THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 28, Issue of July 9, pp. 29278–29285, 2004
Printed in U.S.A.

Hierarchical Requirement of SWI/SNF in Retinoblastoma Tumor Suppressor-mediated Repression of Plk1*

Received for publication, January 14, 2004, and in revised form, March 25, 2004
Published, JBC Papers in Press, April 22, 2004, DOI 10.1074/jbc.M400395200

Ranjaka W. Gunawardena‡, Hasan Siddiqui‡, David A. Solomon, Christopher N. Mayhew§, Justin Held, Steven P. Angus, and Erik S. Knudsen¶

From the Department of Cell Biology, Neurobiology and Anatomy, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45267-0521

Plk1 (Polo-like kinase 1) is a critical regulator of cell cycle progression that harbors oncogenic activity and exhibits aberrant expression in multiple tumors. However, the mechanism through which Plk1 expression is regulated has not been extensively studied. Here we demonstrate that Plk1 is a target of the retinoblastoma tumor suppressor (RB) pathway. Activation of RB and related pocket proteins p107/p130 mediate attenuation of Plk1. Conversely, RB loss deregulates the control of Plk1 expression. RB pathway activation resulted in the repression of Plk1 promoter activity, and this action was dependent on the SWI/SNF chromatin remodeling complex. Although SWI/SNF subunits are lost during tumorigenesis and cooperate with RB for transcriptional repression, the mechanism through which SWI/SNF impinges on RB action is unresolved. Therefore, we delineated the requirement of SWI/SNF for three critical facets of Plk1 promoter regulation: transcription factor binding, corepressor binding, and histone modification. We find that E2F4 and pocket protein association with the Plk1 promoter is independent of SWI/SNF. However, these analyses revealed that SWI/SNF is required for histone deacetylation of the Plk1 promoter. The importance of SWI/SNF-dependent histone deacetylation of the Plk1 promoter was evident, because blockade of this event restored Plk1 expression in the presence of active RB. In summary, these data demonstrate that Plk1 is a target of the RB pathway. Moreover, these findings demonstrate a hierarchical role for SWI/SNF in the control of promoter activity through histone modification.

Progression through the cell cycle is a carefully choreographed process that is often deregulated in cancer cells (1–3). It is believed that deregulation of proliferation control serves to fuel tumor development and progression. Interplay between cell cycle regulatory proteins is increasingly relevant for understanding the underlying basis of appropriate cell cycle control and the development of aberrant proliferation in cancer. Here we demonstrate a novel regulation of the mitotic Plk1 (Polo-like kinase 1) by the retinoblastoma tumor suppressor (RB)[1] pathway.

Initially identified through homology with the *Drosophila polo*, Plk1 governs multiple events associated with G_2/M progression (4–7). For example, Plk1 is a determinant of mitosis promoting factor, CDK1/cyclin B, which stimulates entry into mitosis (6, 8). Additionally, centrosome duplication and assembly of the mitotic spindle apparatus are regulated through the action of Plk1 (6, 9). Recently, a number of studies have implicated a role for Plk1 in cancer. For example, numerous tumor types (*e.g.* colorectal cancer, squamous cell carcinomas of the head and neck, and melanoma) aberrantly express Plk1 (10–13). Consistent with a causative role in tumorigenesis, ectopic expression of Plk1 can transform cells in culture (14). Conversely, Plk1 ablation in tumor cells results in mitotic failure and cell death (9, 15–17).

Plk1 expression is repressed in resting cells and induced only as cells progress through G_1/S (18, 19). The induction of Plk1 protein levels is largely dependent on the regulation Plk1 promoter activity, and *cis*-acting elements that modulate cell cycle dependence have been defined (18, 20). Particularly, the Plk1 promoter is subject to transcriptional repression through cell cycle-dependent element/cell cycle-gene homology region elements that are required for the cell cycle dependence of Plk1 promoter activity (20, 21). However, the mechanism through which silencing of Plk1 expression occurs as cells exit the cell cycle and the source of the deregulated expression in tumor cells is unknown.

A critical regulator of G_1/S-dependent gene expression is the RB pathway. RB is functionally inactivated in the majority of tumors via a number of discrete mechanisms (1–3, 22). It is believed that RB, in concert with the related pocket proteins p107 and p130, functions as a tumor suppressor through its capacity to repress the transcription of critical targets in a cell cycle-dependent manner and thus prevent proliferation (22, 23). In G_0 or early G_1 cells, RB and related proteins are hypophosphorylated and form complexes with the E2F family of transcription factors (24–26). The E2F family of transcription factors are involved in the regulation of numerous genes required for cell cycle progression. RB, p107, and p130 mediate transcriptional repression and subsequent attenuation of E2F-regulated genes by recruiting additional corepressors (*e.g.* histone deacetylases) that modify chromatin structure (25–27). Repression is alleviated when RB family members are phos-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this work.

§ Supported by Training Grant T32CA59268 from the NCI, National Institutes of Health.

¶ Supported by Grant CA106471 from the NCI, National Institutes of Health. To whom correspondence should be addressed: Dept. of Cell Biology, Vontz Center for Molecular Studies, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0521. Tel.: 513-558-8885; Fax: 513-558-4454; E-mail: Erik.Knudsen@UC.edu.

[1] The abbreviations used are: RB, retinoblastoma tumor suppressor; Dox, doxycycline; MAF, mouse adult fibroblast; β-gal, β-galactosidase; GFP, green fluorescent protein; RT, reverse transcriptase; ChIP, chromatin immunoprecipitation; CPT, camptothecin; TSA, trichostatin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PSM, phosphorylation site mutant.

phorylated by CDK complexes in mid G_1, thus enabling progression through the cell cycle (28). Transcriptional repression is viewed as requisite for RB function in tumor suppression based on genetic and biochemical data (29, 30). Additionally, we and others have found that the loss of SWI/SNF chromatin remodeling factors compromises RB-mediated transcriptional repression (31–34).

SWI/SNF is a heterogeneous multisubunit chromatin remodeling complex (35, 36). This complex utilizes the energy of ATP to remodel chromatin structure and contains either BRG1 or BRM as the central ATPase (36). The activity of the core ATPase subunit is required by the SWI/SNF complex to regulate gene transcription (35, 37, 38). Prior studies have demonstrated that the combined losses of BRG1 and BRM result in resistance to the activation of the RB pathway and aberrant cell cycle progression (32, 33). Additionally, the loss of SWI/SNF activity is associated with a failure of RB to elicit transcriptional repression of specific targets (*e.g.* cyclin A). Based on studies in yeast, the loss of SWI/SNF could disrupt virtually any step associated with transcriptional repression (35, 38). For example, SWI/SNF could be required for the assembly of E2F proteins at promoter, for the retention of RB or related proteins at the promoter, or for subsequent modifications of the promoter. However, the mechanism through which SWI/SNF cooperates with RB for transcriptional repression is not understood.

In this study, we specifically focused on elucidating the regulation of Plk1 expression. We show that Plk1 is repressed via activation of the RB pathway and that Plk1 expression is deregulated through targeted RB loss. Moreover, we demonstrate that the repression of Plk1 by RB is dependent on SWI/SNF activity. Analysis of SWI/SNF function demonstrates that chromatin remodeling is not required for the association of E2F or RB family members at the Plk1 promoter. In contrast, histone deacetylation of the Plk1 promoter was dependent on SWI/SNF and critical for the observed transcriptional repression. Thus, this study provides critical insight into the mechanism through which Plk1 transcription is regulated and demonstrates the intricate relationship between SWI/SNF and histone deacetylases during RB-mediated transcriptional repression.

MATERIALS AND METHODS

Cell Culture, Plasmids, Infections, and Transfections—SW13, TSUPr-1, U2OS, and A5-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units of penicillin-streptomycin, 2 mM L-glutamine at 37 °C in 5% CO_2. In addition, A5-1 cells were maintained in G418 (200 μg/ml), hygromycin B (200 μg/ml), and doxycycline (Dox, 1 μg/ml). Primary $Rb^{loxP/loxP}$ mouse adult fibroblasts (MAFs) were isolated from $Rb^{loxP/loxP}$ mice (39). The cells were propagated by routine subculturing in Dulbecco's modified Eagle's medium containing 10% fetal calf serum supplemented with 100 units/ml penicillin-streptomycin and 2 mM L-glutamine. Primary cultures were between passage 2 and 6. Plasmids encoding β-gal, p16ink4a, PSM-RB, and BRG1 have been previously described (20, 31, 40). The reporter constructs 3xE2F-Luc and Plk1-Luc have been previously described (20, 31, 40). Adenoviruses encoding GFP and p16ink4a have been previously described (41). The Cre-encoding adenovirus was obtained from the Iowa University vector core. Infections were performed at a multiplicity of infection of 50–100 for ~95–100% infection efficiency after 24 h as determined by GFP fluorescence.

Reporter Assays, Immunoblotting, and RT-PCR—Immunoblotting was performed using standard techniques. The following antibodies were used: RB-purified mouse anti-human (554136, BD Biosciences), p107 (sc-318, Santa Cruz), p130 (sc-317, Santa Cruz), Plk1 (sc 17783, Santa Cruz; 06–813, Upstate Biotechnology, Inc.), BRG1 (sc-17796, Santa Cruz), p16ink4a (sc-759, Santa Cruz), E2F1 (gift from A. Yee), E2F2 (sc-9967, Santa Cruz), E2F4 (sc-1082, Santa Cruz), Lamin B (sc-6217, Santa Cruz), and Vimentin (gift from Dr. Wallace Ip). All of the immunoblots were repeated at least three times with independent samples. The reporter assays were performed as described previously (42). The reporter assays were performed in triplicate and from three independent experiments. RT-PCR was performed as described previously (42). The following primer pairs were utilized: human Plk1, 5′-CCA GAG GGA GAA GAT GTC CA-3′ and 5′-ATA ACT CGG TTT CGC TGC AG-3′; human GAPDH, 5′-TGG AAA TCC CAT CAC CAT CT-3′ and 5′-TTC ACA CCC ATG ACG AAC AT-3′; and rat Plk1, 5′-TTT GTG TTC GTG GTT TTG GA-3′ and 5′-TTC TTC CGT TCC CCT TCA TA-3′. The rat β-actin primers have been previously described (42). The experiments were performed at least three times, and representative data are shown.

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation (ChIP) assays were performed as previously described (42). The following antibodies were utilized for ChIP: p107 (sc-318, Santa Cruz), p130 (sc-317, Santa Cruz), E2F4 (sc-1082, Santa Cruz), acetylated histone H4 (06–866, Upstate Biotechnology, Inc.), and Dbf4 (sc-11354, Santa Cruz). The following primers were used to amplify regions of the human Plk1 promoter: 5′-GGTTTGGTTTCCCAGGCTAT-3′ and 5′-GCTGGGAACGTTACAAAAGC-3′. The ChIP assays were repeated at least twice with independent samples.

RESULTS

The RB Pathway Regulates Plk1 Expression—The *Plk1* gene is subject to deregulated expression in a variety of tumor types and has been shown to harbor oncogenic activity (14, 43). Additionally, it has been previously shown that Plk1 is a G_1/S-regulated gene (20, 44). However, the mechanisms through which Plk1 expression is controlled or deregulated in human cancers are poorly understood. In a microarray screen we identified Plk1 as a target for RB-mediated repression (45). Therefore, we initially determined the activity of the RB pathway on Plk1 protein levels (Fig. 1). To activate endogenous RB (and the related proteins p107 and p130), U2OS cells were infected with adenoviruses encoding either GFP (control) or p16ink4a, which prevents their phosphorylation by inhibiting CDK4 activity. As expected, the expression of p16ink4a resulted in RB dephosphorylation (Fig. 1*A*), indicating endogenous RB pathway activation. Plk1 protein levels were significantly attenuated in those cells infected with p16ink4a encoding adenovirus, as compared with cells infected with the GFP encoding virus (Fig. 1*A*).

To determine whether targeted RB loss, such as occurs in cancer, influences Plk1 expression, conditional knockout of the *Rb* gene was employed. MAFs of the $Rb^{loxP/loxP}$ genotype were infected with recombinant adenoviruses encoding either GFP or Cre-recombinase. In this system, the endogenous *Rb* locus is subject to recombination through the expression of the Cre-recombinase, and RB protein expression is ablated (not shown). Under this condition, we find that Plk1 protein levels were elevated following RB loss (Fig. 1*B*, compare *lanes 1* and *2*). Similarly, by microarray analysis we observe a 2.4-fold elevation in Plk1 RNA levels with RB loss.[2] Thus, endogenous RB serves to maintain the appropriate levels of Plk1. To determine the subsequent action of endogenous RB on attenuating Plk1 protein levels under physiological stress, we examined the expression of Plk1 following exposure to camptothecin (CPT). CPT induces an RB-dependent checkpoint response, wherein specifically those cells deficient in RB (Cre-infected) continue cell cycle progression in the presence of CPT (Fig. 1*C*). Consistent with protein analyses, MAFs deficient in RB showed upregulation of Plk1 RNA levels compared with MAFs harboring RB (Fig. 1*D*, compare *lanes 1* and *3*). Treatment with CPT resulted in the repression of Plk1 RNA levels in RB-proficient MAFs. (Fig. 1*D*, compare *lanes 1* and *2*). However, Cre-mediated ablation of RB largely relieved the repression of Plk1 transcription following treatment with CPT (Fig. 1*D*, compare *lanes 2* and *4*). These observations were further supported by analysis of Plk1 protein levels (Fig. 1*E*, compare *lanes 2* and *4*). Collectively, these results demonstrate that Plk1 is a target of the RB pathway, and specific RB loss results in deregulation of this critical target.

[2] M. P. Markey, C. N. Mayhew, and E. S. Knudsen, manuscript in preparation.

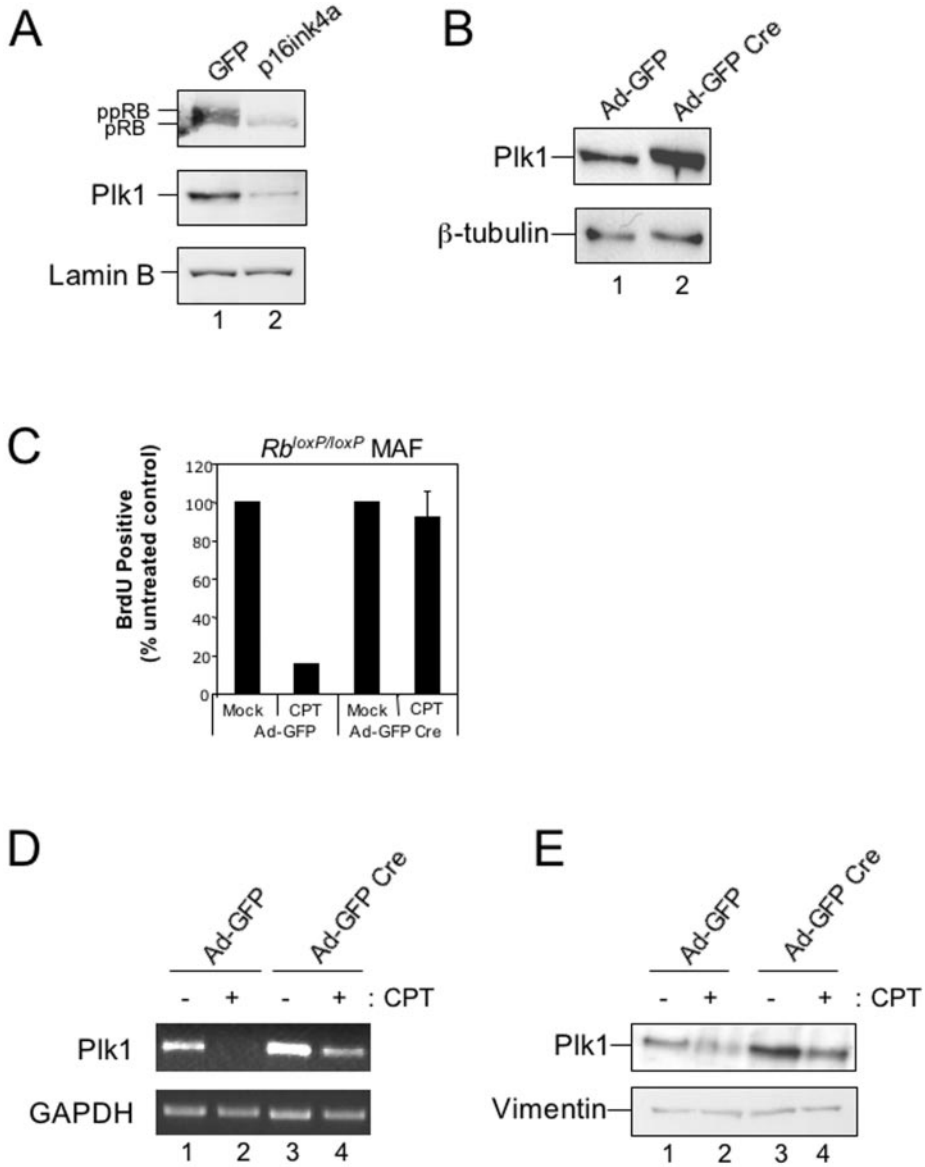

FIG. 1. **RB pathway represses Plk1 expression.** *A*, U2OS cells were infected with either GFP (*lane 1*) or p16ink4a (*lane 2*) encoding adenoviruses. The cells were harvested 36 h post-infection, and total protein was resolved by SDS-PAGE. The indicated proteins were detected by immunoblotting. *B*, $Rb^{loxP/loxP}$ MAFs were infected with GFP- or Cre-encoding adenoviruses. The cells were harvested 6-days post-infection, and immunoblotted for Plk1 and β-tubulin. *C*, Ad-GFP- and Ad-Cre-infected $Rb^{loxP/loxP}$ MAFs were treated with 10 μM camptothecin for 16 h 6 days post-infection and subsequently pulse-labeled with bromodeoxyuridine (*BrdU*) for 12 h. The cells were fixed, and the percentage of bromodeoxyuridine incorporation was determined. Untreated controls were set to 100%. *D*, $Rb^{loxP/loxP}$ MAFs treated with 5 μM camptothecin for 16 h 6 days post-Ad-GFP and Ad-Cre infection were harvested, total RNA was isolated, and RT-PCR was performed. *E*, similar to Fig. 3*C*, except the cells were harvested prior to bromodeoxyuridine addition. Total protein was isolated, resolved by SDS-PAGE, and immunoblotted for Plk1 and Vimentin.

RB-mediated Repression of Plk1 Is Compromised in SWI/SNF-deficient Cells—Having established that Plk1 protein levels are modulated by the RB pathway, we next sought to elucidate the mechanism of this regulation. Because SWI/SNF activity is compromised in specific cancers (33, 46) and known to be required for repression of selected RB target genes (*e.g.* cyclin A) (31, 34), the action of SWI/SNF in Plk1 regulation was investigated. The SW13 cell line does not express the BRG1 and BRM ATPases requisite for SWI/SNF activity, whereas TSUPr-1 cells express BRM and are sensitive to RB-mediated signaling (32). To activate the endogenous RB pathway, adenoviral transduction of p16ink4a that maintains RB in its hypophosphorylated/active state was utilized. As shown, expression of p16ink4a led to the dephosphorylation of RB and related proteins p107 and p130 in both cell types (Fig. 2*A*). To determine the coordinate action of the RB pathway and SWI/SNF upon Plk1 expression, we initially analyzed Plk1 promoter activity. SW13 cells and TSUPr-1 cells were cotransfected with Plk1 reporter plasmid and either vector control or p16ink4a-encoding plasmids (Fig. 2*B*). p16ink4a expression potently repressed Plk1 promoter activity in TSUPr-1 cells. In contrast, p16ink4a failed to repress Plk1 in the SW13 cell line. Consistent with the reporter assays, we observed attenuation of endogenous RNA of Plk1 in TSUPr-1 cells infected with p16ink4a (Fig. 2*C*, compare *lanes 3* and *4*). As expected, expression of GAPDH did not change in either of these cell lines even when infected with p16ink4a. In contrast, p16ink4a failed to attenuate endogenous RNA levels of Plk1 in SW13 cells (Fig. 2*C*, compare *lanes 1* and *2*). The SWI/SNF-dependent reduction in promoter activity and RNA levels were reflected in the specific attenuation of Plk1 protein levels by p16ink4a infection in TSUPr-1 cells (Fig. 2*D*). Taken together, these results indicate that SWI/SNF activity is critical for RB pathway-mediated repression of Plk1. To confirm this observation, BRG1 expression was restored in SW13 cells in combination with p16ink4a, and Plk1 promoter activity was analyzed through reporter analysis (Fig. 2*E*). Similar to our earlier results, p16ink4a alone failed to repress Plk1 promoter activity (Fig. 2*E*). However, cotransfection of BRG1 and p16ink4a significantly repressed Plk1 promoter activity in the BRG1/BRM-deficient SW13 cells. Collectively, these data demonstrate that SWI/SNF activity is required for RB-mediated repression of the Plk1 promoter and attenuation of its RNA and protein levels.

SWI/SNF Is Dispensable for the Assembly of E2F or Pocket Proteins at the Plk1 Promoter—Failure of the RB pathway to repress Plk1 in the absence of SWI/SNF activity could be attributed to multiple functions of this complex. In yeast, it has been shown that SWI/SNF activity is required for the assembly

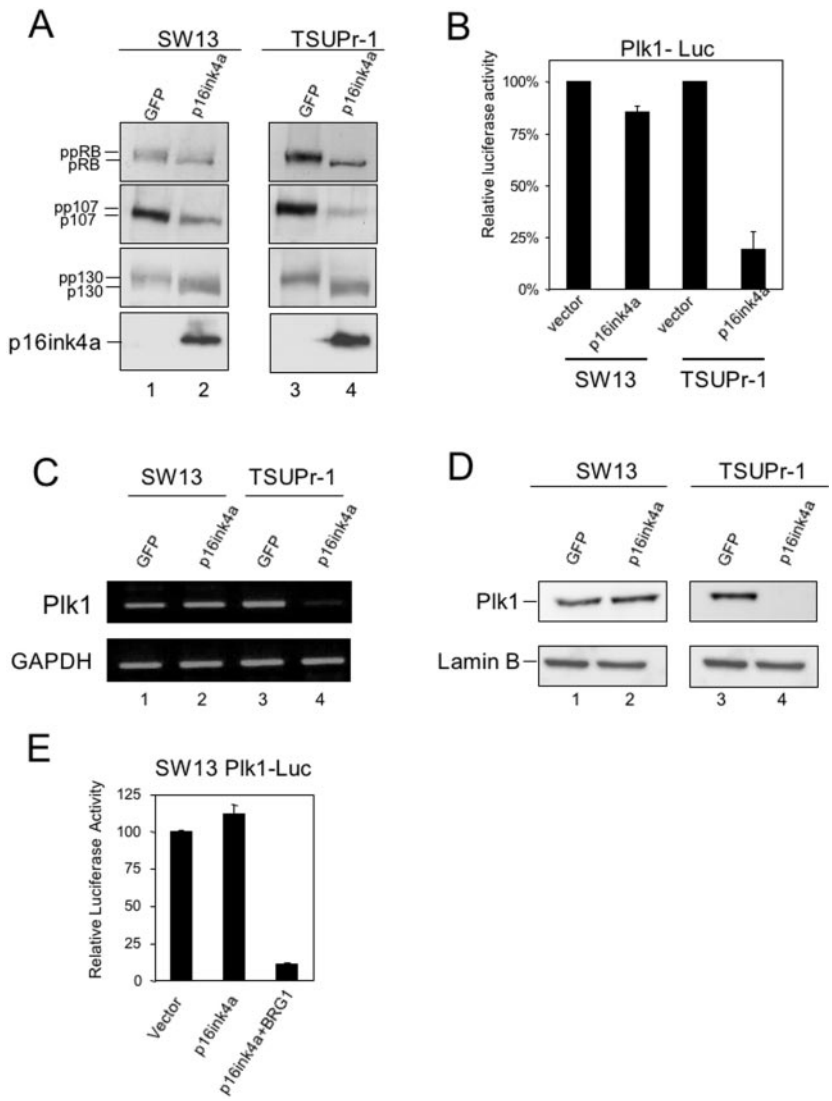

FIG. 2. **SWI/SNF is required for RB-mediated repression of Plk1.** *A*, SW13 (*lanes 1* and *2*) and TSUPr-1 (*lanes 3* and *4*) cells were infected with GFP (*lanes 1* and *3*) or p16ink4a (*lanes 2* and *4*) encoding adenoviruses. The cells were harvested 36 h post-infection, total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. *B*, SW13 and TSUPr-1 cells were cotransfected with cytomegalovirus β-gal and Plk1-Luc reporter plasmids and either vector or p16ink4a expression plasmids. Relative luciferase activity was normalized to β-gal activity for transfection efficiency and vector control was set to 100. *C*, SW13 (*lanes 1* and *2*) and TSUPr-1 (*lanes 3* and *4*) cells were infected with either GFP (*lanes 1* and *3*) or p16ink4a (*lanes 2* and *4*) encoding adenoviruses. Total RNA was extracted 24 h post-infection and subjected to linear RT-PCR amplification with primers specific for the indicated genes. *D*, SW13 (*lanes 1* and *2*) and TSUPr-1 (*lanes 3* and *4*) cells were infected with either GFP (*lanes 1* and *3*) or p16ink4a (*lanes 2* and *4*) encoding adenoviruses. Total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. *E*, SW13 cells were cotransfected with cytomegalovirus β-gal and Plk1-Luc reporter plasmids and either vector, p16ink4a, or p16ink4a and BRG1 expression plasmids. The relative luciferase activity was normalized to β-gal activity for transfection efficiency, and vector control was set to 100.

of transcription factor complexes on chromatin (38, 47). Therefore, SWI/SNF loss could bypass RB-mediated repression through two basic mechanisms. First, SWI/SNF could be required for E2F action. Specifically, SWI/SNF could be required for the expression of E2F family members or for E2F-chromatin interactions that are required for RB pathway-mediated repression (48). Second, SWI/SNF could be required for pocket proteins to associate with E2F factors on target promoters.

Initially, we probed the action of SWI/SNF directly on E2F proteins. The E2F family is broadly defined as activating (E2F1–E2F3) or repressive (E2F4 and E2F5) E2Fs based on their predominant transcriptional role (49). Immunoblot analyses demonstrated that both SW13 and TSUPr-1 cells expressed similar levels of E2F1 ("activating E2F") and E2F4 proteins ("repressive E2F") (Fig. 3*A*). This result is consistent with microarray analyses that did not identify E2F family members as targets of SWI/SNF (35) and suggests that limitation of E2F expression does not underlie the resistance to RB pathway activation in SW13 cells. Therefore, several approaches were utilized to subsequently delineate the role of SWI/SNF on the functional interaction of E2F with chromatin. Initially, we determined whether E2F proteins were compromised for transcriptional activation, because the RB pathway is compromised for transcriptional repression. To perform these analyses we used a synthetic promoter composed of multimerized E2F sites (3xE2F-Luc), wherein promoter activity is dependent on E2F binding for activity. Endogenous E2F activity was readily detected with this reporter (Fig. 3*B*). Additionally, ectopic expression of E2F2 clearly activated the reporter (3-fold) in the absence of SWI/SNF (Fig. 3*B*). These results suggest that E2F binding to a simple promoter element and stimulation of transcription is independent of SWI/SNF action.

Because transcription factors interact with chromatin in a dynamic fashion, we assessed the action of SWI/SNF on E2F2 retention in living cells. In the case of E2F proteins, we have previously shown that the diffusion rate of these proteins in living cells is dependent on chromatin association (50). Here we constructed an expression vector encoding enhanced GFP fused to the N terminus of human E2F2 (GFP-E2F2). Expression of the GFP-E2F2 fusion protein was verified by immunoblotting (Fig. 3*C*). Additionally, the GFP-E2F2 construct efficiently stimulated transcription from the 3xE2F-Luc reporter (not shown). Having validated the functional activity of GFP-E2F2, it was utilized in fluorescence recovery after photobleaching (FRAP) analysis to determine the influence of SWI/SNF on the nuclear retention of E2F2. SW13 and TSUPr-1 cells were transfected with GFP-E2F2 expression plasmids, and fluorescence recovery after photobleaching analysis was performed 18 h post-transfection. Under these conditions, we did not observe a significant difference in the mobility of GFP-E2F2 between the two cells lines (data not shown). To specifically address whether SWI/SNF influenced the mobility of E2F2 in

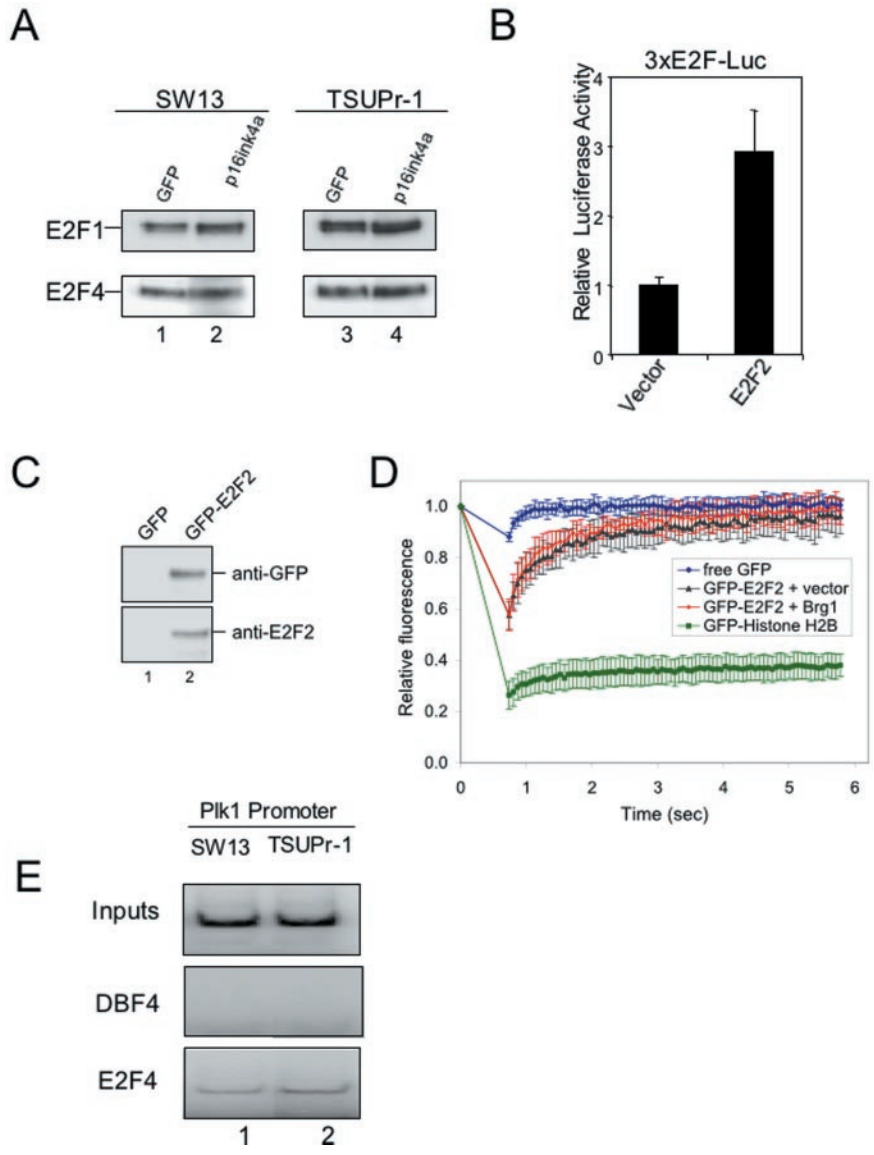

FIG. 3. **SWI/SNF is dispensable for E2F chromatin/promoter association.** *A*, SW13 (*lanes 1* and *2*) and TSUPr-1 (*lanes 3* and *4*) cells were infected with either GFP (*lanes 1* and *3*) or p16ink4a (*lanes 2* and *4*) encoding adenoviruses. Total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. *B*, SW13 cells were transfected with cytomegalovirus β-gal, the reporter construct 3XE2F-Luc, and either vector or E2F2 as indicated. The relative luciferase activity was normalized to β-gal activity for transfection efficiency, and vector control was set to 1. *C*, U2OS cells were transiently transfected with free GFP alone or GFP-E2F2. Total protein was resolved by SDS-PAGE, and GFP-E2F2 protein was detected with antibodies specific for GFP and E2F2. *D*, SW13 cells on 25-mm coverslips were cotransfected with GFP, GFP-Histone H2B, or GFP-E2F2 and either empty vector or wild type BRG1. The coverslips were transferred to live cell imaging chambers, and nuclear fluorescence recovery after photobleaching analysis was performed 18 h post-transfection. *E*, SW13 (*lane 1*) and TSUPr-1 (*lane 2*) cells were infected with p16ink4a encoding adenoviruses. ChIP assays were performed with antibodies for E2F4 and Dbf4 (nonspecific). Input and immunoprecipitated DNA was amplified by PCR with primers specific for the Plk1 promoter.

SW13 cells, we cotransfected these cells with GFP-E2F2 and either vector or BRG1. We failed to observe any difference in fluorescence recovery in the presence or absence of BRG1 (Fig. 3*D*). As expected, freely diffusible GFP rapidly recovered fluorescence after photobleaching, whereas GFP-histone H2B failed to recover because of its tight association with chromatin. Taken together, these findings demonstrate that SWI/SNF does not globally affect E2F2 chromatin retention as assessed by live cell imaging.

To specifically determine whether SWI/SNF is required for the association of an E2F family member with the Plk1 promoter, ChIP assays were performed. SW13 and TSUPr-1 cells were infected with p16ink4a encoding adenovirus, and protein-DNA complexes were cross-linked with formaldehyde and immunoprecipitated with antibodies to E2F4 and Dbf4 (nonspecific antibody control). Immunoprecipitated DNA was purified and utilized in quantitative radioactive PCR using primers flanking the E2F-binding sites present in the Plk1 promoter. Amplification of the DNA was well within the linear range of PCR (data not shown). E2F4 occupancy was observed in both TSUPr-1 and SW13 cells (Fig. 3*E*, compare *lanes 1* and *2*). Together, these results demonstrate that SWI/SNF is not required for the assembly of E2F4 on promoters.

In addition to regulating transcription factor association, SWI/SNF could regulate the ability of pocket proteins to assemble at promoters. As shown in Fig. 2*A*, RB and the related proteins p107 and p130 are expressed in TSUPr-1 and SW13 cells, and these proteins were dephosphorylated following the expression of p16ink4a. Next, we determined whether RB had the capacity to functionally interact with E2F proteins in the absence of SWI/SNF. It is known that RB binding to E2F proteins will inhibit their transactivation function. As such, the 3xE2F-Luc reporter construct was utilized to determine whether RB has the capacity to physically interact with E2F family members on a promoter. In SW13 cells the expression of a constitutively active allele of RB (PSM-RB) inhibited virtually all activation of the reporter (Fig. 4*A*). This result indicates that RB retains the capacity to efficiently interact with E2F in the absence of SWI/SNF activity.

To determine the requirement for SWI/SNF in the assembly of pocket proteins on the Plk1 promoter, ChIP analysis was performed (51, 52). We observed approximately equal recruitment of p130 to promoters in the presence or absence of SWI/SNF (Fig. 4*B*, *lanes 1* and *2*, respectively). In contrast, there was enhanced p107 recruitment in the SWI/SNF-deficient cells (Fig. 4*B*, *lanes 1* and *2*). Thus, SWI/SNF is not required for these pocket proteins to assemble on promoters. Similarly, we detected RB at the Plk1 promoter in the absence of SWI/SNF

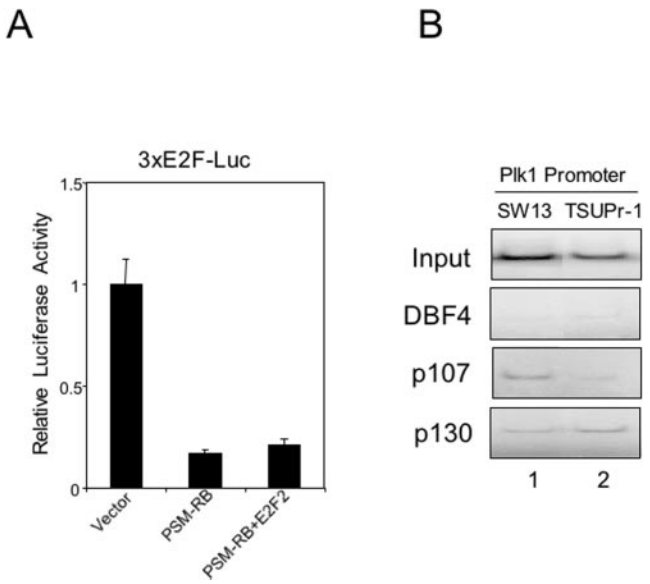

FIG. 4. **SWI/SNF is not required for pocket protein association with the Plk1 promoter.** *A*, SW13 cells were transfected with cytomegalovirus β-gal and 3XE2F-Luc reporter plasmids and either vector, PSM-RB, or PSM-RB and E2F2 expression plasmids as indicated. The relative luciferase activity was normalized to β-gal activity for transfection efficiency. *B*, SW13 (*lane 1*) and TSUPr-1 (*lane 2*) cells were infected with p16ink4a encoding adenoviruses. The ChIP assays were performed with antibodies for p107 and p130. Input and immunoprecipitated DNA was amplified by PCR using primers specific for the Plk1 promoter.

(data not shown). However, in our hands the occupancy of RB at the Plk1 promoter (as well as other promoters) is not consistently detectable. Therefore, similar to the situation with E2F factors, BRG1/BRM was not required for promoter association of p107/p130.

SWI/SNF Is Required for Plk1 Promoter Histone Deacetylation—It is believed that one of the key components of RB-mediated transcriptional repression is not only its recruitment to chromatin but also subsequent histone modifications at the promoter (25). Specifically, RB and related proteins recruit histone deacetylase activities that result in promoter hypoacetylation. We have recently shown that such modifications represent a critical means through which RB functions to repress transcription (42). Therefore, we determined whether SWI/SNF influences promoter histone acetylation. ChIP assays were performed using anti-acetyl histone H4 antibody to determine the acetylation status of histone H4 at the Plk1 promoter (Fig. 5*A*). As shown, infection with p16ink4a resulted in significant histone deacetylation of the Plk1 promoter in TSUPr-1 cells (Fig. 5*A*, *top left panel*, compare *lanes 3* and *4*), which was evident from quantification of independent experiments (Fig. 5*A*, *right panel*). In contrast, p16ink4a infection failed to induce any histone deacetylation of Plk1 promoter in SW13 cells, indicating that SWI/SNF is required for histone deacetylation of the Plk1 promoter.

To determine the effect of histone deacetylation on Plk1 expression, we utilized an immortalized rat fibroblast cell line that expresses PSM-RB in a tet-off inducible fashion (A5-1). In this system, removal of the tetracycline analogue Dox results in the induction of PSM-RB (Fig. 5*B*). Under these conditions, Plk1 protein levels were efficiently down-regulated (Fig. 5*B*). These results were confirmed at the level of endogenous RNA (Fig. 5*C*, *lanes 1* and *2*). To investigate the coordinate action of SWI/SNF and histone deacetylation in this system, two approaches were employed. First, an inducible cell line (A5-1 pTS-dnBRG1) that expresses a dominant negative mutant of BRG1 was utilized to specifically determine the requirement for SWI/SNF during RB-mediated repression in this system. Removal of Dox from the media results in the coordinate induction of both PSM-RB and mutant BRG1 as we have previously reported (42). Under these conditions RB-mediated attenuation of Plk1 RNA levels were compromised (Fig. 5*C*, *lanes 3* and *4*). Therefore, RB-mediated repression of Plk1 is dependent on SWI/SNF activity consistent with what was observed in SW13 cells. To address whether inhibition of histone deacetylase activity specifically has the potential to block RB-mediated attenuation of Plk1, a pharmacological inhibitor of deacetylase enzymes, trichostatin A (TSA), was utilized. As shown, TSA significantly reversed the RB-mediated attenuation of Plk1 RNA levels (Fig. 5*C*, *lanes 5* and *6*). To directly investigate Plk1 promoter activity, the Plk1 reporter construct was integrated into A5-1 cells. In this system, there was a 10-fold reduction in Plk1 promoter activity in presence of active RB (Fig. 5*D*). Treatment with the histone deacetylase inhibitor TSA partially alleviated RB-mediated repression of Plk1 (Fig. 5*D*). Collectively, these data demonstrate that SWI/SNF is required for the deacetylation of the Plk1 promoter and that this event is critical for repression mediated by the RB pathway. Our findings support a model wherein both SWI/SNF and histone deacetylation are required for RB-mediated repression of Plk1 (Fig. 6). Moreover, histone deacetylation of Plk1 promoter requires SWI/SNF activity, thus placing SWI/SNF and histone deacetylation in a hierarchical order for the repression of Plk1 expression.

DISCUSSION

Plk1 plays critical roles in progression through the cell cycle. Specifically, Plk1 is implicated in a variety of processes associated with mitotic progression. These activities range from roles in centrosome duplication (which occurs at the G_1/S transition) to spindle pole maturation required for a productive nuclear division (6, 7). As such, the regulation of Plk1 expression is tightly controlled, and modification of Plk1 levels is associated with diverse effects on cell biology. For example, loss of Plk1 is inconsistent with cellular viability and induces catastrophic events in mitosis (15). In contrast, ectopic expression of Plk1 is associated with cellular transformation and is deregulated in human cancers (14, 19, 43). It has been previously demonstrated that Plk1 expression is stimulated as cells progress toward the G_1/S transition (20, 44). Here we show that this gene regulation is manifested through the activity of the RB pathway. Because the RB pathway is compromised in the majority of tumors, these analyses provide a likely mechanism for the deregulation of Plk1 expression observed in tumors.

The mechanism through which RB regulates critical downstream target gene expression has been hypothesized to involve the recruitment of corepressors (25, 26). Prior studies have demonstrated that RB can recruit a myriad of corepressors to facilitate transcriptional repression (31, 53). Here we show that the activity of SWI/SNF is required for the attenuation of Plk1 with RB pathway activation. Such a result is consistent with the requirement for SWI/SNF in the repression of several additional RB target genes including cyclin A, Cdc2, and cyclin E (34, 54). These findings suggest that repression by RB may be generally dependent on SWI/SNF activity, underscoring the ability of SWI/SNF deficiency to render cells resistant to the acute cell cycle arrest elicited by activation of the RB pathway. In the context of RB-mediated repression of the Plk1 promoter, there are several possible requisite actions for SWI/SNF. First, the transcription factor responsible for recruiting RB and related proteins could fail to associate with its cognate response element in the absence of SWI/SNF activity. Such a phenomenon is observed in yeast, wherein the Gal4 transcription factor requires SWI/SNF activity to associate with chromatin *in vitro* (47). Similarly, the recruitment of GCN5-containing complexes

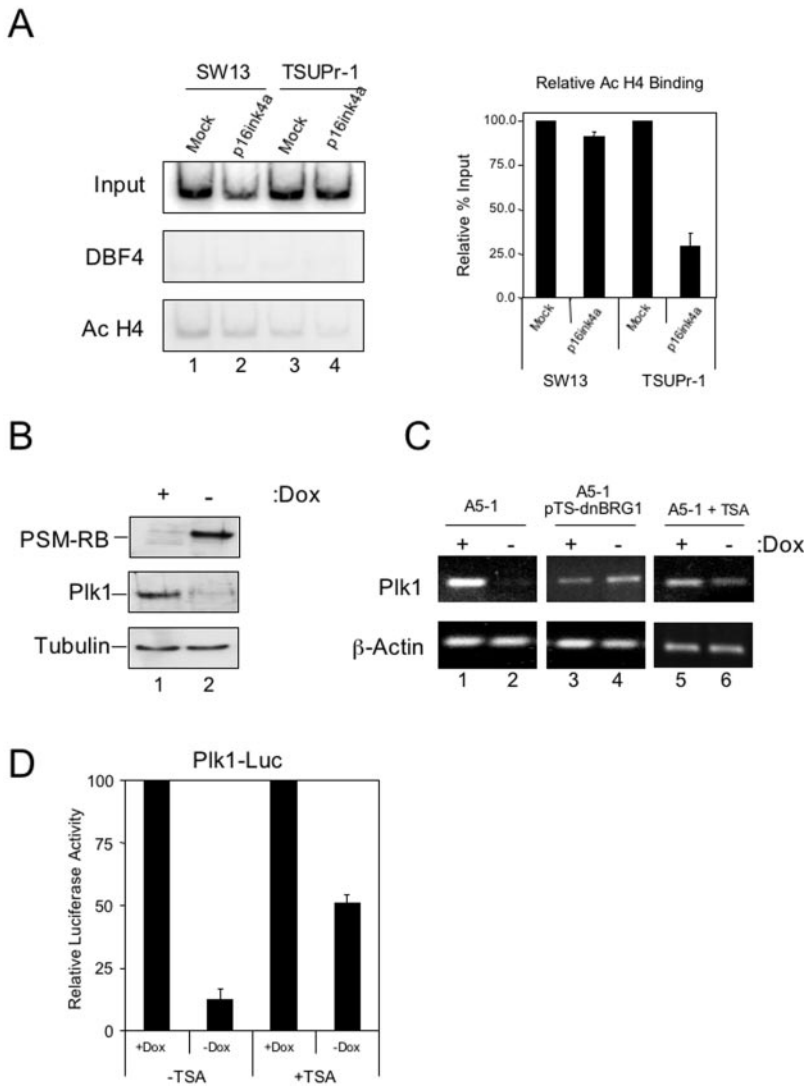

FIG. 5. **SWI/SNF is required for histone deacetylase-mediated repression of the Plk1 promoter.** *A*, *left panel* SW13 (*lanes 1* and *2*) and TSUPr-1 (*lanes 3* and *4*) cells were either mock infected (*lanes 1* and *3*) or infected with p16ink4a (*lanes 2* and *4*) encoding adenoviruses. The ChIP assays were performed with antibodies for acetylated histone H4. Input and immunoprecipitated DNA was amplified by PCR using primers specific for the Plk1 promoter. *Right panel*, quantitative analyses of histone acetylation of the Plk1 promoter from two independent experiments. *B*, A5-1 cells were cultured in the presence (*lane 1*) or absence (*lane 2*) of doxycycline for 24 h. Total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. *C*, A5-1 (*lanes 1*, *2*, *5*, and *6*) or A5-1 pTS-dnBRG1 (*lanes 3* and *4*) cells were cultured in the presence (*lanes 1*, *3*, and *5*) or absence (*lanes 2*, *4*, and *6*) of Dox and the addition of 100 nM TSA (*lanes 5* and *6*). Total RNA was isolated 24 h post-treatment, and the indicated RNA levels were determined by RT-PCR analyses. *D*, the Plk1-Luc reporter construct was stably integrated into A5-1 cells, and three clones were selected. The cells were cultured with or without Dox in the presence or absence of TSA. The relative luciferase activity was determined from three independent experiments with the *+Dox* condition set to 100.

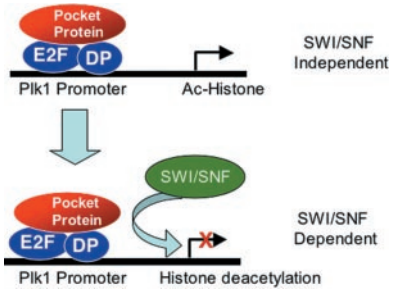

FIG. 6. **Schematic diagram of transcriptional regulation of Plk1.** Here we show that activation of the Plk1 promoter is regulated by E2F and pocket proteins. In SWI/SNF-deficient cells, E2F4 and pocket proteins are recruited to the Plk1 promoter. Despite the presence of the pocket proteins, histones remain acetylated and the promoter retains activity. By contrast, in the presence of SWI/SNF, the E2F4 and pocket protein recruitment results in histone deacetylation at the Plk1 promoter and subsequent promoter repression. In summary, our data support a model of hierarchy between SWI/SNF and histone deacetylation of the Plk1 promoter.

to specific promoters is dependent on SWI/SNF activity (38). In the case of the RB pathway this transcription factor would be the E2F family of transcription factors, and it is well known that disruption of E2F-chromatin association represents one means to bypass RB-mediated arrest (30, 55). However, we show here that SWI/SNF is not required for the basic retention of E2F proteins on chromatin, using both reporter assays and a live cell imaging approach. Additionally, we can readily detect E2F4 at the Plk1 promoter in the absence of SWI/SNF activity. Second, RB and related proteins could fail to stably interact with E2F at the promoter in the absence of SWI/SNF. We addressed this possibility by delineating the binding of RB to E2F in a simple functional interaction study, which revealed that RB does retain the capacity to interact with E2F proteins to inhibit transactivation. Additionally, we could clearly detect the RB-related proteins p107 and p130 at the Plk1 promoters by ChIP. Thus, assembly of potential repressor complexes at target promoters occurs independently of SWI/SNF. Last, SWI/SNF activity could in fact be required for chromatin modifications leading to repression. It has been previously established that RB-repressor complexes can utilize histone deacetylation as a means to facilitate transcriptional repression (34, 42). Analyses of the Plk1 promoter clearly demonstrated histone deacetylation during repression. However, this deacetylation was dependent on SWI/SNF activity. In the case of the RB family of proteins, histone deacetylation plays an important role in transcriptional repression of specific genes. For example, the cyclin E, TopoIIα, TS, and Cdc2 genes are repressed via the RB pathway in a histone deacetylase-dependent manner (34, 42). Our results suggest that the requisite action of SWI/SNF in the repression of these genes could be solely through the control of the histone deacetylation of these promoters. Such a possibility is demonstrated in the case of Plk1 where the

inhibitor of histone deacetylation, TSA, significantly reversed RB-mediated repression.

In conclusion, these studies delineate a critical mechanism through which Plk1 is transcriptionally regulated. Specifically, the RB pathway is responsible for repression of the Plk1 promoter. This repression is dependent on SWI/SNF functioning in a hierarchical manner to control histone deacetylation of the Plk1 promoter.

Acknowledgments—We thank Dr. Karen Knudsen and all members of both Knudsen laboratories for thought-provoking discussion and assistance with manuscript preparation. Additionally, we were assisted by the generous contributions of reagents from Dr. Peter Stambrook, Dr. Anthony Imbalzano, Dr. Igor Roninson, and Dr. Takeshi Uchiumi.

REFERENCES

1. Sherr, C. J., and McCormick, F. (2002) *Cancer Cell* **2,** 103–112
2. Sherr, C. J. (2000) *Harvey Lect.* **96,** 73–92
3. Sears, R. C., and Nevins, J. R. (2002) *J. Biol. Chem.* **277,** 11617–11620
4. Golsteyn, R. M., Schultz, S. J., Bartek, J., Ziemiecki, A., Ried, T., and Nigg, E. A. (1994) *J. Cell Sci.* **107,** 1509–1517
5. Golsteyn, R. M., Lane, H. A., Mundt, K. E., Arnaud, L., and Nigg, E. A. (1996) *Prog. Cell Cycle Res.* **2,** 107–114
6. Leung, G. C., and Sicheri, F. (2003) *Cell* **115,** 3–4
7. Nigg, E. A. (1998) *Curr. Opin. Cell Biol.* **10,** 776–783
8. Toyoshima-Morimoto, F., Taniguchi, E., Shinya, N., Iwamatsu, A., and Nishida, E. (2001) *Nature* **410,** 215–220
9. Lane, H. A., and Nigg, E. A. (1996) *J. Cell Biol.* **135,** 1701–1713
10. Holtrich, U., Wolf, G., Brauninger, A., Karn, T., Bohme, B., Rubsamen-Waigmann, H., and Strebhardt, K. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1736–1740
11. Knecht, R., Elez, R., Oechler, M., Solbach, C., von Ilberg, C., and Strebhardt, K. (1999) *Cancer Res.* **59,** 2794–2797
12. Kneisel, L., Strebhardt, K., Bernd, A., Wolter, M., Binder, A., and Kaufmann, R. (2002) *J. Cutan. Pathol.* **29,** 354–358
13. Takahashi, T., Sano, B., Nagata, T., Kato, H., Sugiyama, Y., Kunieda, K., Kimura, M., Okano, Y., and Saji, S. (2003) *Cancer Sci.* **94,** 148–152
14. Smith, M. R., Wilson, M. L., Hamanaka, R., Chase, D., Kung, H., Longo, D. L., and Ferris, D. K. (1997) *Biochem. Biophys. Res. Commun.* **234,** 397–405
15. Liu, X., and Erikson, R. L. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 5789–5794
16. Spankuch-Schmitt, B., Bereiter-Hahn, J., Kaufmann, M., and Strebhardt, K. (2002) *J. Natl. Cancer Inst.* **94,** 1863–1877
17. Cogswell, J. P., Brown, C. E., Bisi, J. E., and Neill, S. D. (2000) *Cell Growth Differ.* **11,** 615–623
18. Lee, K. S., Yuan, Y. L., Kuriyama, R., and Erikson, R. L. (1995) *Mol. Cell. Biol.* **15,** 7143–7151
19. Wolf, G., Elez, R., Doermer, A., Holtrich, U., Ackermann, H., Stutte, H. J., Altmannsberger, H. M., Rubsamen-Waigmann, H., and Strebhardt, K. (1997) *Oncogene* **14,** 543–549
20. Uchiumi, T., Longo, D. L., and Ferris, D. K. (1997) *J. Biol. Chem.* **272,** 9166–9174
21. Zhu, H., Chang, B. D., Uchiumi, T., and Roninson, I. B. (2002) *Cell Cycle* **1,** 59–66
22. Nevins, J. R. (2001) *Hum. Mol. Genet.* **10,** 699–703
23. Cam, H., and Dynlacht, B. D. (2003) *Cancer Cell* **3,** 311–316
24. Nevins, J. R. (1998) *Cell Growth Differ.* **9,** 585–593
25. Harbour, J. W., and Dean, D. C. (2000) *Curr. Opin. Cell Biol.* **12,** 685–689
26. Harbour, J. W., and Dean, D. C. (2001) *Curr. Top. Microbiol. Immunol.* **254,** 137–144
27. Muchardt, C., and Yaniv, M. (2001) *Oncogene* **20,** 3067–3075
28. Mittnacht, S. (1998) *Curr. Opin. Genet. Dev.* **8,** 21–27
29. Harbour, J. W., and Dean, D. C. (2000) *Genes Dev.* **14,** 2393–2409
30. Zhang, H. S., Postigo, A. A., and Dean, D. C. (1999) *Cell* **97,** 53–61
31. Strobeck, M. W., Knudsen, K. E., Fribourg, A. F., DeCristofaro, M. F., Weissman, B. E., Imbalzano, A. N., and Knudsen, E. S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7748–7753
32. Strobeck, M. W., Reisman, D. N., Gunawardena, R. W., Betz, B. L., Angus, S. P., Knudsen, K. E., Kowalik, T. F., Weissman, B. E., and Knudsen, E. S. (2002) *J. Biol. Chem.* **277,** 4782–4789
33. Reisman, D. N., Strobeck, M. W., Betz, B. L., Sciariotta, J., Funkhouser, W., Jr., Murchardt, C., Yaniv, M., Sherman, L. S., Knudsen, E. S., and Weissman, B. E. (2002) *Oncogene* **21,** 1196–1207
34. Zhang, H. S., Gavin, M., Dahiya, A., Postigo, A. A., Ma, D., Luo, R. X.,1 Harbour, J. W., and Dean, D. C. (2000) *Cell* **101,** 79–89
35. Peterson, C. L., and Workman, J. L. (2000) *Curr. Opin. Genet. Dev.* **10,** 187–192
36. Narlikar, G. J., Fan, H. Y., and Kingston, R. E. (2002) *Cell* **108,** 475–487
37. Kingston, R. E., and Narlikar, G. J. (1999) *Genes Dev.* **13,** 2339–2352
38. Fry, C. J., and Peterson, C. L. (2001) *Curr. Biol.* **11,** R185–R197
39. Vooijs, M., Jonkers, J., Lyons, S., and Berns, A. (2002) *Cancer Res.* **62,** 1862–1867
40. Strobeck, M. W., Fribourg, A. F., Puga, A., and Knudsen, E. S. (2000) *Oncogene* **19,** 1857–1867
41. Angus, S. P., Fribourg, A. F., Markey, M. P., Williams, S. L., Horn, H. F., DeGregori, J., Kowalik, T. F., Fukasawa, K., and Knudsen, E. S. (2002) *Exp. Cell Res.* **276,** 201–213
42. Siddiqui, H., Solomon, D. A., Gunawardena, R. W., Wang, Y., and Knudsen, E. S. (2003) *Mol. Cell. Biol.* **23,** 7719–7731
43. Yuan, J., Horlin, A., Hock, B., Stutte, H. J., Rubsamen-Waigmann, H., and Strebhardt, K. (1997) *Am. J. Pathol.* **150,** 1165–1172
44. Hamanaka, R., Smith, M. R., O'Connor, P. M., Maloid, S., Mihalic, K., Spivak, J. L., Longo, D. L., and Ferris, D. K. (1995) *J. Biol. Chem.* **270,** 21086–21091
45. Markey, M. P., Angus, S. P., Strobeck, M. W., Williams, S. L., Gunawardena, R. W., Aronow, B. J., and Knudsen, E. S. (2002) *Cancer Res.* **62,** 6587–6597
46. Reisman, D. N., Strobeck, M. W., Betz, B. L., Sciariotta, J., Funkhouser, W., Jr., Murchardt, C., Yaniv, M., Sherman, L. S., Knudsen, E. S., and Weissman, B. E. (2003) *Cancer Res.* **63,** 560–566
47. Burns, L. G., and Peterson, C. L. (1997) *Mol. Cell. Biol.* **17,** 4811–4819
48. Wu, C. L., Classon, M., Dyson, N., and Harlow, E. (1996) *Mol. Cell. Biol.* **16,** 3698–3706
49. Trimarchi, J. M., and Lees, J. A. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 11–20
50. Angus, S. P., Solomon, D. A., Kuschel, L., Hennigan, R. F., and Knudsen, E. S. (2003) *Mol. Cell. Biol.* **23,** 8172–8188
51. Wells, J., Boyd, K. E., Fry, C. J., Bartley, S. M., and Farnham, P. J. (2000) *Mol. Cell. Biol.* **20,** 5797–5807
52. Takahashi, Y., Rayman, J. B., and Dynlacht, B. D. (2000) *Genes Dev.* **14,** 804–816
53. Luo, R. X., Postigo, A. A., and Dean, D. C. (1998) *Cell* **92,** 463–473
54. Strobeck, M. W., DeCristofaro, M. F., Banine, F., Weissman, B. E., Sherman, L. S., and Knudsen, E. S. (2001) *J. Biol. Chem.* **276,** 9273–9278
55. Rowland, B. D., Denissov, S. G., Douma, S., Stunnenberg, H. G., Bernards, R., and Peeper, D. S. (2002) *Cancer Cell* **2,** 55–65