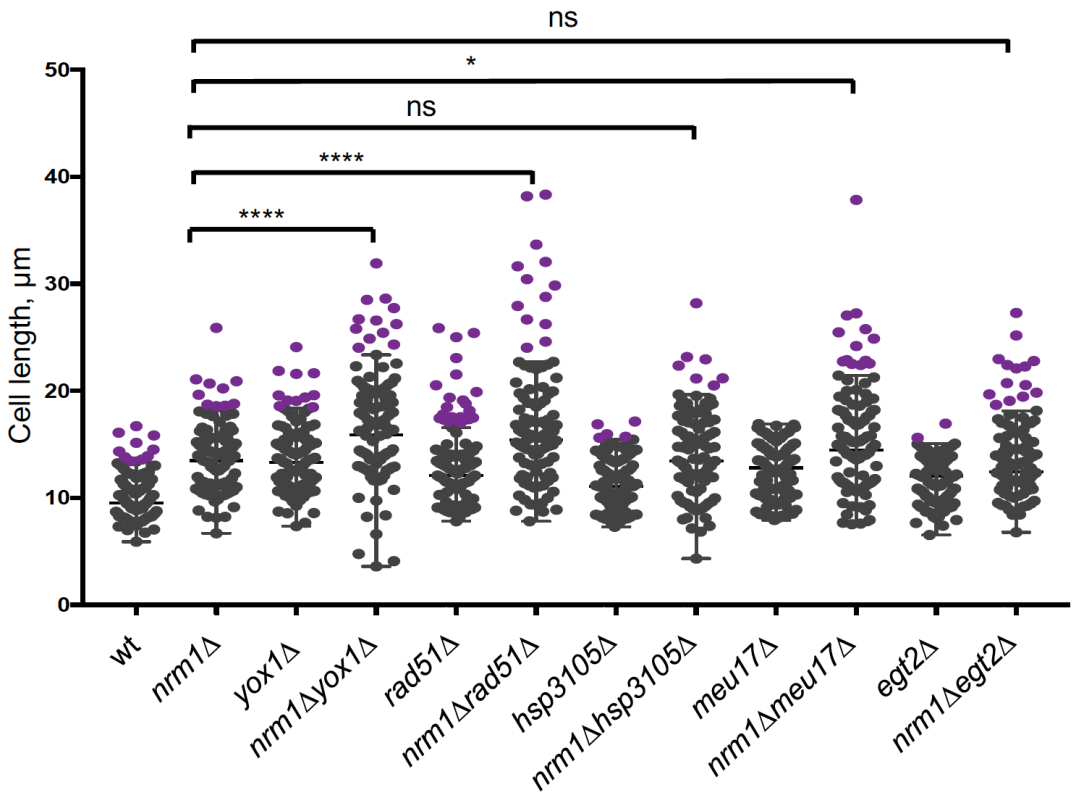


Figure 32. Deletion of MBF target genes in *nrm1*Δ background results in cell length increase. Quantification of cell length in wt, *nrm1*Δ and single and double deletion cells. At least 100 cells for each strain were imaged with light microscope and measured with ImageJ. Outliers (in purple) were calculated using Tukey's rule, with upper fence = First Quartile – 1.5* Inner Quartile Range, lower fence = Third Quartile + 1.5 * Inner Quartile Range (**** = P-value < 0.0001, * = P-value ≤ 0.05, ns = P-value > 0.05).

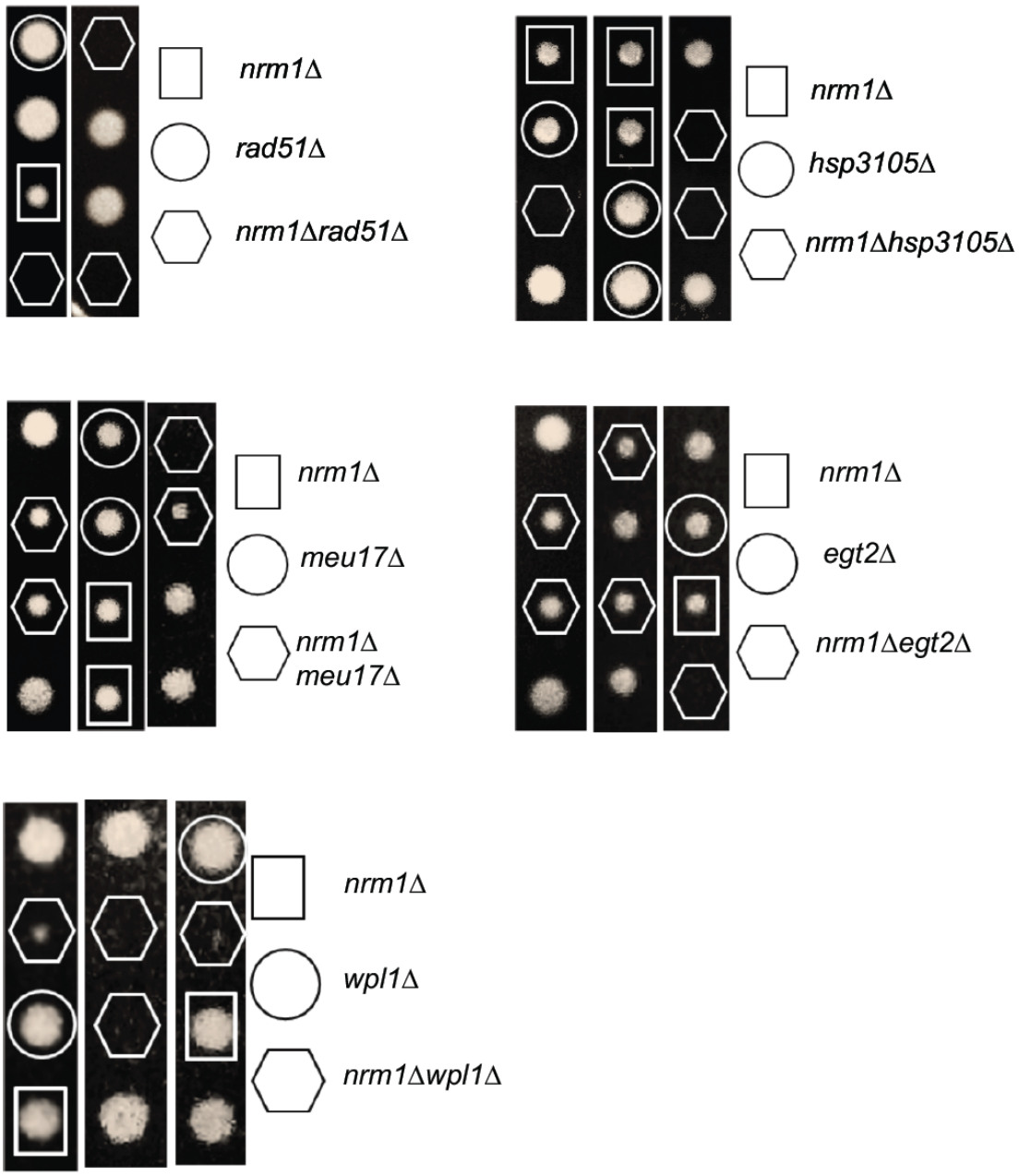


Figure 33. Deletion of MBF target genes in *nrm1*Δ background leads to cell death. Results of tetrad dissection analysis, where each colony represents progeny of single spore with either parental or recombinant genotype.

3.4.3. Creation and characterisation of thiamine repressible system.

Synthetic genetic array and further validation of the hits by tetrad dissection analysis allow us to establish, which genes are critical for survival of cells with deregulated transcription. The most interesting ones, with respect to cancer treatment, are the interactions that are synthetic lethal, meaning that double deletion cells cannot be investigated. This makes it difficult to establish the role of the candidate protein for cell viability, when G1/S transcription is deregulated. To overcome this issue of lethality of double deletion cells, I have been developing a system, where expression of *nrm1* can be chemically repressed to create an inducible *nrm1* deletion. The promoter of the *nmt1* gene in fission can be repressed by thiamine (Maundrell, 1990). Putting *nrm1* under inducible nmt1 promoter will allow repression of *nrm1* levels by addition of thiamine. If these levels are low enough to inactivate Nrm1 function I will be able to turn off Nrm1 function in the background of a mutant showing synthetic lethality with *nrm1*Δ and study the consequences of a double deletion. The *P1nmt-nrm1::CloNat* strain was available in the de Bruin laboratory collection. I have analysed the expression levels of *nrm1* in this strain by RT-qPCR. Yeast culture was grown in Edinburgh Minimum Media (EMM) until OD_{595} 0.3-0.5 and 25 ml of the culture were collected for RNA extraction. The rest of the culture was diluted into 25 ml of fresh EMM containing 5 μg/ml of thiamine and incubated overnight at 30°C. The next morning 25 ml of the culture were collected for RNA extraction. The transcript levels of *nrm1* were analysed by RT-qPCR using Ct value method and *act1* levels for normalisation. I have also tested the levels of MBF target genes *cdc22* and *cdc18*, which in the absence of *nrm1* should be upregulated. As expected transcript levels of *nrm1* were not detectable in *nmr1*Δ cells, while both *cdc22* and *cdc18* were upregulated in both treated and untreated conditions in comparison to wt (Fig.34). In contrast in *P1nmt-nrm1::CloNat* strain levels of *nrm1* were upregulated and levels of *cdc18* and *cdc22* were downregulated in untreated conditions in comparison to wt. Unfortunately, after treatment with thiamine the levels of *nrm1* were almost 10 times higher than in wt (Fig.34). Thus, P1nmt-promoter does not repress transcription of *nrm1* below wt levels.

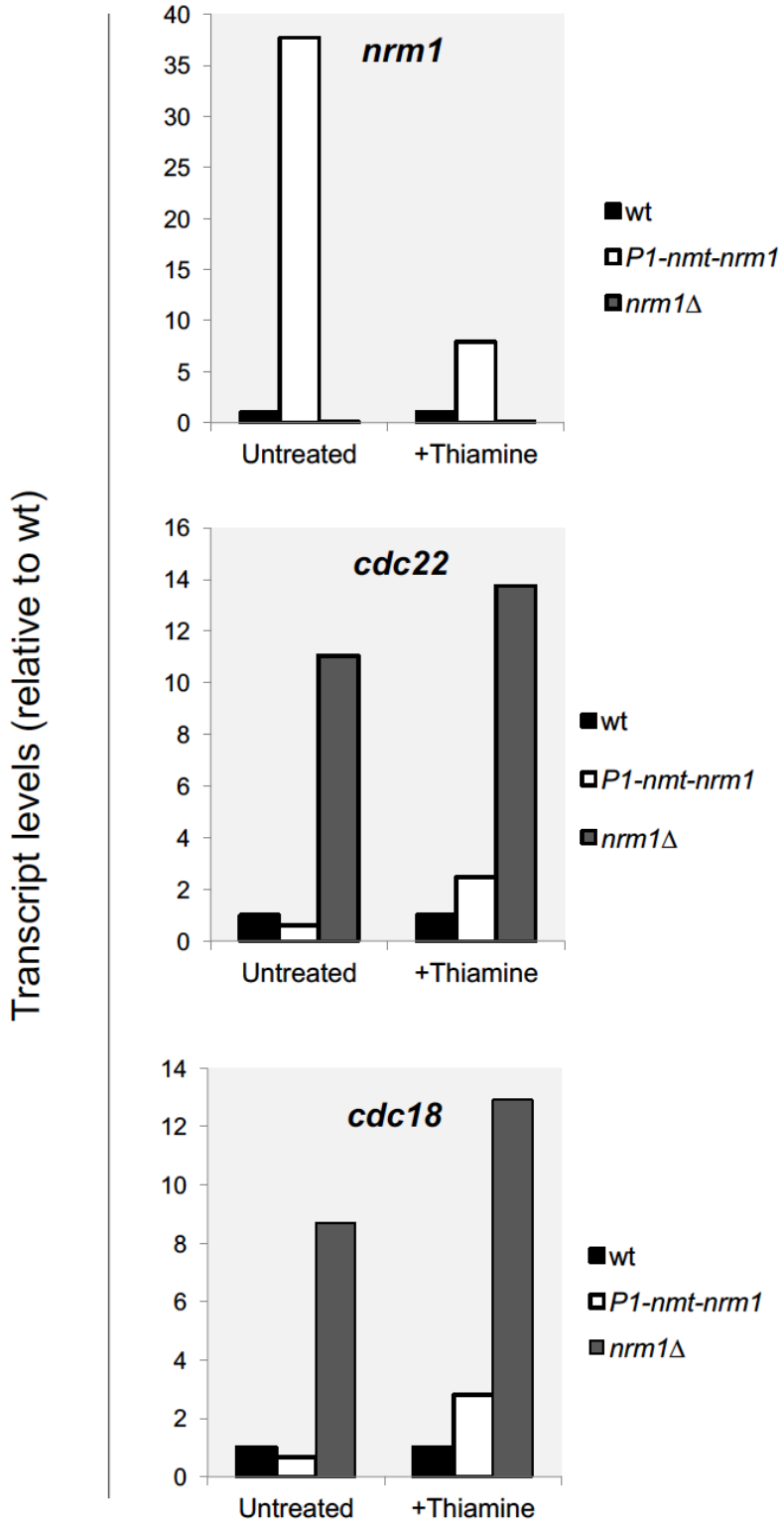


Figure 34. Comparison of transcription levels of MBF targets *nrm1*, *cdc22* and *cdc18* in wt, *nrm1*Δ and *P1nmt-nrm1::CloNat* strains. Yeast culture was grown in EMM without (Untreated) and with 5 µg/ml of thiamine (+Thiamine) and transcript levels were analysed by RT-qPCR with primers, specific to *nrm1*, *cdc18* and *cdc22* open reading frame.

Two attenuated forms of nmt-promoter, *P41-nmt* and *P81-nmt*, are also available. These promoters are compromised for their ability to activate transcription, but therefore also repress transcription upon addition of thiamine more than the *P1-nmt* promoter. Therefore, I decided to swap the native *nrm1* promoter driving the *nrm1* gene with *P41-nmt* and *P81-nmt*. The constructs carrying these nmt-promoters and homology arms to *nrm1* gene were created from the plasmids *P41nmt-pFa6a-natMX6* and *P81nmt-pFa6a-natMX6* by PCR (Bähler *et al.*, 1998). Wild type fission yeast cells were transformed with the PCR product according to the protocol. Positive colonies were selected on media containing nourseothricin (NAT). Selected clones were subjected to the thiamine treatment as described above and levels of *nrm1*, *cdc22* and *cdc18* were analysed by RT-qPCR. I found, that P41-nmt promoter allows higher levels of *nrm1* than wt in untreated conditions and upon addition of thiamine nrm1 transcript levels are still twice higher than in wt (Fig.35). At the same time levels of other MBF targets *cdc22* and *cdc18* were higher than in wt, which is unexpected, since high levels of *nrm1* in *P41-nmt-nrm1::CloNat* clones should repress transcription of *cdc22* and *cdc18* below wt. When transcription of *nrm1* was driven by P81-nmt-promoter, the levels of *nrm1* were highly upregulated and *cdc22* and *cdc18* were downregulated in untreated conditions in comparison to wt as expected (Fig.36). Upon addition of thiamine *nrm1* levels were 1.2 times higher than in wt (clone 1), but *cdc22* and *cdc18* levels were not as upregulated as in *nrm1*Δ. Still, this result is quite promising and clone 1 will be analysed again to confirm *nrm1*, *cdc22* and *cdc18* transcription levels.

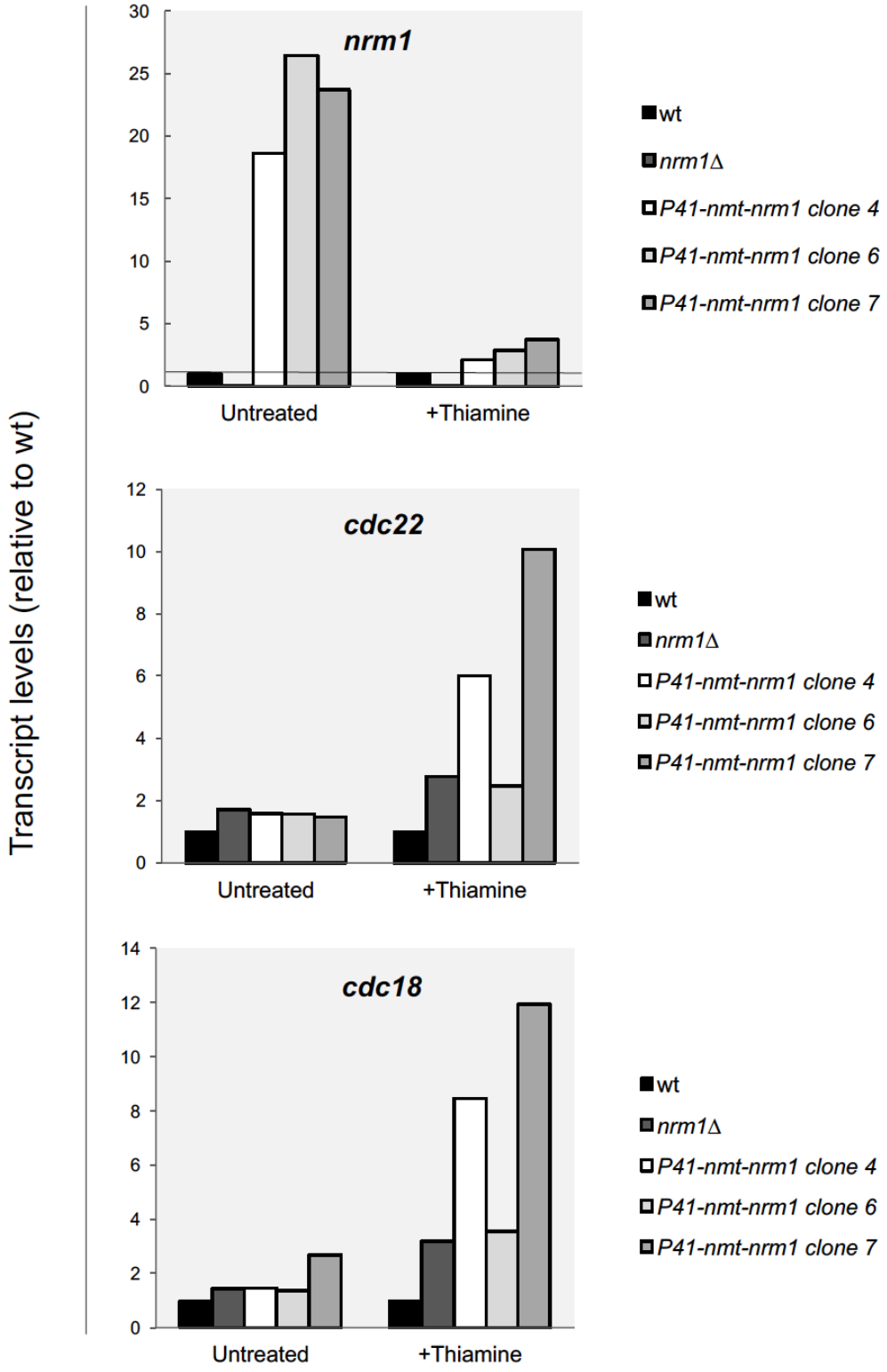


Figure 35. Comparison of transcription levels of MBF targets *nrm1*, *cdc22* and *cdc18* in wt, *nrm1*Δ and *P41nmt-nrm1::CloNat* strains. Yeast culture was grown in EMM without (Untreated) and with 5 μg/ml of thiamine (+Thiamine) and transcript levels were analysed by RT-qPCR with primers, specific to *nrm1*, *cdc18* and *cdc22* open reading frame.

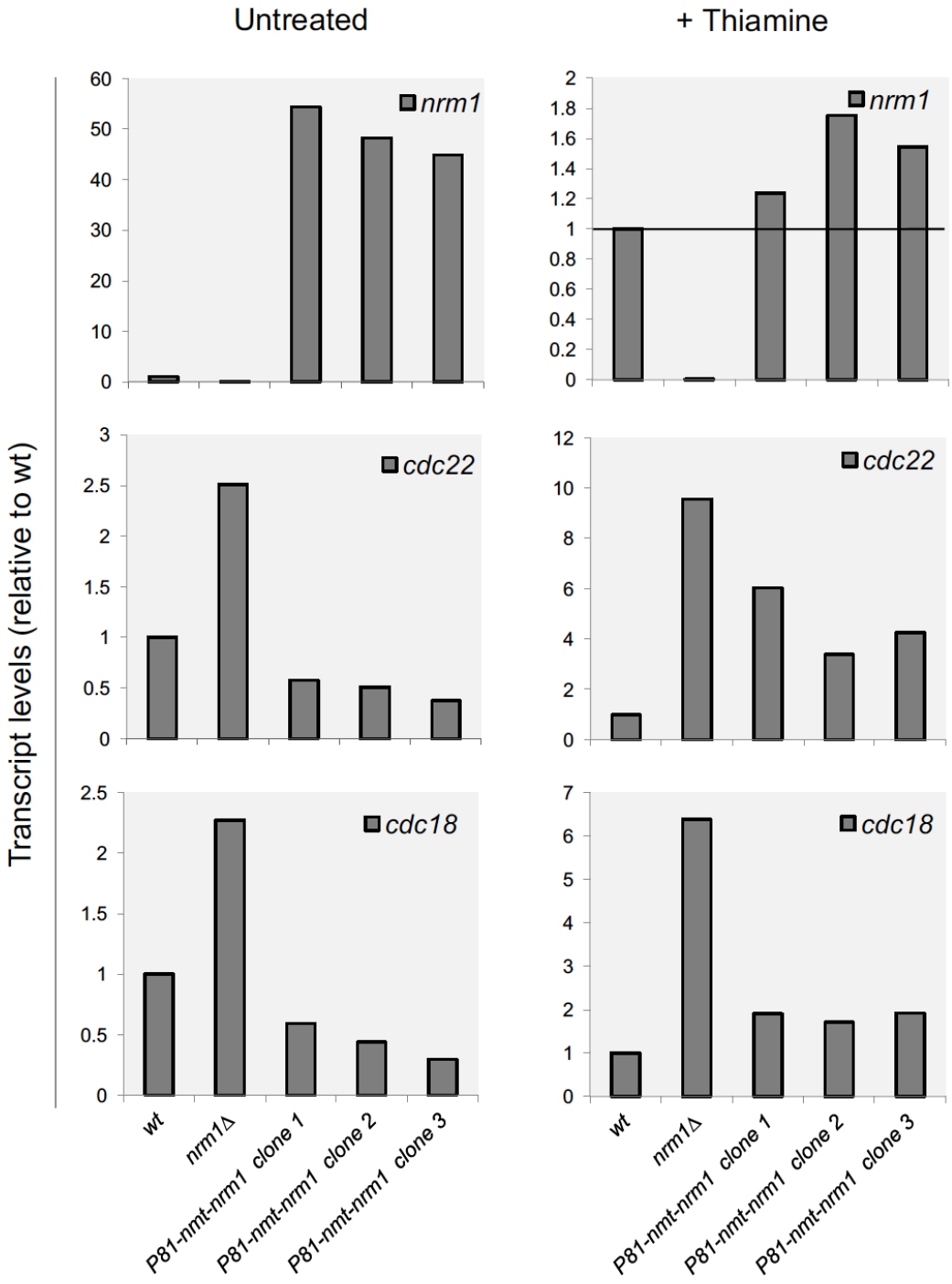


Figure 36. Comparison of transcription levels of MBF targets *nrm1*, *cdc22* and *cdc18* in wt, *nrm1*Δ and *P81nmt-nrm1::CloNat* strains. Yeast culture was grown in EMM without (Untreated) and with 5 μg/ml of thiamine (+Thiamine) and transcript levels were analysed by RT-qPCR with primers, specific to *nrm1*, *cdc18* and *cdc22* open reading frame.

3.4.4. Summary.

In *nrm1*Δ fission yeast cells G1/S transcription is constitutively active, which results in high levels of replication stress-induced DNA damage. This resembles the human model of oncogene-induced deregulated G1/S transcription, which has a central role in oncogene-induced replication stress. To establish specific cellular dependencies of cells experiencing deregulated G1/S transcription we have performed a Synthetic Genetic Array in fission yeast to identify non-essential genes, which become essential in cells with *nrm1* deletion (synthetic lethality or sickness). These genes are dependencies of cells with deregulated G1/S transcription and represent potential dependencies of cancer cells and therefore targets for anti-cancer therapy. The SGA revealed that *nrm1*Δ cells become highly dependent on pathways, which provide stress support in cancer cells, such as DNA damage stress, mitotic stress, metabolic stress, proteotoxic stress and oxidative stress. The hits from the SGA showing a strong negative interaction are being validated by phenotypic analysis of the double mutants. However, we are unable to do this for the most interesting candidates that show synthetic lethal interactions. Therefore, I developed a thiamine repressible system, which would allow me to repress *nrm1* transcription and track changes in cells with a deletion of a gene of interest. The system with *P81-nmt-nrm1::CloNat* clone 1 showed the most promising result with *nrm1* transcript levels being very close to wt and will be further confirmed.

4. DISCUSSION

4.1. The role of histone acetylation and deacetylation in modulation of G1/S cell cycle transcription in *S. cerevisiae*.

G1/S transcription drives cells cycle entry. In budding yeast G1/S transcription is regulated by SBF and MBF transcription factor complexes. Previously published data suggest that HATs and HDACs are also involved in regulation (Cosma *et al*., 1999; Fazzio *et al*., 2001; Robert *et al*., 2004; Stefan & Koch, 2009; Takahata *et al*., 2009). We have established that acetylation at G1/S target promoters is cell cycle regulated (Fig.4 and 5). I have also established, that HDAC Rpd3 is involved in full repression of G1/S targets and HAT Gcn5 is likely to be required for full induction of mostly SBF target genes. However, in the context of G1/S transcriptional wave the effect of deletion of *RPD3* is not significant, since levels of de-repression in *rpd3*Δ cells are still much lower than maximum levels at G1/S transition. In case of *GCN5* deletion, transcript levels at G1/S transition were lower in *gcn5*Δ cells than in wt, but still these levels were much higher than those in G1 and S phases in *gcn5*Δ cells. Deletion of *GCN5* results in lower levels of G1 cyclin *CLN2* gene, which might cause cell cycle delay and affects transcription levels of other G1/S target genes. However, this transcriptional effect could be due to loss of synchrony. To overcome the synchrony issue, I am planning to test the effect of *GCN5* deletion in *whi5*Δ background. In this case Swi4 will not be repressed and G1/S transcription will be activated in G1 phase straight away.

Overall, I have shown that in unperturbed conditions Rdp3 and Gcn5 are not required for regulation, but provide tuning of transcript levels, and regulation is performed by transcription factors. However, it is possible that there is a redundancy in HDACs and HATs and deletion of a single enzyme does not cause deregulation of G1/S transcription. In this case only knock out of several histone-modifying enzymes in combination will significantly affect transcription. It is also interesting to test the effect of simultaneous *RPD3* and *GCN5* deletion. Double deletion may result in loss of regulation of G1/S transcription. This

would suggest that the balance between hypo- and hyper-acetylation is required for maintaining G1/S transcriptional wave.

Involvement of HDACs, specifically human homologue of Rdp3 HDAC1, in G1/S transition was shown in human cells (Brehm *et al*., 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al*., 1998). Moreover, in human cells depletion of Gcn5 leads to the cell cycle delay and down-regulation of E2F targets (Kikuchi *et al*., 2005). Together with my results in budding yeast, these studies suggest, that regulation of G1/S transcription by HDACs and HATs can be more important in complex multicellular organisms. And in budding yeast SBF and MBF transcription factors are robust to provide transcriptional regulation. However, more detailed analysis of cell cycle progression of *gcn5*Δ cells by FACS together with transcription analysis in *whi5*Δ background will establish, whether Gcn5 is required for proper cell cycle progression in budding yeast.

Another possibility is that the modulation in budding yeast may become important upon stress conditions and when the amount of mRNA and then protein is crucial for cell fitness. This possibility could be further investigated in the group.

4.2. The role of histone methyltransferase Set2 in regulation of G1/S transcription in *S. pombe* upon genotoxic stress.

The outcome for transcription of histones lysine methylation depends on the chromatin context and the number of methyl groups. Set2-dependent H3K36me3 is required transcriptional elongation in both yeast and human cells (Kizer *et al*., 2005). In fission yeast H3K36me2 is also a mark of active transcription (Morris *et al*., 2005). However, the role of Set2 in transcriptional regulation in response to replication stress is not well established.

I showed that Set2 is involved in activation of MBF-dependent transcription in response to genotoxic stress, caused by bleomycin (Fig.12) and activation and maintenance in response to replication stress, caused by HU treatment (Fig.13). Moreover, Set2 provides proper activation of replication licensing factors (Fig.14) and thus proper cell cycle progression. My results are in line with previous studies, showing that Set2-dependent H3K36me2 and H3K36me3 correlate with activated transcription (Fig.15).

Set2-dependent methylation at promoter regions may provide a surface for transcription factors binding, and deletion of *set2*Δ should decrease binding efficiency of MBF components Res1 (DNA binding) and Cdc10 (activation). However, ChIP results do not support this hypothesis, since levels in Res1-Myc binding are comparable in wt and *set2*Δ in both treated and untreated, except *cdc22* (this can be due to the short treatment time) (Fig.16A). Deletion of *set2* reduces Cdc10-Myc binding (Fig.16B) even more than Res1 binding. This reduction may be due to decrease in Res1 binding which is required for Cdc10 binding to target promoters. These results suggest that Set2 regulates activation of MBF-dependent transcription via different from TF recruitment mechanism.

4.3. Expansion of G1/S transcriptional network in yeast.

The size of G1/S transcriptional network is different in different yeast species: from 80 in fission yeast to more than 200 in budding yeast (Rustici *et al*., 2004). Moreover, while in *S. cerevisiae* and closely related species (clade 1) G1/S transcription is regulated by two transcription factor complexes SBF and MBF with Swi4 and Mbp1 DNA binding subunits, more distantly related species possess only MBF (DNA binding subunits which recognize MCB motifs) (Koch *et al*., 1993; Galagan *et al*., 2003; Bähler, 2005; Côte *et al*., 2009; Ofir *et al*., 2012).

These differences raise a question of how the network evolved and was regulated in ancestral fungi. We, and our collaborators, performed a functional study combined with bioinformatics analysis to investigate whether SBF or MBF is an ancestral transcription factor. We have established that Swi4 DNA binding domain from distant species can bind a critical subset of SBF target genes. Closely related species were able to regulate larger subsets of genes than more distant species. These genes possess MCB-like binding motifs, which can be bound by hybrid Swi4 with comparable affinity to native Swi4. And genes which can be exclusively regulated by native *S. cerevisiae* Swi4 (*CLN2*, *PCL1*, *PRY2*) carry SCB motifs, are present only in clade 1. Our findings suggest that ancestral fungi species possessed MCB-like motif, and the SCB motif is a result of optimization during network expansion.

Our work represents a case study of the evolution of transcriptional network via expansion. Mutations occur in URS of genes, and new motifs cannot be recognised by an old TF. This old TF is also optimised to recognise and preferentially bind new motifs. In case of G1/S transcriptional network, new MCB and SCB motifs evolved in budding yeast from MCB-like motif present in other species. While in distantly related species Mbp1/Res provide both activation and repression of G1/S transcription, budding yeast and closely related species evolved to possess activator SBF and repressor MBF. At the same time, budding yeast Swi4 and Mbp1 share sequence similarity with Mbp1 and Res proteins in other species. This raises a question why budding yeast obtained two distinct modes of regulation and what defines SBF as an activator

and MBF as a repressor. One may think, that *S. cerevisiae* life cycle is less complicated then parasitic *C. albicans* or *N. crassa*, and complicated regulation of G1/S transition and transcription seems to be unnecessary. One of the possible explanations is that there simply was a capacity/possibility for network expansion and this expansion would not interfere with other existing networks. While in case of more complicated life cycle further evolution and optimisation was not possible because of pre-existing networks. This possibility to expansion may be also explained by limited DNA capacity to encode new transcription factors, since lots of DNA capacity is engaged in carrying genetic information about specialised proteins and transcription factors, which allow transcriptional regulation in different conditions.

Another question what makes SBF an activator and MBF a repressor will be addressed in further functional studies in our group.

4.4. Fission yeast with deregulated G1/S transcription become dependent on cancer stress support pathways.

Activation of oncogenes such as Ras and Myc leads to deregulation of G1/S transcription, which is observed in all types of cancer (Almasan *et al.*, 1995; Mittnacht *et al*., 1997; Peeper *et al*., 1997; Sage *et al.*, 2000; Sheen & Dickson, 2002; Chen *et al*., 2006; Robinson *et al*., 2009). This transcriptional deregulation results in premature S phase entry, replication stress and DNA damage. Replication stress triggers a cellular response to prevent replication stress-induced DNA damage. When oncogene-induced replication stress does result in high levels of DNA damage, the DNA damage checkpoint will prevent proliferation either by inducing apoptosis or senescence serving as a tumorigenic barrier (Bartkova *et al*., 2006; Halazonetis *et al*., 2008). However, when the checkpoint is compromised and cells are allowed to proliferate this will lead to transformation and cancer initiation. Cancer cells experience high levels of replication stress, which drives genomic instability. These levels would be detrimental for normal proliferating cells so cancer cells rely on tolerance mechanisms to prevent catastrophic genomic instability. Deregulation of G1/S transcription in fission yeast, via inactivation of Nrm1, also results high levels of replication stress, inducing DNA damage, resembling a human model of cancer development. As in cancer cells, fission yeast cells tolerate replication stress induced by deregulated G1/S transcription as well. We therefore decided to use *nrm1*Δ fission yeast as a model system for establishing the tolerance mechanism to deregulated G1/S transcription.

We used our *nrm1*Δ system to perform Synthetic Genetic Array to identify genes, not essential in wt cells that become essential for survival of *nrm1*Δ cells. My screen revealed, that cells with deregulated G1/S transcription become dependent on stress response pathways that are hallmarks of cancer. Now potential hits are validated by other members of the group.

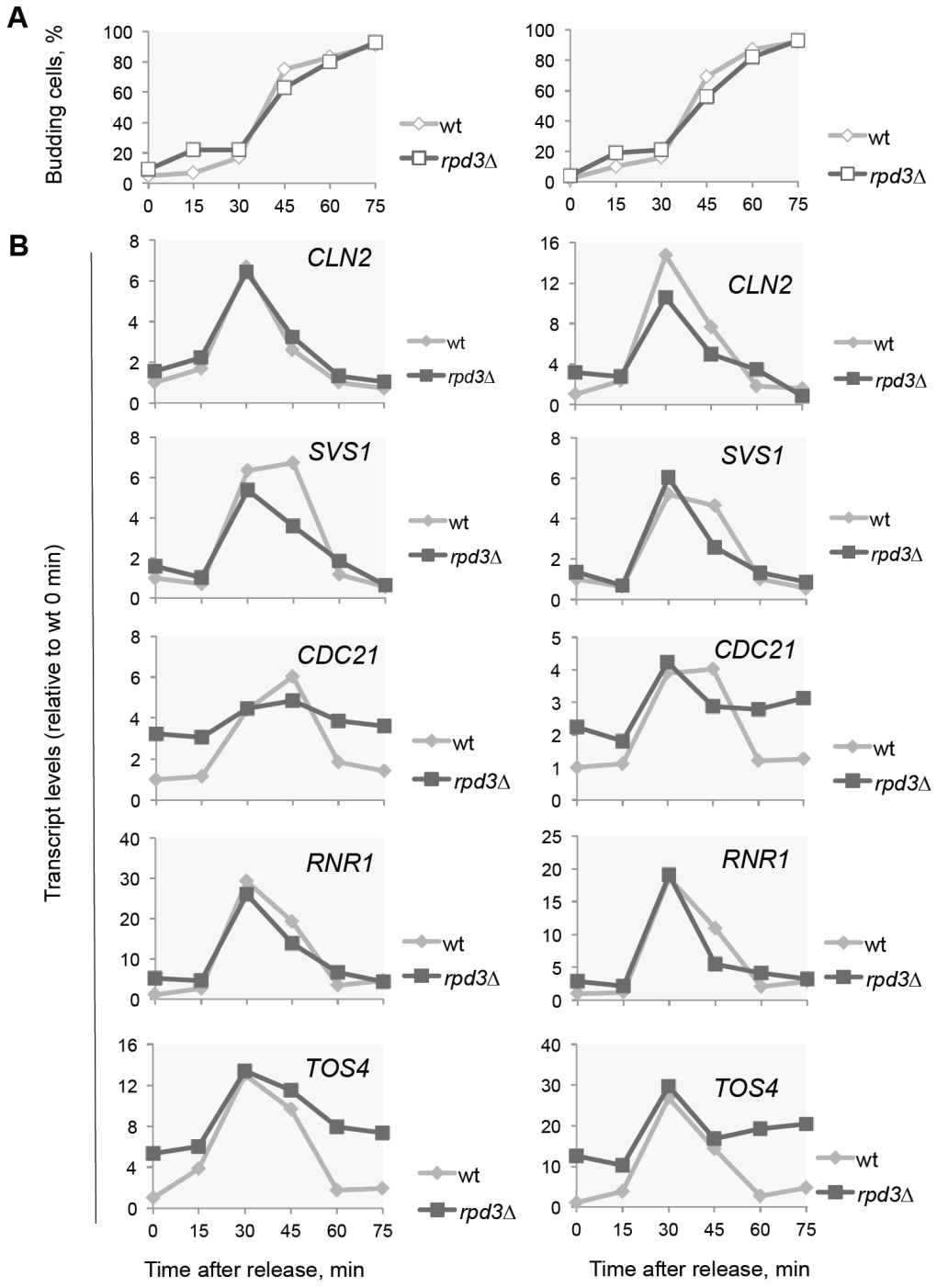
Several studies were already performed to establish synthetic lethal interactions in cancer cells lines (Luo *et al*., 2008; Luo *et al*., 2009; Cowley *et al*., 2014; Yu *et al*., 2016). In these studies cancer cell lines were screened to identify cell dependencies. Among advantages of our model system is that we

are assessing the direct consequences of transcriptional deregulation, unlike in cancer cell lines, where oncogene activation affects not only G1/S transcription. Thus, our model system specifically investigates the consequence of deregulation of G1/S transcription, which is common to most if not all cancer types. Another advantage of using fission yeast as a model is a relatively fast way to confirm synthetic lethal or sick interaction via tetrad spore analysis and analysis of the cell length, which are described in this thesis. In addition, fission yeast allows for quick dissection of the molecular mechanism and cellular processes that are important for cell viability in cells experiencing deregulated G1/S transcription. An important tool for this is the ability to manipulate synthetic lethal interactions via a repressible system, where levels of *nrm1* can be decreased by thiamine. This system allows us to track development of cell defects over the time without killing cells. The results of P81-nmt system are already promising, but I can enhance the system using auxin-based degron system. In this case, Nrm1 protein will contain sequence, which in the presence of plant hormone auxin will be recognises by SCF E3 ubiquitin ligase and Nrm1 protein will be targeted to degradation (Nishimura *et al*., 2009).

Overall, my screen established that cells with deregulated G1/S transcription become dependent on stress support pathways involved in the tolerance to stresses that are hallmarks of cancer. The hits, which belong to certain pathways, will be validated further. Since *nrm1*Δ fission yeast cells resemble precancerous cells, analogous of identified genes in human cells represent cancer cells addictions and may provide new directions of development of anti-cancer therapy.

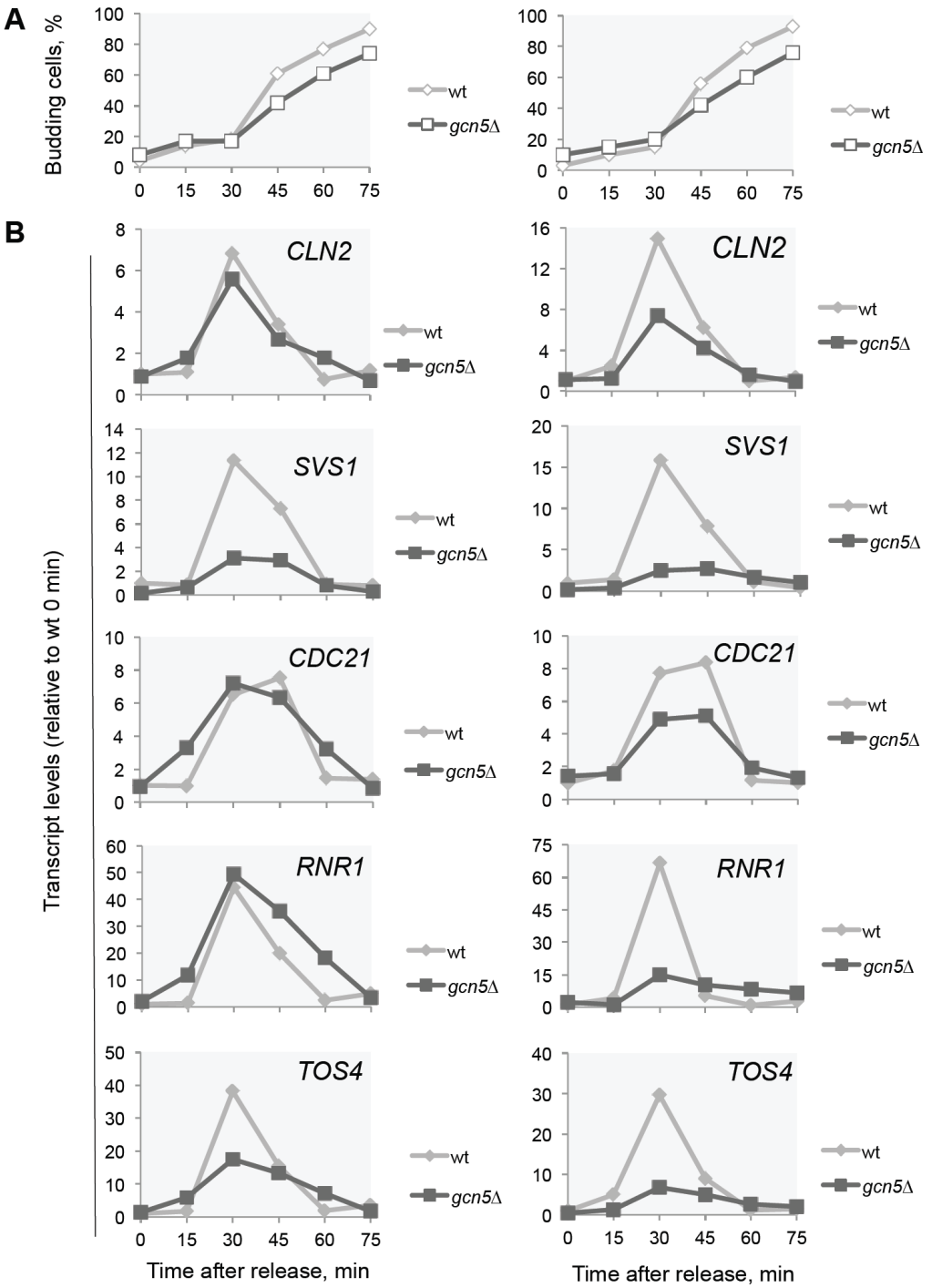
5. SUPPLEMENTARY

S.3.1. The role of histone acetylation and deacetylation in modulation of G1/S cell cycle transcription in *S. cerevisiae*.

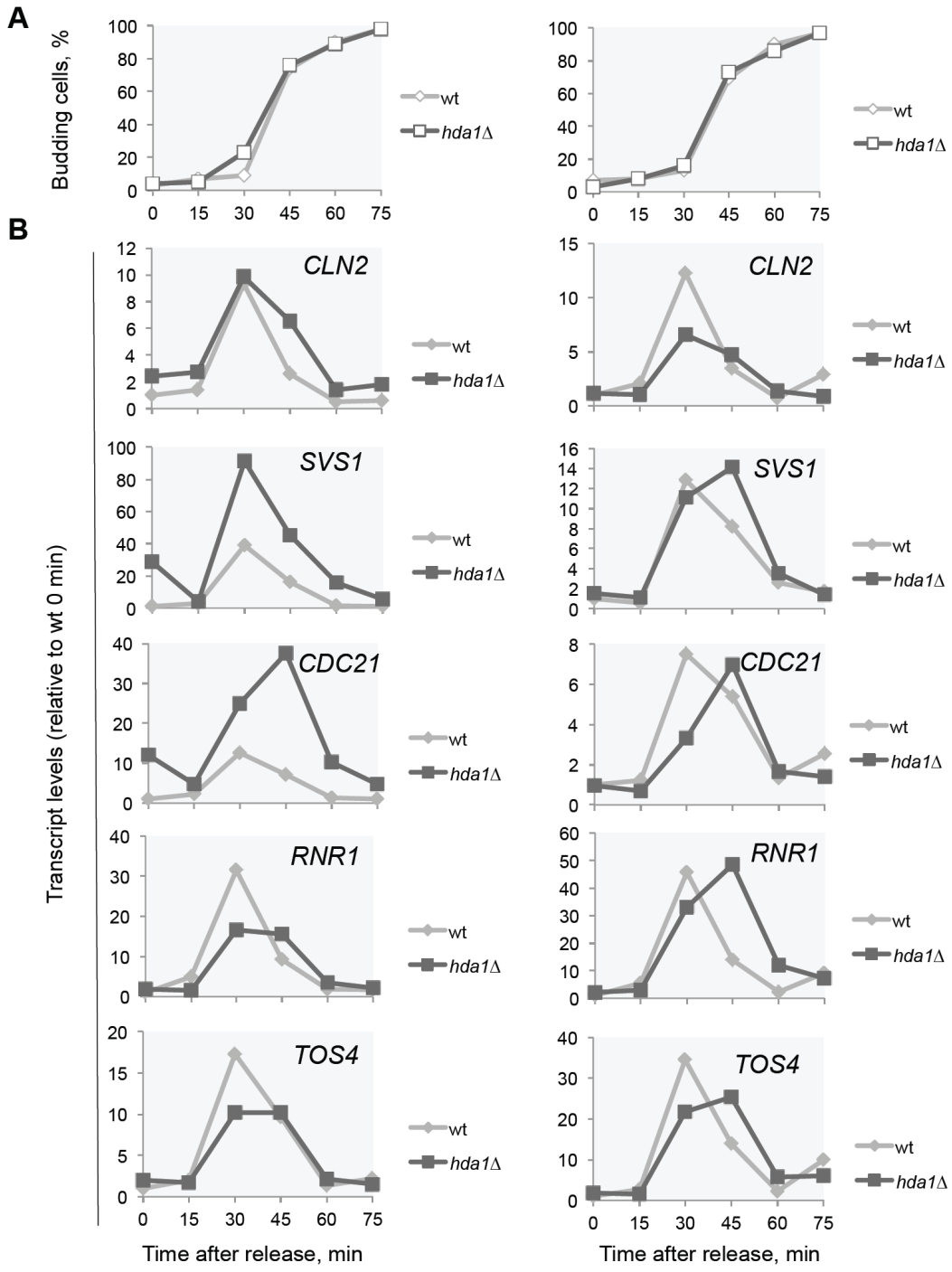


Supplementary figure 1. Progression through the cell cycle and G1/S transcriptional wave are not affected in the absence of Rpd3 (2 other biological

(continued) repeats). (A) Budding indexes. Exponentially growing wt and *rpd3*Δ cultures were arrested in G1 phase and released. Number of budding cells was counted under the light microscope at each time point, 100 cells were counted in total. (B) Transcript levels in wt and *rpd3*Δ cells during cell cycle. Exponentially growing wt and *rpd3*Δ cultures were arrested in G1 phase and released. Transcript levels were established by RT-qPCR using ACT1 as a reference gene.

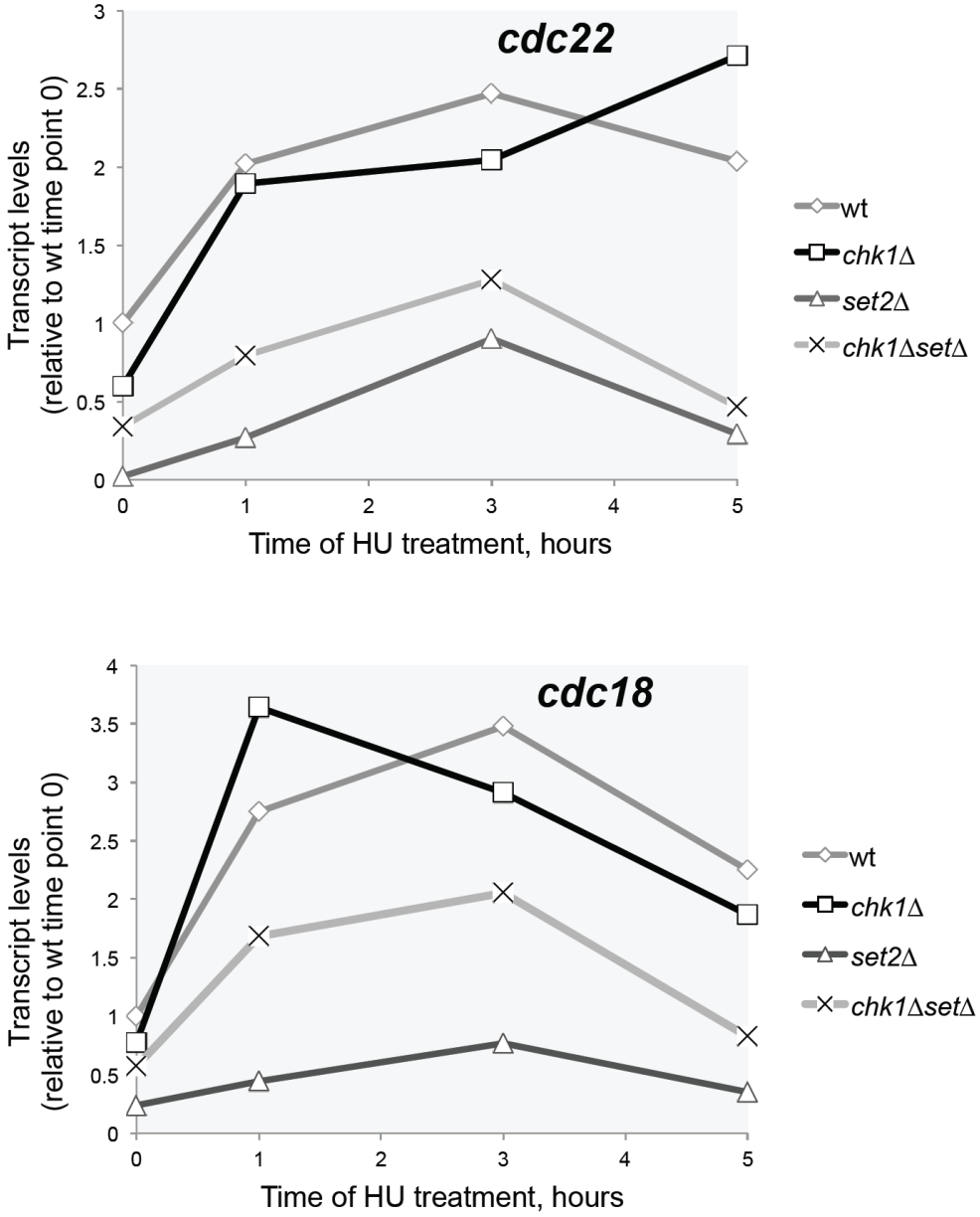


Supplementary figure 2. Deletion of *GCN5* results in the cell cycle delay and lower levels of G1/S transcription. (2 other biological repeats) (A) Budding indexes. Exponentially growing wt and *gcn5*Δ cultures were arrested in G1 phase and released. Number of budding cells was counted under the light microscope at each time point, 100 cells were counted in total. (B) Transcript levels in wt and *gcn5*Δ cells during cell cycle. Exponentially growing wt and *gcn5*Δ cultures were arrested in G1 phase and released. Transcript levels were established by RT-qPCR using *ACT1* as a reference gene.



Supplementary figure 3. HDAC Hda1 is not involved in repression of G1/S transcription in S phase. (2 other biological repeats) (A) Budding indexes. Exponentially growing wt and *hda1*Δ cultures were arrested in G1 phase and released. Number of budding cells was counted under (continued) the light microscope at each time point, 100 cells were counted in total. (B) Transcript levels in wt and *hda1*Δ cells during cell cycle. Exponentially growing wt and *hda1*Δ cultures were arrested in G1 phase and released. Transcript levels were established by RT-qPCR using *ACT1* as a reference gene.

S.3.2. The role of histone methyltransferase Set2 in regulation of G1/S transcription in *S. pombe* upon genotoxic stress.



Supplementary figure 4. MBF transcription is not fully activated and maintained in *set2*Δ cells in response to HU. Fission yeast wt, *chk1*Δ, *set2*Δ and *chk1*Δ*set2*Δ cells were grown to early exponential phase and treated with 12 mM HU for 5 hours. Samples were collected every hour, and transcript levels were quantified by RT-qPCR with primers, specific to *cdc22* and *cdc18* coding regions. Transcript levels are relative to wt time point 0.

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