Published in final edited form as:

Biochim Biophys Acta. 2012 January ; 1819(1): 16–27. doi:10.1016/j.bbagr.2011.09.003.

The mitotic Clb cyclins are required to alleviate HIR-mediated repression of the yeast histone genes at the G1/S transition

Amit Dipak Amin¹, Dessislava K. Dimova², Monica E. Ferreira¹, Nidhi Vishnoi¹, Leandria C. Hancock¹, Mary Ann Osley^{3,*}, and Philippe Prochasson^{1,*}

¹Department of Pathology and Laboratory Medicine, University of Kansas Medical School, Kansas City KS, USA

²Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway NJ, USA

³Department of Molecular Genetics and Microbiology, University of New Mexico, Albuquerque NM, USA

Abstract

The histone genes are an important group of cell cycle regulated genes whose transcription is activated during the G1/S transition and repressed in early G1, late S, and G2/M. The HIR complex, comprised of Hir1, Hir2, Hir3 and Hpc2, regulates three of the four histone gene loci. While relief of repression at the G1/S boundary involves the HIR complex, as well as other cofactors, the mechanism by which this derepression occurs remains unknown. To better understand how transcriptional repression contributes to periodic expression in the cell cycle, we sought to identify the cell cycle signals required to alleviate HIR-mediated repression of the histone genes. By measuring histone gene transcription in strains with various combinations of *clb* mutations, we found that the mitotic Clb1/Clb2 cyclins are required to alleviate Hir-mediated repression during the G1/S transition and that Clb2 physically interacts with the HIR complex. While the HIR complex regulates histone genes transcription in combination with two other histone H3/H4 chaperones, Asf1 and Rtt106, our data demonstrate that the mitotic Clb cyclins are necessary to specifically alleviate the repressive action of the HIR complex itself in order to allow proper expression of the histone genes in late G1/early S phase.

Keywords

Clb cyclins; HIR complex; Asf1; Rtt106; histone genes; cell cycle

1. INTRODUCTION

Progression through the eukaryotic cell cycle is accompanied by periodic fluctuations in the expression of numerous genes [1]. The products of most of these genes are either required for cell cycle progression or the control of the various cell cycle transitions [2-6]. A number of transcription factors that control periodic transcription have been identified in yeast and

© 2011 Elsevier B.V. All rights reserved.

***Corresponding Authors:** Philippe Prochasson and Mary Ann Osley, Mailing address: Department of Pathology and Laboratory Medicine, University of Kansas, Medical School, 3901 Rainbow Blvd, MS 1053, Kansas City KS, USA, mosley@salud.unm.edu (MAO), pprochasson@kumc.edu (PP), **Phone:** 913-588-4739, **Fax:** 913-588-7073 .

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

mammals [2, 3, 7]. In most cases, the function of these factors is controlled by different cyclin-dependent kinases (CDKs, e.g. Cdk1/Cdc28) [1], generally by phosphorylation. In mammals, for example, Cyclin A/Cdk2-mediated phosphorylation stimulates the activity of p53 and B-myb and inhibits the activity of E2F [3]. In budding yeast, a genome-wide analysis revealed that half of the 800 cell cycle regulated genes are responsive to either elevated Cln3-Cdk1 or Clb2-Cdk1 activity, or to both activities [6]. For example, the SBF and MBF transcription factors that regulate genes required for G1 and S phase progression are activated by Cln3/Cdk1, but inhibited by Clb2/Cdk1 kinase activity [6, 8, 9]. Other transcription factors in yeast with roles in periodic transcription, such as Mcm1/SFF and Swi5, are subject to positive and negative regulation, respectively, by Clb2-Cdk1 [6-8, 10-13].

In budding yeast, most transcription factors regulated by CDKs are activators, and transcriptional activation is postulated to be the predominant mechanism controlling periodic transcription. In contrast, transcriptional repression is a major mechanism of gene regulation in the vertebrate cell cycle. For example, a number of genes required for S-phase entry are transcribed at the G1/S transition, and periodic control is achieved via E2F binding sites in their promoters [14-17]. The binding of the retinoblastoma tumor suppressor pRb to E2F alters its activity, and as a result, E2F-regulated genes are not transcribed [14, 15, 17]. Association of pRb with E2F negatively affects transcription in two ways: (i) it prevents E2F from activating transcription at some promoters and (ii) when recruited by E2F, pRb actively represses transcription at others [15, 18]. E2F-RB complexes are prevalent in G0 and early G1 phase of the cell cycle and are disrupted in late G1. This is attributed primarily to the phosphorylation of pRb by the G1 CDK's, cyclin D/cdk4,6 and cyclin E/cdk2 [19, 20]. The disruption of E2F-pRB complexes at the G1/S transition leads to both E2F-dependent trans-activation and relief of pRB-dependent repression. While the regulation of periodic transcription in yeast appears to be regulated mainly via transcriptional activation, it is interesting to note that the recently characterized protein Whi5 acts as an SBF-associated transcriptional repressor [21, 22]. Inactivation of *WHI5* leads to premature activation of G1-specific transcription in G1 phase. Whi5 associates with G1-specific promoters in an SBF-dependent manner and is released from the DNA concurrent with transcriptional activation. Similar to CDK phosphorylation of pRb leading to activation of E2F-responsive genes, Cln3-associated Cdk1 phosphorylation of Whi5 promotes its release from SBF, resulting in derepression of SBF-dependent transcription [1, 21, 22].

The histone genes are an important group of cell-cycle-regulated genes. Transcription of these genes is tightly regulated and occurs during late G1 and S phases of the cell cycle to produce large quantities of new histones during DNA replication in S phase (reviewed in [23, 24]). Aberrant expression of the histone genes outside of S phase can be toxic and lead to alterations in chromatin structure [23]. In budding yeast, both positive and negative regulation contributes to the pattern of histone gene transcription during the cell cycle. In addition to conserved upstream activation elements (UAS) in their promoters, six of the eight histone genes (*HTA1-HTB1*, *HHT1-HHF1* and *HHT2-HHF2*) contain a negative regulatory site, named the NEG or CCR region, which is in close proximity to the UAS elements [25, 26]. The cell cycle regulated UAS elements are required to activate transcription at the G1/S transition [27], while the negative element is required to repress transcription in early G1, late S and G2 and M phases of the cell cycle [25, 26, 28]. In the absence of the negative element, while histone mRNA levels still peak in early S-phase, transcription occurs inappropriately in G1, G2 and M phases [26, 28]. The *HTA1-HTB1* negative site functions in an UAS- and activator-independent manner and confers periodic transcription to a constitutive promoter [26, 28].

Several trans-acting factors that act at the negative site to repress transcription were identified through genetic screens [29, 30]. Among these were the four evolutionarily conserved *HIR1*, *HIR2*, *HIR3* and *HPC2* genes [28, 30-32]. We have previously shown that these four proteins stably assemble to form the HIR corepressor complex, which constitutes a novel histone chaperone complex ([33, 34] and reviewed in [35]). Although it was initially reported that the Hir/Hpc proteins did not possess intrinsic DNA binding activity [32, 36], the HIR complex was later observed to bind to DNA in a non-specific manner [34]. Thus, the HIR complex is postulated to be recruited to the negative site of the histone gene promoters by an as yet uncharacterized sequence-specific DNA binding factor [37]. However, the constitutively expressed Hir1 and Hir2 proteins can bypass the requirement for the NEG site when artificially recruited to DNA and repress transcription at the appropriate time in the cell cycle [28]. Recently, we have shown that the CDI domain located within the N-terminal region of Hpc2 is essential for the recruitment of the HIR complex to the HIR-dependent histone gene loci [38], strongly suggesting that the Hpc2 CDI domain could directly bind to the unknown NEG sequence-specific DNA binding factor. Neither the levels of the HIR proteins nor their presence at the histone gene promoters appear to be cell cycle-regulated [28, 32, 39], which suggests that the repressive activity of the HIR complex is antagonized during the cell cycle to allow the histone genes to be transcribed [28].

The role of the HIR complex in repressing transcription of the histone genes outside of S phase has recently been linked to its association and interplay with two other H3/H4 histone chaperones – Asf1 and Rtt106 [40]. The recruitment of Asf1 to the histone genes is dependent on the HIR complex, while Rtt106 recruitment is dependent on both Asf1 and the HIR complex [40]. This suggests that it is the concerted actions of the HIR complex, Asf1 and Rtt106 that is required to maintain a repressive chromatin structure in order to prevent inappropriate transcription of the histone genes outside of S phase. We have recently shown that Rtt106 also plays an essential role in the recruitment of both SWI/SNF and RSC chromatin remodeling complexes to the HIR-dependent histone genes [41]. While RSC is present at the histone genes outside of S phase concomitant with their repression [42], SWI/SNF is recruited to the histone genes in late G1/early S phase [41] in order to activate their expression [39]. Therefore, Rtt106 is both a co-repressor and a co-activator of histone gene transcription [41].

To gain a better understanding of the mechanisms by which transcriptional repression contributes to periodic expression in the cell cycle, we identified the cell cycle signals required to alleviate HIR-mediated repression of the histone genes at the G1/S transition. We found that the mitotic Clb1/Clb2 cyclins are required for maximal transcription of the histone genes by alleviating Hir-mediated repression at the *HTA1-HTB1* promoter. Furthermore, we showed that Clb2 physically interacts with the HIR complex *in vivo*. We propose that the role of the mitotic Clb cyclins is to specifically alleviate repression exerted by the HIR complex at the histone gene loci, as the cyclins do not appear to relieve repression mediated by Asf1 or Rtt106 at the *HTA1-HTB1* promoter or to regulate the recruitment of the SWI/SNF complex to this promoter.

2. MATERIALS AND METHODS

2.1. Genetic methods and growth conditions

Yeast strains were grown in rich YEPD medium (1% yeast extract, 2% peptone and 2% dextrose) or in minimal synthetic dextrose (SD) medium (0.67% yeast nitrogen base, 2% dextrose and appropriate drop-out mixtures of amino acids and bases) and genetically manipulated by standard techniques. Cells were transformed with plasmids using the DMSO-enhanced whole cell transformation protocol as described by Hill and coworkers [43].

2.2. Yeast strains

The yeast strains used in this study are listed in Table 1. All strains are isogenic or congeneric to W303-1A or BY4741.

2.3. Plasmids

Plasmids for *HIR1-HA*, *HIR2-HA* and *Cib2-HA* fusion genes were constructed by sequential cloning of a PCR amplified product from the native promoter (500 bp upstream of +1 ATG) and coding sequences into YCp:*URA3* (pRS416) or YCp:*LEU2* (pRS415) vectors that contain three HA-tag sequences followed by an *ADHI* terminator sequence. All plasmids were verified by DNA sequencing. Details of constructs used are available upon request.

2.4. Cell cycle synchronization, arrest and release

For cell synchronization, cells were grown in YEPD medium and $\sim 5 \times 10^6$ cells/ml were incubated with α -factor (Sigma) at 5 μ g/ml for *BAR1*⁺ strains or 0.05 μ g/ml for *bar1* Δ strains for 2.5 – 3 hours at 24°C, or until >97% of cells exhibited a characteristic G1 arrest morphology. Mating pheromone was removed by centrifugation, the cells were washed once with dH₂O, resuspended in appropriate prewarmed medium in the presence of 5mM CaCl₂ and 40 μ g/ml pronase, and then incubated at 24°C or 37°C. To assay the degree of synchronization, the percentage of budded cells was scored over time after G1 release by phase-contrast microscopy and the expression of the cell cycle regulated *CLN2* gene was monitored by RT-qPCR. Samples for RNA or protein analysis were removed at regular intervals after release and placed in tubes containing 100 μ l of a 10% sodium azide solution. The cells were centrifuged and the resulting cell pellets were frozen in dry-ice/ethanol bath and stored at –80°C.

2.5. Quantitative S1 nuclease protection assay

Total RNA was extracted from synchronized or exponentially growing cells as previously described [44, 45] and used in S1 nuclease protection assays. S1-nuclease hybridization probes to detect *HTB1*, *HTB2* and *RP51A* mRNAs were prepared by end-labeling 0.7 Kb SacI/HindIII (*HTB1*), 0.7 Kb HindIII/AccI (*HTB2*), or 0.6 Kb AvaII/SalI (*RP51A*) double-stranded DNA fragments with ³²P ATP in the presence of T4 polynucleotide kinase as previously described [45]. Ten nanograms of each probe were hybridized to 20 μ g of total RNA. Forty μ g of total RNA were used when expression levels were predicted to be low. Hybridization was performed at 47°C for at least 12 hours, and S1 nuclease digestion was performed for 1 hour at 15°C at an enzyme concentration of 1000 U/ml as previously described [45]. Products were resolved on 8M urea-4% polyacrilamide gels and exposed to X-ray film for autoradiography. Quantitation was performed on a FUJI Bio-Imaging analyzer using MACBAS software.

2.6. RT-qPCR analysis

Total RNA was extracted from cells using the E.Z.N.A. Total RNA Kit I (Omega, R6834-02) as described in the manufacturer's instructions. cDNA was then synthesized from 400ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems product # 4368814). Quantitative PCR was used to analyze the cDNA using Power SYBR PCR Master Mix (Applied Biosystems, product # 4367659). qPCR was carried out using the MyiQ (BioRad) machine. The amplified DNA was normalized to DNA amplified from *ACT1* or *SCR1* RNA samples, as indicated.

2.7. Chromatin Immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as described previously [41], except that samples taken at the indicated time-points after release at 24°C or 37°C from α -

factor arrest were diluted 1:1 with Milli-Q water at room temperature or 10°C, respectively, to obtain the same temperature in the samples prior to addition of formaldehyde. Samples were cross-linked for 1 h at room temperature. SWI/SNF complex in cross-linked chromatin extract was immunoprecipitated using an antibody specific for the N-terminal end of Swi2/Snf2 by over-night incubation at 4°C, followed by 1 h incubation with Protein A beads. Relative levels of immunoprecipitated *HTA1-HTB1* promoter sequence were quantified by qPCR. An intergenic region of chromosome V (*INTV*) was used as a non-target control. Primer sequences are available upon request.

2.8. Co-immunoprecipitation assays and Western blot analysis

Yeast whole cell extracts were prepared in lysis buffer (40 mM HEPES-KOH pH 7.5, 350 mM NaCl, 10% Glycerol, 0.1% Tween-20, 1 µg/ml pepstatin, 2 µg/ml leupeptin, 0.5 mM DTT and 1 mM PMSF). One milligram of protein extract was immunoprecipitated overnight at 4°C with 15 µl of IgG Sepharose beads (GE Healthcare Life Science, product # 17096901) that binds to the Protein A tag of the TAP tagged subunit. The beads were washed three times with lysis buffer and samples were prepared for Western blot analysis. The immunoprecipitates were resolved on 8 or 10% SDS-PAGE and blotted to a PVDF membrane (Thermo Scientific, product # 88518). Hybridization was carried out using antibodies against HA (Roche product # 12013819001) or rabbit PAP (Peroxidase-Anti-Peroxidase) antibody (Sigma-211 Aldrich, product # P1291) to detect the TAP tag.

3. RESULTS

3.1. Transcription of the genes coding for histones H2A and H2B is dependent upon the CDC4 gene product

All four histone loci are transcriptionally activated in late G1 [46, 47], and the transcription of the *HTA1* gene has been shown to require a functional *CDC4* gene product ([32, 48], and Osley, unpublished observations). The Cdc4, Cdc34, Cdc53 and Skp1 proteins form a complex, SCF^{CDC4}, which is required for the ubiquitin-dependent proteolysis of a number of proteins that regulate cell cycle progression [49-53]. *CDC4* is critical for the progression of yeast cells into S phase, as, in the absence of *CDC4* function, cells perform 'START'-related events but fail to perform subsequent events such as DNA replication, spindle formation, and cytokinesis (for a review, see reference [54]). These observations raised the possibility that both the initiation of DNA replication and histone synthesis may depend on the same regulatory step. We asked whether *CDC4* played a role in transcription of the two *HTA-HTB* loci coding for histones H2A and H2B. We examined the transcription of *HTA2* and *HTB2* genes in a strain carrying a temperature-sensitive (*Ts*⁻) allele of *CDC4* and compared it to *HTA1* and *HTB1* transcription in the same *cdc4^{ts}* mutant. Although the *HTA2* and *HTB2* genes do not contain a negative element in their promoter and are not regulated by the HIR complex [24, 55], both are activated at the same time in late G1 as the *HTA1* and *HTB1* genes (Fig. 1A). As shown in Fig. 1B, after release of a *cdc4^{ts}* mutant from α -factor arrest into nonpermissive (37°C) conditions, both *HTA2* and *HTB2* mRNA levels were greatly reduced and showed delayed accumulation when compared to permissive conditions (24°C), in which a normal periodic accumulation of transcripts occurred in S phase. As previously reported [32], *HTA1* and *HTB1* mRNA levels were reduced by at least 3 fold in S phase when *cdc4^{ts}* cells were released at the restrictive temperature (Fig. 1B). However, a low level of *HTA-HTB* transcription persisted in cells blocked at the *CDC4*-dependent step (Fig. 1B, 37°C). This is consistent with the fact that at the non-permissive temperature, *cdc4^{ts}* cells arrest in late G1/early S phase as monitored by the expression of *CLN2*, which is activated but then remains highly transcribed (data not shown). In contrast, in a wild-type strain, there was no difference in the levels of *HTA1*, *HTB1* and *HTB2* mRNAs between 24°C and 37°C, except for a slight reduction in the levels of *HTA2* mRNAs. However, the

timing of the first cell cycle in the wild-type strain appears to be slightly earlier at 37°C than at 24°C (Fig. 1A).

These results showed that activation of both the *HTA1-HTB1* and *HTA2-HTB2* loci is dependent on events regulated by *CDC4*. However, the low level of transcripts and delayed expression of the histone genes seen in the *cdc4^{ts}* mutant at the nonpermissive temperature suggested that there is also a *CDC4*-independent step in the regulation of these loci.

3.2. Transcription of the HTB genes is not dependent on the S-phase Clb5/Clb6 cyclins

A major role for the SCF^{CDC4} complex is the ubiquitination of p40^{SIC1}, which targets it for degradation [49]. p40^{SIC1} is a potent Cdk1 inhibitor, and its destruction at the G1/S transition leads to the activation of all six Clb/Cdk1 complexes (reviewed in [56-58]). To determine whether the *CDC4*-dependence of *HTA-HTB* transcription reflected a requirement for Clb kinases, we monitored the expression of the two loci in synchronized cells of different *clb* mutants. The coupling of histone synthesis to DNA replication suggested that the S-phase cyclins *CLB5* and *CLB6*, which are involved in the initiation of DNA replication [59-61], might also regulate histone gene transcription. We examined *HTB1* and *HTB2* mRNA levels during the cell cycle in a strain carrying deletions of both cyclin genes. Such a strain is viable due to the redundancy among Clb cyclins, but shows a delayed entry into S-phase [52, 61, 62]. As can be seen in Figure 2A and 2B, there was no difference between the wild type and *clb5Δclb6Δ* strains in either the timing or levels of *HTB1* and *HTB2* mRNAs. The only difference was a delay of 15-30 min in *HTB* expression during the second cell cycle in the mutant strain, which corresponded to its delayed entry into S-phase (Figure 2C and 2D) [61]. Thus, these results show that the Clb5/Clb6 cyclins and their associated kinase activities are not directly involved in the transcriptional control of either *HTA-HTB* locus.

3.3. Transcription of both HTA1-HTB1 and HTA2-HTB2 loci is dependent on the mitotic Clb1/Clb2 cyclins

New histone synthesis occurs at the G1/S boundary and is required for the G2/M transition [23, 24]. Cells unable to synthesize histones H2B and H4 arrest in G2 presumably due to the abnormal chromatin structure of their chromosomes, which are duplicated in the absence of histone synthesis [63, 64]. Interestingly, a strain carrying mutations in all four mitotic cyclin genes, *CLB1*, *CLB2*, *CLB3*, and *CLB4*, arrests in G2 with duplicated but unsegregated chromosomes [8, 65]. We therefore asked if the *CDC4*-dependence of histone gene transcription reflected a specific requirement for the mitotic Clb cyclins.

We monitored histone gene expression during the cell cycle in a strain deleted of *CLB1*, *CLB3*, and *CLB4* genes and carrying a temperature sensitive allele of *CLB2* [8] after release of cells from α -factor arrest to permissive (24°C) and nonpermissive temperatures (37°C) (Fig. 3). The mRNA levels produced from all four *HTA* and *HTB* genes were reduced between 2.7- to 5-fold in S phase when cells were shifted to 37°C compared to 24°C, but were not completely eliminated (Fig. 3 and data not shown). We also observed a similar reduction in the expression of the *HHT1*, *HHF1* and *HHT2* genes when cells were shifted to 37°C (data not shown). As discussed above, the synthesis of new histones is imperative for passage through the G2/M transition [23, 24]. Therefore, as expected, cells placed at the nonpermissive temperature (37°C) suffer from G2/M arrest, which is apparent by the absence of peak expression during the second cell cycle. The expression of *CLN2*, which was used as a control for cell cycle progression, showed that cells were able to enter S phase at 37°C, but arrested shortly thereafter as indicated by the constitutive levels of *CLN2* RNA compared to permissive conditions (24°C) (Fig. 3D). These results indicate that the mitotic Clb cyclins are required for the full transcriptional activation of both the *HTA1-HTB1* and *HTA2-HTB2* loci.

Clb1/Clb2 and Clb3/Clb4 constitute two distinct pairs of mitotic cyclins, each of which is presumed to confer specialized functions upon association with the cyclin-dependent kinase Cdk1 [65-67]. However, due to the redundancy among the mitotic cyclins [52, 61, 62], the results shown in Fig. 3 could not distinguish between a role for one or the other of the two mitotic cyclin pairs. We therefore measured expression of both *HTA-HTB* loci during the cell cycle in a strain carrying deletions of the *CLB3* and *CLB4* genes. No significant differences in either *HTA1*, *HTB1* or *HTA2* expression were observed between the *clb3Δclb4Δ* mutant and a wild type (WT) strain (Fig. 4A), suggesting that Clb3/Clb4 are not responsible for histone gene expression.

We then monitored expression of the histone genes during the cell cycle in a strain deleted for the *CLB1* gene and carrying a temperature sensitive allele of *CLB2*, but containing functional *CLB3/CLB4* cyclin genes (Fig. 4B). There was a 2- to 3-fold reduction in the levels of *HTA1*, *HTB1* and *HTA2* RNAs in the *clb1Δclb2^{ts}* mutant at the nonpermissive temperature compared to the permissive temperature (Fig. 4B). However, the reduction in the *HTA* and *HTB* RNA levels in the *clb1Δclb2^{ts}* mutant strain was not totally equivalent to the reduction observed in the *clb1Δ clb2^{ts} clb3Δ clb4Δ* mutant strain (Fig. 3). We propose that this could be due to the redundancy among the mitotic cyclins [52, 61, 62], and thus Clb3/Clb4 may be able to partially compensate for the loss of Clb1 and Clb2 at the nonpermissive temperature in the *clb1Δclb2^{ts}* mutant strain. Nevertheless, because the expression of the histone genes was not affected in the *clb3Δ clb4Δ* mutant, these results strongly suggest that the Clb1/Clb2 cyclins play a major role in the control of transcription of both the *HTA1-HTB1* and *HTA2-HTB2* loci. Because the reduction in mRNA production from the *HTA1-HTB1* and *HTA2-HTB2* loci in the absence of functional mitotic Clb cyclins resembled the effect observed in the *cdc4^{ts}* mutant, we conclude that both the Cdc4- and the Clb1/Clb2-dependence of their transcription reflects a requirement for an active mitotic Clb1/Clb2-Cdk1 kinase.

3.4. HIR-mediated repression of *HTA1-HTB1* transcription confers dependence upon the mitotic Clb cyclins

Unpublished observations by Dimova and Osley showed that the *HTA1-HTB1* negative element confers mitotic Clb cyclin-dependence on expression of a heterologous promoter [68]. We therefore asked whether the HIR complex, which negatively regulates *HTA1-HTB1* transcription through the negative site, is responsible for the Clb1/Clb2-dependence of this locus. We disrupted the *HIR1* gene in a *clb1Δclb2^{ts}clb3Δclb4Δ* strain and examined *HTA1* and *HTB1* mRNA levels in synchronized cultures at both 24°C and 37°C (Fig. 5A and B). As previously shown [26, 29](and data not shown), deletion of *HIR1*, similar to removal of the negative site, resulted in a higher level of mRNA accumulation in G1, G2, and M phases, although peak levels were still achieved in early S-phase under permissive conditions (Fig. 5A and B, 24°C). Strikingly, under nonpermissive conditions (37°C) (Fig. 5A and B, 37°C), *HTA1* and *HTB1* mRNA accumulated to the same levels as under permissive conditions (24°C) in the first cell cycle in a *clb1Δclb2^{ts}clb3Δclb4Δhir1Δ* strain. However, due to subsequent G2/M arrest at the nonpermissive temperature, as shown by continuous *CLN2* expression (Fig. 5D), no *HTA1* or *HTB1* RNAs accumulated during the second cell cycle at 37°C. Contrary to the effect on *HTA1-HTB1* expression, *HIR1* deletion did not restore *HTA2* mRNA levels at the nonpermissive temperature (Fig. 5C). This was expected, as transcription of the *HTA2-HTB2* locus is HIR-independent [29, 30]. These results indicate that deletion of *HIR1*, and hence disruption of the HIR complex, can completely bypass the requirement for the mitotic Clb cyclins to restore the expression of *HTA1-HTB1* under nonpermissive conditions. Thus, these results indicate that Clb1/Clb2 is required for the relief of HIR-mediated repression of the *HTA1-HTB1* locus to allow maximal transcription of these genes to occur at the proper time in the cell cycle.

Because of the functional interactions between the HIR complex and the mitotic Clb cyclins, we asked whether Clb2 and the HIR complex might be physically associated in yeast using a co-immunoprecipitation assay with epitope tagged Clb2, Hir1 and Hir2 proteins. As shown in Fig. 6A, immunoprecipitated Hir1-TAP and Hir2-TAP specifically associated with Clb2-HA (Fig. 6A, lane 3 and 5 of the IP panel), while no Clb2-HA was detected in an untagged strain (Fig. 6A, lane 1 IP panel). We performed the reciprocal immunoprecipitation, and showed that Clb2-TAP co-precipitated Hir1-HA and Hir2-HA (Fig. 6B and C, IP panel, lanes 3). These data demonstrate that the HIR complex specifically associates with the mitotic Clb2 cyclin *in vivo*, and suggest that the association might reflect a subsequent modification of the complex by the Clb1/Clb2-Cdk1 kinase. Clb1/Clb2 dependent relief of HIR-mediated repression at the *HTA1-HTB1* locus might occur, for example, through the targeting of the HIR co-repressor complex for inactivation by the Clb1/Clb2-dependent Cdk1 kinase or through the recruitment of the SWI/SNF chromatin remodeling complex which is required for activation of the histone genes [39].

3.5. The mitotic Clb cyclins activate *HTA1-HTB1* transcription by specifically alleviating repression mediated by the HIR complex

We have recently shown that the SWI/SNF complex is recruited in late G1 to the *HTA1-HTB1* promoter by Rtt106 [41] and is required for the activation of histone gene transcription at the G1/S phase boundary [39]. We therefore asked whether the Clb1/Clb2 cyclins could alleviate HIR-mediated repression by controlling the recruitment of SWI/SNF to the *HTA1-HTB1* locus. We monitored the presence of the SWI/SNF complex at the *HTA1-HTB1* promoter by chromatin immunoprecipitation (ChIP) using an antibody against the N-terminal domain of the Swi2/Snf2 catalytic subunit in both a wild-type and *clb1Δclb2^{ts}clb3Δclb4Δ* strain released from G1 arrest at both the permissive (24°C) and nonpermissive temperature (37°C). As seen in Figure 7, there were no differences in the recruitment of SWI/SNF to the *HTA1-HTB1* promoter in the *clb1Δclb2^{ts}clb3Δclb4Δ* strain compared to a wild-type strain at either temperature. However, we observed a slight delay and decrease in SWI/SNF recruitment to this promoter at 37°C in both strains compared to 24°C (Fig. 7). Thus, these results indicate that the decrease in expression of the *HTA1-HTB1* genes at the nonpermissive (37°C) temperature in the *clb1Δclb2^{ts}clb3Δclb4Δ* strain (Fig. 3) is not due to an alteration in recruitment of the SWI/SNF complex. These data suggest that the function of the mitotic Clb cyclins could be to directly turn off HIR-mediated repression in late G1 to allow expression of *HTA1-HTB1* locus.

As discussed in the Introduction, the HIR complex associates with the H3/H4 histone chaperones Asf1 and Rtt106 [40], both of which play a role in the transcriptional repression of the HIR-dependent histone genes [40, 69]. Fillingham and colleagues [40] reported that the presence of Rtt106 at the *HTA1-HTB1* promoter was dependent on the HIR complex and Asf1, while the presence of Asf1 was only dependent on the HIR complex, and that all of these factors were present at the HIR-dependent histone genes throughout the cell cycle. Interestingly, deletion of *ASF1*, *RTT106* or genes encoding HIR complex subunits results in nucleosome depletion at the *HTA1-HTB1* promoter [40], suggesting that the repressive chromatin structure established by Asf1/HIR/Rtt106 represents the main mechanism to repress the histone genes. To better understand the mechanism by which the mitotic Clb cyclins alleviate HIR-dependent repression of the *HTA1-HTB1* locus, we sought to determine which of these three factors is the main target of the mitotic Clb cyclins. We first asked whether deletion of *RTT106* or *ASF1* in the *clb1Δclb2^{ts}clb3Δclb4Δ* strain could bypass the requirement for the mitotic Clb cyclins at the nonpermissive temperature as observed when *HIR1* was deleted (Fig 5). As shown in Figure 8, deletion of either *RTT106* or *ASF1* in the quadruple *clb* strain did not significantly restore the peak of expression of *HTA1* as observed upon deletion of *HIR1* in the *clb1Δclb2^{ts}clb3Δclb4Δ* strain at the nonpermissive

temperature (Fig. 8 compare panels B and C to D). However, a slight but persistent accumulation of *HTA1* RNA was observed after S phase when the *asf1Δ* or *rtt106Δ* cells entered G2/M phase at the nonpermissive temperature (Fig. 8B and C, 60, 75 and 90min) when compared to the *clb1Δclb2^{ts}clb3Δclb4Δ* strain (Fig. 8 panel A). Similar data were obtained for *HTB1* RNA levels (data not shown). Because the presence of the HIR complex at the *HTA1-HTB1* promoter has been shown to be independent of *ASF1* and *RTT106* [40], we conclude that the reduced *HTA1* expression in lateG1/early S phase at the nonpermissive temperature in the *clb1Δclb2^{ts}clb3Δclb4Δ* strain primarily reflects the repressive action exerted by the HIR complex alone. Taken together, these results strongly suggest that the role of the mitotic Clb cyclins, and in particular Clb1/Clb2, is to alleviate the repressive effects of the HIR complex itself at the *HTA1-HTB1* locus in late G1/early S phase to allow proper histone gene expression. Thus, our results strongly suggest that in addition to the repressive activity of Asf1 and Rtt106, likely through their roles in nucleosome assembly, the HIR complex represses histone gene transcription outside of S phase by an independent mechanism that is alleviated by the mitotic Clb cyclins in late G1 to allow the full expression of HIR-dependent histone genes (Fig. 9).

4. DISCUSSION

The yeast histone genes are subject to both positive and negative regulation to achieve maximal transcription in S phase of the cell cycle. Conserved cell cycle regulated UAS elements found in all of the histone gene promoters are required to activate transcription at the G1/S transition through the binding of activators that include SBF, MBF, Spt10 and Spt21 [24, 26, 27, 70-77]. Negative regulation is achieved by the constitutive binding of the evolutionarily conserved HIR complex to a negative regulatory element in the promoters of three of the four histone gene loci (reviewed in [24, 35]). In addition to the action of activators, HIR-dependent repression of these promoters must be relieved at the G1/S boundary to achieve maximal levels of histone gene-specific RNAs in S phase. In this study, we have shown that the transcriptional activation of the four genes encoding histones H2A and H2B (the *HTA1-HTB1* and *HTA2-HTB2* loci) is dependent upon completion of late G1 events controlled by the *CDC4* gene product. Furthermore, our results indicate that in late G1/early S phase, full transcriptional activation of the HIR-dependent histone genes *HTA1*, and *HTB1*, as well as the HIR-independent histone genes *HTA2* and *HTB2*, requires the mitotic Clb1/Clb2 cyclins, which is likely to account for the Cdc4-dependence of histone gene transcription. Additionally, we found that the two other Hir-dependent histone gene loci, *HHT1-HHF1* and *HHT2-HHF2*, also require the mitotic Clb cyclins as well as Cdc4 to become fully expressed in G1/S phase (data not shown). A strain carrying mutations in all four mitotic cyclin genes, *CLB1*, *CLB2*, *CLB3*, and *CLB4*, arrests in G2 with duplicated but unsegregated chromosomes [8, 65]. G2 arrest is also seen in cells that are unable to synthesize histones H2B and H4, and this has been attributed to an abnormal chromatin structure of the duplicated chromosomes [63, 64]. Thus, our data showing that the mitotic Clb cyclins are necessary for the correct expression of all four histone gene loci in late G1/early S phase may partially explain the G2 arrest phenotype observed in the absence of functional mitotic Clb cyclins in a *clb1Δclb2^{ts}clb3Δclb4Δ* strain [8, 65].

The observation that the transcription of the histone genes is dependent upon the mitotic Clb cyclins was at first surprising given that histone gene transcription is coupled to S phase and the mitotic Clb cyclin-associated Cdk1 kinase does not become fully active until G2 phase [52, 66, 78]. However, at the G1/S transition when histone gene transcription is activated [47], the Clb1/Clb2-associated Cdk1 kinase has been shown to be active, although kinase levels are significantly lower than in G2 phase [79, 80]. Interestingly, we found that *in vivo* Clb2 associates with the HIR complex (Fig. 6). Moreover, our preliminary data show that the Clb2-Cdk1 complex can phosphorylate all four subunits of the HIR complex *in vitro*

(data not shown). Additionally, a global study to identify substrates of Clb2-Cdk1 reported that Hpc2 was among the 200 proteins found in this screen [81], thus further corroborating our findings. Analysis of the protein sequences of the four subunits of the HIR complex reveals that they all contain at least one RXL motif, a feature found in Cdk1 kinase substrates that mediates an interaction with the hydrophobic patch found on cyclins [82] (data not shown), as well as multiple putative Cdk1 phosphorylation sites [83]. In addition, the human homolog of Hir1 and Hir2, HIRA, was reported to be phosphorylated in S phase by cyclin A-cdk2 [84]. Consistent with the notion that human HIRA is itself a cell cycle regulator, ectopic expression of HIRA causes cell cycle arrest in S phase [84]. Confirmation of the yeast HIR complex as a direct target of the Clb-Cdk1 kinase and identification of the HIR complex residues phosphorylated by this kinase will ultimately shed light on the role played by the Clb cyclins in the function of the HIR complex to control transcription of the histone genes. Interestingly, we also found that the expression of the HIR-independent histone genes, *HTA2* and *HTB2*, is also dependent on the Clb1/Clb2 cyclins. However, no repressors for *HTA2-HTB2* locus are currently known, so this suggests that the mitotic Clb cyclins could be required either to alleviate the repression of an unknown repressor or to directly activate the transcription of this gene pair. Thus, additional work is needed to understand the specific role of the mitotic Clb cyclins in the transcriptional regulation of the *HTA2-HTB2* locus.

We have shown here that inactivation of the negative regulatory system by deletion of the *HIR1* gene relieved the dependence of *HTA1-HTB1* transcription on the mitotic Clb1/Cb2 cyclins (Fig. 5). The HIR regulatory system includes not only the HIR complex proteins but several additional factors that associate with the HIR complex at histone gene promoters to regulate periodic transcription. These include the histone chaperones Asf1 and Rtt106, which establish a repressive chromatin structure at the histone gene loci, and the chromatin remodelers RSC and SWI/SNF (reviewed in [35]). While the former remodeler represses histone gene transcription in G2/M and G1, the latter acts as a transcriptional co-activator at the G1/S phase boundary. We showed that the recruitment of the SWI/SNF complex to a HIR-dependent promoter is not affected by the inactivation of the Clb cyclins (Fig. 7). More importantly, unlike the deletion of *HIR1*, deletion of *ASF1* or *RTT106* in the absence of functional Clb cyclins only partially relieved the defect in histone gene transcription, leading to an intermediate phenotype (Fig. 8). These results strongly suggest that in addition to the repressive activity of Asf1 and Rtt106, likely through their roles in nucleosome assembly, the HIR complex represses histone gene transcription outside of S phase by an independent mechanism that is alleviated by the mitotic Clb cyclins in late G1 to allow the full expression of HIR-dependent histone genes (Fig. 9). These results additionally suggest that the HIR complex itself, and not its associated factors, is the primary target of the mitotic Clb cyclins. Thus, the putative regulation of the HIR complex by Clb cyclin-dependent phosphorylation is unlikely to play a role in the association of these factors with histone gene promoters. Instead, the activity of some of these factors could be affected by HIR complex modification. We have previously shown that the HIR complex, when bound to a nucleosome template *in vitro* inhibits SWI/SNF chromatin remodeling activity [34]. This inhibition could result from: (i) a direct inhibition of the SWI/SNF catalytic activity by the HIR complex; or (ii) the non-specific DNA binding of the HIR complex to nucleosomes, which generates a distinct protein/DNA complex that is resistant to remodeling by SWI/SNF. Thus, a potential mechanism for the mitotic Clb1/Clb2 cyclins and the putative Clb2-Cdk1-dependent phosphorylation of the HIR complex could be to alleviate this HIR-dependent inhibition of SWI/SNF chromatin remodeling activity. Future studies exploring these different hypotheses will shed light on understanding the specific mechanisms by which the mitotic Clb cyclins control histone gene transcription during the cell cycle, which is essential for cell viability, as well as further our understanding of the mechanism by which the HIR complex represses gene transcription.

Acknowledgments

The authors thank Amanda Raff for her excellent technical support for some of the experiments. This work was supported by grants NIH GM40118 to M.A.O. and NIH P20 RR016475 from the Kansas INBRE Program of the National Center for Research Resources to P.P. This work was also supported by a Leukemia and Lymphoma Society Special Fellowship to P.P., the Kansas Bioscience Authority (KBA), and the Kansas Medical Center Research Institute (KMCR).

References

- [1]. Wittenberg C, Reed SI. Cell cycle-dependent transcription in yeast: promoters, transcription factors, and transcriptomes. *Oncogene*. 2005; 24:2746–2755. [PubMed: 15838511]
- [2]. Bahler J. Cell-cycle control of gene expression in budding and fission yeast. *Annu Rev Genet*. 2005; 39:69–94. [PubMed: 16285853]
- [3]. Dynlacht BD. Regulation of transcription by proteins that control the cell cycle. *Nature*. 1997; 389:149–152. [PubMed: 9296491]
- [4]. Koch C, Nasmyth K. Cell cycle regulated transcription in yeast. *Curr Opin Cell Biol*. 1994; 6:451–459. [PubMed: 7917338]
- [5]. Nasmyth K. At the heart of the budding yeast cell cycle. *Trends Genet*. 1996; 12:405–412. [PubMed: 8909137]
- [6]. Spellman PT, Sherlock G, Zhang MQ, Iyer VR, Anders K, Eisen MB, Brown PO, Botstein D, Futcher B. Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol Biol Cell*. 1998; 9:3273–3297. [PubMed: 9843569]
- [7]. Andrews B, Measday V. The cyclin family of budding yeast: abundant use of a good idea. *Trends Genet*. 1998; 14:66–72. [PubMed: 9520600]
- [8]. Amon A, Tyers M, Futcher B, Nasmyth K. Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. *Cell*. 1993; 74:993–1007. [PubMed: 8402888]
- [9]. Koch C, Schleiffer A, Ammerer G, Nasmyth K. Switching transcription on and off during the yeast cell cycle: Cln/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at start, whereas Clb/Cdc28 kinases displace it from the promoter in G2. *Genes Dev*. 1996; 10:129–141. [PubMed: 8566747]
- [10]. Althoefer H, Schleiffer A, Wassmann K, Nordheim A, Ammerer G. Mcm1 is required to coordinate G2-specific transcription in *Saccharomyces cerevisiae*. *Mol Cell Biol*. 1995; 15:5917–5928. [PubMed: 7565744]
- [11]. Maher M, Cong F, Kindelberger D, Nasmyth K, Dalton S. Cell cycle-regulated transcription of the CLB2 gene is dependent on Mcm1 and a ternary complex factor. *Mol Cell Biol*. 1995; 15:3129–3137. [PubMed: 7760809]
- [12]. Moll T, Tebb G, Surana U, Robitsch H, Nasmyth K. The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the *S. cerevisiae* transcription factor SWI5. *Cell*. 1991; 66:743–758. [PubMed: 1652372]
- [13]. Sanders SL, Herskowitz I. The BUD4 protein of yeast, required for axial budding, is localized to the mother/BUD neck in a cell cycle-dependent manner. *J Cell Biol*. 1996; 134:413–427. [PubMed: 8707826]
- [14]. Chan HM, Shikama N, La Thangue NB. Control of gene expression and the cell cycle. *Essays Biochem*. 2001; 37:87–96. [PubMed: 11758459]
- [15]. Dyson N. The regulation of E2F by pRB-family proteins. *Genes Dev*. 1998; 12:2245–2262. [PubMed: 9694791]
- [16]. Frolov MV, Dyson NJ. Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. *J Cell Sci*. 2004; 117:2173–2181. [PubMed: 15126619]
- [17]. Helin K. Regulation of cell proliferation by the E2F transcription factors. *Curr Opin Genet Dev*. 1998; 8:28–35. [PubMed: 9529602]
- [18]. Zwicker J, Muller R. Cell-cycle regulation of gene expression by transcriptional repression. *Trends Genet*. 1997; 13:3–6. [PubMed: 9009839]

- [19]. Adams PD, Li X, Sellers WR, Baker KB, Leng X, Harper JW, Taya Y, Kaelin WG Jr. Retinoblastoma protein contains a C-terminal motif that targets it for phosphorylation by cyclin-cdk complexes. *Mol Cell Biol.* 1999; 19:1068–1080. [PubMed: 9891042]
- [20]. Kato J, Matsushime H, Hiebert SW, Ewen ME, Sherr CJ. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev.* 1993; 7:331–342. [PubMed: 8449399]
- [21]. Costanzo M, Nishikawa JL, Tang X, Millman JS, Schub O, Breikreuz K, Dewar D, Rupes I, Andrews B, Tyers M. CDK activity antagonizes Whi5, an inhibitor of G1/S transcription in yeast. *Cell.* 2004; 117:899–913. [PubMed: 15210111]
- [22]. de Bruin RA, McDonald WH, Kalashnikova TI, Yates J 3rd, Wittenberg C. Cln3 activates G1-specific transcription via phosphorylation of the SBF bound repressor Whi5. *Cell.* 2004; 117:887–898. [PubMed: 15210110]
- [23]. Gunjan A, Paik J, Verreault A. Regulation of histone synthesis and nucleosome assembly. *Biochimie.* 2005; 87:625–635. [PubMed: 15989979]
- [24]. Osley MA. The regulation of histone synthesis in the cell cycle. *Annu Rev Biochem.* 1991; 60:827–861. [PubMed: 1883210]
- [25]. Freeman KB, Karns LR, Lutz KA, Smith MM. Histone H3 transcription in *Saccharomyces cerevisiae* is controlled by multiple cell cycle activation sites and a constitutive negative regulatory element. *Mol Cell Biol.* 1992; 12:5455–5463. [PubMed: 1448078]
- [26]. Osley MA, Gould J, Kim S, Kane MY, Hereford L. Identification of sequences in a yeast histone promoter involved in periodic transcription. *Cell.* 1986; 45:537–544. [PubMed: 3518945]
- [27]. Eriksson PR, Ganguli D, Clark DJ. Spt10 and Swi4 control the timing of histone H2A/H2B gene activation in budding yeast. *Mol Cell Biol.* 2011; 31:557–572. [PubMed: 21115727]
- [28]. Spector MS, Raff A, DeSilva H, Lee K, Osley MA. Hir1p and Hir2p function as transcriptional corepressors to regulate histone gene transcription in the *Saccharomyces cerevisiae* cell cycle. *Mol Cell Biol.* 1997; 17:545–552. [PubMed: 9001207]
- [29]. Osley MA, Lycan D. Trans-acting regulatory mutations that alter transcription of *Saccharomyces cerevisiae* histone genes. *Mol Cell Biol.* 1987; 7:4204–4210. [PubMed: 3125420]
- [30]. Xu H, Kim UJ, Schuster T, Grunstein M. Identification of a new set of cell cycle-regulatory genes that regulate S-phase transcription of histone genes in *Saccharomyces cerevisiae*. *Mol Cell Biol.* 1992; 12:5249–5259. [PubMed: 1406694]
- [31]. DeSilva H, Lee K, Osley MA. Functional dissection of yeast Hir1p, a WD repeat-containing transcriptional corepressor. *Genetics.* 1998; 148:657–667. [PubMed: 9504914]
- [32]. Sherwood PW, Tsang SV, Osley MA. Characterization of HIR1 and HIR2, two genes required for regulation of histone gene transcription in *Saccharomyces cerevisiae*. *Mol Cell Biol.* 1993; 13:28–38. [PubMed: 8417331]
- [33]. Green EM, Antczak AJ, Bailey AO, Franco AA, Wu KJ, Yates JR 3rd, Kaufman PD. Replication-independent histone deposition by the HIR complex and Asf1. *Curr Biol.* 2005; 15:2044–2049. [PubMed: 16303565]
- [34]. Prochasson P, Florens L, Swanson SK, Washburn MP, Workman JL. The HIR corepressor complex binds to nucleosomes generating a distinct protein/DNA complex resistant to remodeling by SWI/SNF. *Genes Dev.* 2005; 19:2534–2539. [PubMed: 16264190]
- [35]. Amin AD, Vishnoi N, Prochasson P. A global requirement for the HIR complex in the assembly of chromatin. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms.* 2011 doi: 10.1016/j.bbagr.2011.07.008.
- [36]. Lamour V, Lecluse Y, Desmaze C, Spector M, Bodescot M, Aurias A, Osley MA, Lipinski M. A human homolog of the *S. cerevisiae* HIR1 and HIR2 transcriptional repressors cloned from the DiGeorge syndrome critical region. *Hum Mol Genet.* 1995; 4:791–799. [PubMed: 7633437]
- [37]. Moran, L.; Cornell University. Ph.D. Thesis. New York: 1994. Factors involved in temporal and autogenous control of histone synthesis in *Saccharomyces cerevisiae*.
- [38]. Vishnoi N, Flaherty K, Hancock LC, Ferreira ME, Amin AD, Prochasson P. Separation-of-function mutation in HPC2, a member of the HIR complex in *S. cerevisiae*, results in derepression of the histone genes but does not confer cryptic TATA phenotypes. *Biochimica et*

Biophysica Acta (BBA) - Gene Regulatory Mechanisms. 2011 doi:10.1016/j.bbagrm.2011.07.004.

- [39]. Dimova D, Nackerdien Z, Furgeson S, Eguchi S, Osley MA. A role for transcriptional repressors in targeting the yeast Swi/Snf complex. *Mol Cell*. 1999; 4:75–83. [PubMed: 10445029]
- [40]. Fillingham J, Kainth P, Lambert JP, van Bakel H, Tsui K, Pena-Castillo L, Nislow C, Figeys D, Hughes TR, Greenblatt J, Andrews BJ. Two-color cell array screen reveals interdependent roles for histone chaperones and a chromatin boundary regulator in histone gene repression. *Mol Cell*. 2009; 35:340–351. [PubMed: 19683497]
- [41]. Ferreira ME, Flaherty K, Prochasson P. The *Saccharomyces cerevisiae* Histone Chaperone Rtt106 Mediates the Cell Cycle Recruitment of SWI/SNF and RSC to the HIR-Dependent Histone Genes. *PLoS One*. 2011; 6:e21113. [PubMed: 21698254]
- [42]. Ng HH, Robert F, Young RA, Struhl K. Genome-wide location and regulated recruitment of the RSC nucleosome-remodeling complex. *Genes Dev*. 2002; 16:806–819. [PubMed: 11937489]
- [43]. Hill J, Donald KA, Griffiths DE. DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Res*. 1991; 19:5791. [PubMed: 1945859]
- [44]. Hereford LM, Osley MA, Ludwig TR 2nd, McLaughlin CS. Cell-cycle regulation of yeast histone mRNA. *Cell*. 1981; 24:367–375. [PubMed: 7016339]
- [45]. Moran L, Norris D, Osley MA. A yeast H2A-H2B promoter can be regulated by changes in histone gene copy number. *Genes Dev*. 1990; 4:752–763. [PubMed: 2199321]
- [46]. Cross SL, Smith MM. Comparison of the structure and cell cycle expression of mRNAs encoded by two histone H3-H4 loci in *Saccharomyces cerevisiae*. *Mol Cell Biol*. 1988; 8:945–954. [PubMed: 3280973]
- [47]. Hereford L, Bromley S, Osley MA. Periodic transcription of yeast histone genes. *Cell*. 1982; 30:305–310. [PubMed: 6751560]
- [48]. White JH, Green SR, Barker DG, Dumas LB, Johnston LH. The CDC8 transcript is cell cycle regulated in yeast and is expressed coordinately with CDC9 and CDC21 at a point preceding histone transcription. *Exp Cell Res*. 1987; 171:223–231. [PubMed: 3305044]
- [49]. Feldman RM, Correll CC, Kaplan KB, Deshaies RJ. A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell*. 1997; 91:221–230. [PubMed: 9346239]
- [50]. Mathias N, Johnson SL, Winey M, Adams AE, Goetsch L, Pringle JR, Byers B, Goebel MG. Cdc53p acts in concert with Cdc4p and Cdc34p to control the G1-to-S-phase transition and identifies a conserved family of proteins. *Mol Cell Biol*. 1996; 16:6634–6643. [PubMed: 8943317]
- [51]. Schneider BL, Yang QH, Futcher AB. Linkage of replication to start by the Cdk inhibitor Sic1. *Science*. 1996; 272:560–562. [PubMed: 8614808]
- [52]. Schwob E, Bohm T, Mendenhall MD, Nasmyth K. The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae*. *Cell*. 1994; 79:233–244. [PubMed: 7954792]
- [53]. Skowyra D, Craig KL, Tyers M, Elledge SJ, Harper JW. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell*. 1997; 91:209–219. [PubMed: 9346238]
- [54]. DeSalle LM, Pagano M. Regulation of the G1 to S transition by the ubiquitin pathway. *FEBS Lett*. 2001; 490:179–189. [PubMed: 11223033]
- [55]. Breeden L. Cell cycle-regulated promoters in budding yeast. *Trends Genet*. 1988; 4:249–253. [PubMed: 3070871]
- [56]. Patton EE, Willems AR, Tyers M. Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. *Trends Genet*. 1998; 14:236–243. [PubMed: 9635407]
- [57]. Peters JM. SCF and APC: the Yin and Yang of cell cycle regulated proteolysis. *Curr Opin Cell Biol*. 1998; 10:759–768. [PubMed: 9914180]
- [58]. Willems AR, Goh T, Taylor L, Chernushevich I, Shevchenko A, Tyers M. SCF ubiquitin protein ligases and phosphorylation-dependent proteolysis. *Philos Trans R Soc Lond B Biol Sci*. 1999; 354:1533–1550. [PubMed: 10582239]
- [59]. Epstein CB, Cross FR. CLB5: a novel B cyclin from budding yeast with a role in S phase. *Genes Dev*. 1992; 6:1695–1706. [PubMed: 1387626]

- [60]. Kuhne C, Linder P. A new pair of B-type cyclins from *Saccharomyces cerevisiae* that function early in the cell cycle. *EMBO J.* 1993; 12:3437–3447. [PubMed: 8253070]
- [61]. Schwob E, Nasmyth K. CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*. *Genes Dev.* 1993; 7:1160–1175. [PubMed: 8319908]
- [62]. Amon A, Irniger S, Nasmyth K. Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. *Cell.* 1994; 77:1037–1050. [PubMed: 8020094]
- [63]. Han M, Grunstein M. Nucleosome loss activates yeast downstream promoters in vivo. *Cell.* 1988; 55:1137–1145. [PubMed: 2849508]
- [64]. Kim UJ, Han M, Kayne P, Grunstein M. Effects of histone H4 depletion on the cell cycle and transcription of *Saccharomyces cerevisiae*. *EMBO J.* 1988; 7:2211–2219. [PubMed: 3046933]
- [65]. Fitch I, Dahmann C, Surana U, Amon A, Nasmyth K, Goetsch L, Byers B, Futcher B. Characterization of four B-type cyclin genes of the budding yeast *Saccharomyces cerevisiae*. *Mol Biol Cell.* 1992; 3:805–818. [PubMed: 1387566]
- [66]. Richardson H, Lew DJ, Henze M, Sugimoto K, Reed SI. Cyclin-B homologs in *Saccharomyces cerevisiae* function in S phase and in G2. *Genes Dev.* 1992; 6:2021–2034. [PubMed: 1427070]
- [67]. Bloom J, Cross FR. Multiple levels of cyclin specificity in cell-cycle control. *Nat Rev Mol Cell Biol.* 2007; 8:149–160. [PubMed: 17245415]
- [68]. Dimova, D.; Cornell University. Ph.D. Thesis. New York: 2000. Antagonizing transcriptional repression of a cell cycle regulated yeast promoter.
- [69]. Sutton A, Bucaria J, Osley MA, Sternglanz R. Yeast ASF1 protein is required for cell cycle regulation of histone gene transcription. *Genetics.* 2001; 158:587–596. [PubMed: 11404324]
- [70]. Eriksson PR, Mendiratta G, McLaughlin NB, Wolfsberg TG, Marino-Ramirez L, Pompa TA, Jainierin M, Landsman D, Shen CH, Clark DJ. Global regulation by the yeast Spt10 protein is mediated through chromatin structure and the histone upstream activating sequence elements. *Mol Cell Biol.* 2005; 25:9127–9137. [PubMed: 16199888]
- [71]. Hess D, Winston F. Evidence that Spt10 and Spt21 of *Saccharomyces cerevisiae* play distinct roles in vivo and functionally interact with MCB-binding factor, SCB-binding factor and Snf1. *Genetics.* 2005; 170:87–94. [PubMed: 15744051]
- [72]. Hierck BP, Molin DG, Boot MJ, Poelmann RE, Gittenberger-de Groot AC. A chicken model for DGCR6 as a modifier gene in the DiGeorge critical region. *Pediatr Res.* 2004; 56:440–448. [PubMed: 15333760]
- [73]. Iyer VR, Horak CE, Scafe CS, Botstein D, Snyder M, Brown PO. Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF. *Nature.* 2001; 409:533–538. [PubMed: 11206552]
- [74]. Koch C, Moll T, Neuberg M, Ahorn H, Nasmyth K. A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase. *Science.* 1993; 261:1551–1557. [PubMed: 8372350]
- [75]. Moll T, Schwob E, Koch C, Moore A, Auer H, Nasmyth K. Transcription factors important for starting the cell cycle in yeast. *Philos Trans R Soc Lond B Biol Sci.* 1993; 340:351–360. [PubMed: 8103939]
- [76]. Simon I, Barnett J, Hannett N, Harbison CT, Rinaldi NJ, Volkert TL, Wyrick JJ, Zeitlinger J, Gifford DK, Jaakkola TS, Young RA. Serial regulation of transcriptional regulators in the yeast cell cycle. *Cell.* 2001; 106:697–708. [PubMed: 11572776]
- [77]. Xu F, Zhang K, Grunstein M. Acetylation in histone H3 globular domain regulates gene expression in yeast. *Cell.* 2005; 121:375–385. [PubMed: 15882620]
- [78]. Surana U, Robitsch H, Price C, Schuster T, Fitch I, Futcher AB, Nasmyth K. The role of CDC28 and cyclins during mitosis in the budding yeast *S. cerevisiae*. *Cell.* 1991; 65:145–161. [PubMed: 1849457]
- [79]. Amon A, Surana U, Muroff I, Nasmyth K. Regulation of p34CDC28 tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae*. *Nature.* 1992; 355:368–371. [PubMed: 1731251]
- [80]. Kellogg DR, Murray AW. NAP1 acts with Clb1 to perform mitotic functions and to suppress polar bud growth in budding yeast. *J Cell Biol.* 1995; 130:675–685. [PubMed: 7622567]

- [81]. Ubersax JA, Woodbury EL, Quang PN, Paraz M, Blethrow JD, Shah K, Shokat KM, Morgan DO. Targets of the cyclin-dependent kinase Cdk1. *Nature*. 2003; 425:859–864. [PubMed: 14574415]
- [82]. Cross FR, Schroeder L, Bean JM. Phosphorylation of the Sic1 inhibitor of B-type cyclins in *Saccharomyces cerevisiae* is not essential but contributes to cell cycle robustness. *Genetics*. 2007; 176:1541–1555. [PubMed: 17483408]
- [83]. Loog M, Morgan DO. Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. *Nature*. 2005; 434:104–108. [PubMed: 15744308]
- [84]. Hall C, Nelson DM, Ye X, Baker K, DeCaprio JA, Seeholzer S, Lipinski M, Adams PD. HIRA, the human homologue of yeast Hir1p and Hir2p, is a novel cyclin-cdk2 substrate whose expression blocks S-phase progression. *Mol Cell Biol*. 2001; 21:1854–1865. [PubMed: 11238922]
- [85]. Lambert JP, Fillingham J, Siahbazi M, Greenblatt J, Baetz K, Figeys D. Defining the budding yeast chromatin-associated interactome. *Mol Syst Biol*. 2010; 6:448. [PubMed: 21179020]

Figure 1. Expression of the *HTA1-HTB1* and *HTA2-HTB2* loci during the cell cycle depends on the *CDC4* gene product

Strains W303-1A (wild-type) (A) and DY8-10C (*cdc4-1 bar1::URA*) (B) were arrested in G1 with α -factor at 24°C, and then released from the block at either 24°C or 37°C (corresponding to permissive and nonpermissive temperature for the *cdc4-1* strain). Samples were removed at regular intervals after release for RNA extraction. The *HTA1*, *HTB1*, *HTA2* and *HTB2* mRNA levels were analyzed by RT-qPCR and normalized to mRNA levels corresponding to *SCR1* for strain W303-1A (A) and to *ACT1* for strain DY8-10C (B). Averages and standard errors of two biological replicates are shown.

Figure 2. Histone gene transcription is not dependent on the *CLB5* and *CLB6* cyclin genes
 Strains YEL106-LU (WT; *CLB+*) and DY15 (*clb5Δ clb6Δ*) were arrested with α -factor at 24°C. After release from the G1 block, cultures were grown at 24°C and samples were removed at 15 minutes intervals for RNA analysis. The *HTB1* (A) and *HTB2* (B) mRNA levels were measured by an S1 nuclease protection assay and compared to *RP51A* mRNA levels, which served as an internal loading control. Results are represented as the ratio of (C) *HTB1* or (D) *HTB2* mRNA to *RP51A* mRNA. Results of one representative experiment are shown.

Figure 3. Transcription of the histone genes is dependent on the mitotic Clb cyclins
Strain A544 (*clb1Δ clb2^{ts} clb3Δ clb4Δ*) was arrested with α -factor at 24°C and released at either the permissive (24°C) or nonpermissive (37°C) temperature. Samples were removed at regular intervals for RNA extraction. The mRNA levels of *HTA1* (A), *HTB1* (B) and *HTA2* (C) were analyzed by RT-qPCR and normalized to *ACT1* mRNA levels. *CLN2* mRNA (D) levels served as a control for cell cycle progression. Averages and standard errors of two biological replicates are shown.

Figure 4. Expression of the histone genes is dependent on *CLB1* and *CLB2* but not on *CLB3* and *CLB4*

(A) Strains W303-1A (WT; *CLB*⁺) and K3385 (*clb3Δ clb4Δ*) were arrested with α -factor at 24°C. After release from the G1 block, each culture was grown at 24°C, and samples were removed at 15 minutes intervals for RNA extraction. The *HTA1*, *HTB1* and *HTA2* mRNA levels were analyzed by RT-qPCR and normalized to *SCR1* mRNA levels. (B) Strain DY23 (*clb1Δ clb2^{ts}*) was arrested with α -factor at 24°C and then released from the G1 block at either 24°C or 37°C. Samples were removed at 15 minutes intervals for RNA extraction. The *HTA1*, *HTB1* and *HTA2* mRNA levels were measured by RT-qPCR and normalized to *ACT1* mRNA levels. Averages and standard errors of two biological replicates are shown.

Figure 5. Deletion of the *HIR1* gene relieves the dependence of HIR-dependent histone gene transcription on the mitotic Clb cyclins

Strain A544Δ1 (*clb1Δ clb2^{ts} clb3Δ clb4Δ hir1Δ*) was arrested with α -factor at 24°C and released from the block at either 24°C or 37°C. Samples were removed at 10 or 15 minutes intervals for RNA extraction and subsequent analysis by RT-qPCR. The *HTA1*, *HTB1* and *HTA2* mRNA levels were analyzed by RT-qPCR and normalized to *ACT1* mRNA levels. *CLN2* mRNA levels served as a control for cell cycle progression. Averages and standard errors of two biological replicates are shown.

Figure 6. Co-immunoprecipitation of Hir1 and Hir2 with the mitotic Clb2 cyclin

(A) Strains YPP993 (*HIR1-TAP*), YPP251 (*HIR2-TAP*), and BY4741 (untagged *HIR*) were transformed with a plasmid carrying a *CLB2-HA* fusion gene where indicated. (B) Strain YPP727 (*CLB2-TAP*) was transformed with a plasmid carrying *HIR1-HA* (B) or *HIR2-HA* (C) genes. Hir1-TAP (A), Hir2-TAP (A), or Clb2-TAP (B and C) were immunoprecipitated (IP) from 1 mg of whole-cell lysates by incubation with IgG-sepharose beads. Fifty μ g of whole-cell lysate and 100% of the immunoprecipitated material were loaded as Input and IP, respectively, resolved by SDS-PAGE, and analyzed by blotting with anti-HA monoclonal antibody (to probe for the HA tagged protein) and a peroxidase anti-peroxidase (α -PAP) antibody (to probe for the indicated TAP tag protein).

Figure 7. The cell cycle dependent recruitment of SWI/SNF to the *HTA1-HTB1* promoter is independent of the mitotic Clb cyclins

SWI/SNF binding to the *HTA1-HTB1* promoter in synchronized cultures of a wild-type (WT-dashed lines) and *clb1Δ clb2^{ts} clb3Δ clb4Δ* strain (solid lines) was analyzed by chromatin immunoprecipitation (ChIP) on samples taken at the indicated time points after release at 24°C (dark grey lines) or 37°C (light grey lines) from α -factor arrest. SWI/SNF was immunoprecipitated using an antibody against the N-terminal end of the Swi2/Snf2 subunit. Enrichment of the *HTA1-HTB1* promoter relative to a control region (*INTV*) was quantified by qPCR. Averages and ranges of duplicate ChIPs from two biological replicates are shown.

Figure 9. Model for the role of the Clb1/2-Cdk1 kinase in the activation of *HTA1-HTB1* transcription in late G1

Rtt106 and Asf1 associate with the HIR complex and are found at the *HTA1-HTB1* promoter throughout the cell cycle. The HIR complex is recruited by an as yet unknown factor (“?”) binding to the NEG site. (A) In early G1, the RSC complex, which acts as a co-repressor, is found at the promoter in an Rtt106-dependent manner [41], and along with the HIR complex represses histone gene transcription. (B) In late G1, SWI/SNF is recruited by Rtt106 and the RSC complex is displaced from the histone gene promoter [41]. We propose that the mitotic Clb1/2-Cdk1 kinase binds to and phosphorylates the HIR complex, thereby alleviating HIR-mediated repression of the *HTA1-HTB1* locus. (C) In Late G1/early S phase, after Clb1/2-Cdk1-dependent inactivation of HIR-mediated repression, SWI/SNF activates transcription of *HTA1-HTB1* locus together with the sequence specific DNA binding proteins Swi4 (SBF) and Spt10, which are bound to the UAS elements [27].

TABLE 1

Saccharomyces cerevisiae strains

Strain	Genotype	Source
W303-1A	<i>MATa ura3-1 his3-11,15 trp1-1 ade2-1 leu2-3,112 can1-100 ssd1</i>	R. Rothstein
BY4741	<i>Mat a, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Open Biosystems
A544	<i>MATa clb1Δ clb2-VI clb3::TRP1 clb4::HIS3 ura3-1 his3-11,15 trp1-1 ade2-1 leu2-3,112 can1-100 ssd1</i>	K. Nasmyth
A544Δ1	<i>MATa clb1Δ clb2-VI clb3::TRP1 clb4::HIS3 hir1::LEU2 ura3-1 his3-11,15 trp1-1 ade2-1 leu2-3,112 can1-100 ssd1</i>	A. Raff
K3385	<i>MATa clb3::TRP1 clb4::HIS3 ura3-1 his3-11,15 trp1-1 ade2-1 leu2-3,112 can1-100 ssd1</i>	K. Nasmyth
DY8-10C	<i>MATa cdc4-1 bar1::URA3 ura3-1 his3-11,15 trp1-1 ade2-1 leu2-3,112 can1-100 ssd1</i>	This study
DY15	<i>MATa clb5::URA3 clb6::LEU2 bar1::HIS3 ura3-1 his3-11,15 trp1-1 ade2-1 leu2-3,112 can1-100 ssd1</i>	This study
DY23	<i>MATa clb1Δ clb2-VI ura3-1 his3-11,15 trp1-1 ade2-1 leu2-3,112 can1-100 ssd1 bar1::HIS3</i>	This study
YPP252	<i>MATa HIR1-TAP::His3MX6: his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Open Biosystems
YPP727	<i>MATa CLB2-TAP::HIS3MX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Open Biosystems
YPP993	<i>MATa HIR2-TAP::His3MX6 ura3-1 trp1-1 his3-11,15 leu2-3,112 can1-100, ade2-1</i>	This study
YPP 1201	<i>MATa clb1Δ clb2-VI clb3::TRP1 clb4::HIS3 asf1::kanMX6 ura3-1 his3-11,15 trp1-1 ade2-1 leu2-3,112 can1-100 ssd1</i>	This study
YPP 1203	<i>MATa clb1Δ clb2-VI clb3::TRP1 clb4::HIS3 rtt106::kanMX6 ura3-1 his3-11,15 trp1-1 ade2-1 leu2-3,112 can1-100 ssd1</i>	This study