SWI/SNF Activity Is Required for the Repression of Deoxyribonucleotide Triphosphate Metabolic Enzymes via the Recruitment of mSin3B*

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The SWI/SNF chromatin remodeling complex plays a critical role in the coordination of gene expression with physiological stimuli. The synthetic enzymes ribonucleotide reductase, dihydrofolate reductase, and thymidylate synthase are coordinately regulated to ensure appropriate deoxyribonucleotide triphosphate levels. Particularly, these enzymes are actively repressed as cells exit the cell cycle through the action of E2F transcription factors and the retinoblastoma tumor suppressor/p107/p130 family of pocket proteins. This process is found to be highly dependent on SWI/SNF activity as cells deficient in BRG-1 and Brm subunits fail to repress these genes with activation of pocket proteins, and this deficit in repression can be complemented, via the ectopic expression of BRG-1. The failure to repress transcription does not involve a blockade in the association of E2F or pocket proteins p107 and p130 with promoter elements. Rather, the deficit in repression is due to a failure to mediate histone deacetylation of ribonucleotide reductase, dihydrofolate reductase, and thymidylate synthase promoters in the absence of SWI/SNF activity. The basis for this is found to be a failure to recruit mSin3B and histone deacetylase proteins to promoters. Thus, the coordinate repression of deoxyribonucleotide triphosphate metabolic enzymes is dependent on the action of SWI/SNF in facilitating the assembly of repressor complexes at the promoter.

Specifically, SNF5 is a tumor suppressor involved in rhabdoid tumors (8–10), while sporadic loss of multiple other subunits is observed in a litany of tumor types (11). In general, the loss of these factors has been implicated in deregulation of target genes associated with cell cycle control, particularly those regulated by the RB/E2F signaling axis (12–14).

The retinoblastoma tumor suppressor (RB)2 was identified based on loss in the pediatric eye tumor of the same name (15–18) and demonstrated to function as a transcriptional modulator. Specifically, RB and related pocket proteins p107/p130 bind to members of the E2F family of transcription factors and mediate transcriptional repression (19–23). These effects on transcription are mediated by the co-recruitment of a myriad of co-repressors to E2F-regulated promoters (24, 25). In this context, SWI/SNF activity is critically important, and deficiency of SWI/SNF activity has been associated with deregulated E2F-dependent gene expression (26).

A critical gene expression program involved in cell cycle control is the regulation of dNTP synthetic enzymes. As cells progress into S-phase there is a coordinated stimulation in the levels of ribonucleotide reductase subunit II (RNRII), thymidylate synthase (TS), and dihydrofolate reductase (DHFR). It is believed that the up-regulation of such synthetic enzymes is important for expanding the pool of dNTPs for DNA synthesis (27, 28). Consistent with this overall idea, multiple DNA tumor viruses harbor oncoproteins that up-regulate the same dNTP metabolic enzymes, presumably to promote viral replication (29). Importantly, these oncoproteins disrupt the function of RB, and it is known that expression of each of these genes is regulated via the E2F family of transcription factors. Conversely, as cells exit cell cycle these genes are repressed by the action of RB and related proteins. Here we define the mechanism through which SWI/SNF participates in the regulation of RNRII, TS, and DHFR in the context of RB-mediated repression.

Experimental Procedures

Cell Culture, Plasmids, Infections, and Transfections—SW13 and TSUPr-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, ...
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100 units of penicillin-streptomycin, 2 mm L-glutamine at 37 °C in 5% CO₂. Plasmids encoding β-galactosidase, p16INK4a, CMV-NeoBam, and BRG-1 have been previously described (30, 31). The reporter constructs RNRII-luc, TS-luc, and DHFR-luc have been previously described (30, 31).

Adenoviruses encoding GFP, p16INK4a, and BRG-1 have been previously described (32, 33). Infections were performed at a multiplicity of infection of 50–100 for ~95–100% infection efficiency after 24–36 h as determined by GFP fluorescence.

Reporter Assays, Immunoblotting, Reverse Transcriptase PCR, and Quantitative PCR—Immunoblotting was performed using standard techniques. The following antibodies were used, purified mouse anti-human Rb (catalogue number 554136; BD Biosciences), p107 (SC-318; Santa Cruz Biotechnology), p130 (SC-317; Santa Cruz Biotechnology), RNRII (SC-10848), TS (gift from Masakazu Fukushima), DHFR (610696; BD Transduction Laboratories), p16INK4a (SC-759; Santa Cruz), E2F4 (SC-1082; Santa Cruz), Lamin B (SC-6217; Santa Cruz). All of the immunoblots were repeated at least two times with independent samples.

The reporter assays were performed as described previously (34). The data reflect the average of at least three independent experiments.

Reverse Transcriptase PCR was performed as described previously (34). The following primer pairs were utilized: human RNRII 5′-CCT CTC CAA GGA CAT TCA GC-3′ and 5′-GGC AAT TTT GAA GCC ATA GA-3′, human TS 5′-TTG GAA GCC ATA GA-3′, human TS 5′-ATT TGG AAT GGC AGT TGA CC-3′ and 5′-CAT GTC TCC CGA TCT CTG GT-3′, human DHFR 5′-GGT TCG CTA ACG TCT GT-3′ and 5′-TGA GCT CCT TGT GGA GGT TC-3′. The experiments were performed at least three times, and representative data are shown. Reverse transcriptase PCR was also performed on RNA extracted from TSUPr-1 cells that had been treated with 400 nM histone deacetylase (HDAC) inhibitors trichostatin A (TSA) (Sigma) for 18–24 h in the presence of adenovirus encoding GFP and p16INK4a.

Real-time PCR—Real-time PCR was performed using SYBR Green PCR master mix (Applied Biosystem). Absolute DNA quantities were measured by using the Applied Biosystem Fast Real-time PCR, and data were normalized to account for variation in inputs to determine the relative quantities. The following primers were used to amplify regions of the human RNRII 5′-GGG AGA TTT AAA GGC TGC TGG AGT GA-3′ and 5′-ACA CCG AGG GAG AGC ATA GTG GA-3′, TS 5′-TAA GAC TCT CAG CTG TGG CCC TG-3′ and 5′-AGA GAG GCA GGC GAA GTG CTG GCA CAA ATG ACC-3′. The experiments were performed at least three times with independent samples, with the following conditions: 95 °C, 10 min; 59 °C, 1 min; 60 °C, 1 min, for 40 cycles.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed as previously described (34). The following antibodies were utilized for ChIP: p107 (SC-318; Santa Cruz Biotechnology), p130 (SC-317; Santa Cruz), E2F4 (SC-1082; Santa Cruz), anti-HDAC1 (05–614; Upstate Biotechnology, Inc.), Brm (15597; Abcam), acetylated histone H4 (06–866; Upstate Biotechnology, Inc.), Dbf4 (SC-11354; Santa Cruz), and mSin3B (SC-768; Santa Cruz). The following primers were used to amplify regions of the human RNRII 5′-GAG GCA TGC ACA GCC AAT-3′ and 5′-GAC ACG GAG GGA GAG CAT AG-3′, TS 5′-TCC GGT CTG TGC CAC ACC-3′ and 5′-TGG ATC TGCC CCA AGA GT-3′, DHFR 5′-AAG GAT GAG GAG GCC AAC AGT-3′ and 5′-GCC GCT CGT TAC AGC AGA-3′. The ChIP assays were repeated twice with independent samples.

RESULTS

The RB Pathway Regulates RNRII, TS, and DHFR Gene Expression in a SWI/SNF-dependent Manner—The dNTP metabolic genes RNRII, TS, and DHFR are tightly regulated to ensure the appropriate coordination of dNTP production with cell cycle control. To define the mechanisms through which this regulation occurs, we challenged the response of such enzymes to activation of the RB pathway. For these analyses we initially utilized the SW13 cell line, which is deficient in BRG-1 and Brm ATPase requisite for SWI/SNF activity, and TSUPr-1, which is proficient in Brm (35). To activate the endogenous RB pathway, SW13 and TSUPr-1 cells were infected with adenoviruses encoding either GFP (control) or p16INK4a (lanes 1 and 3) or p16INK4a (lanes 2 and 4)-encoding adenoviruses. A, cells were harvested 36 h post-infection, total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. B, total RNA was extracted 36 h post-infection and subjected to linear reverse transcriptase PCR amplification with primers specific for the indicated genes. C, total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting.

FIGURE 1. RB pathway represses RNRII, TS, and DHFR expression. 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. A, cells were harvested 36 h post-infection, total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. B, total RNA was extracted 36 h post-infection and subjected to linear reverse transcriptase PCR amplification with primers specific for the indicated genes. C, total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting.
pRB are also decreased, which is consistent with previously published models of p16ink4a-mediated cell cycle arrest (32, 37). The expression of p16ink4a resulted in highly significant diminution of the RNA levels of RNRII, TS, and DHFR in TSUPr-1 cells (Fig. 1B, compare lanes 3 and 4). In contrast, we observed no attenuation of endogenous RNA levels of RNRII, TS, and DHFR in SW13 cells infected with p16ink4a, even though the pocket proteins were amply dephosphorylated (Fig. 1B, compare lanes 1 and 2). These observations were further strengthened by analyses of RNRII, TS, and DHFR protein levels in SW13 and TSUPr-1 cells. Consistent with RNA analyses, RNRII, TS, and DHFR protein levels were significantly attenuated in TSUPr-1 cells in the presence of p16ink4a (Fig. 1C, lanes 3 and 4), in contrast to SW13 cells (Fig. 1C, lanes 1 and 2). These results indicate that RNRII, TS, and DHFR expressions are regulated by the RB pathway in a manner that is dependent on SWI/SNF activity.

Having confirmed that RNRII, TS, and DHFR genes are regulated via the RB pathway, the specific impact on their respective promoters was investigated. SW13 and TSUPr-1 cells were cotransfected with RNRII, TS, and DHFR promoter plasmid, respectively, and either vector control or p16ink4a-encoding plasmids. Overexpression of p16ink4a significantly repressed the promoter activity of RNRII, TS, and DHFR in SWI/SNF-proficient TSUPr-1 cells. In contrast, p16ink4a failed to repress promoter activity in SWI/SNF-deficient SW13 cells (Fig. 2A). To functionally demonstrate the requirement for SWI/SNF activity, BRG-1 expression was restored in SW13 cells in combination with p16ink4a, and RNRII, TS, and DHFR promoter activity was analyzed through reporter analysis. Similar to our earlier observation, p16ink4a alone failed to repress RNRII, TS, and DHFR promoter activity. However, cotransfction of BRG-1 and p16ink4a significantly repressed these promoters in SWI/SNF-deficient SW13 cells (Fig. 2B). Consistent with reporter assays, we observed attenuation of endogenous RNA (Fig. 2C) and protein levels (Fig. 2D) of RNRII, TS, and DHFR upon restoring

FIGURE 3. SWI/SNF is dispensable for RB-related pocket proteins and E2F4 association at RNRII, TS, and DHFR promoters. All experiments utilized SW13 (lanes 1 and 2) and TSUPr-1 (lanes 3 and 4) cells infected with either GFP (lanes 1 and 2) or p16ink4a (lanes 2 and 4)-encoding adenoviruses. ChIP assays were performed with antibodies for E2F4 and Dbf4 (A) (nonspecific), p107 and Dbf4 (B), p130 and Dbf4 (C), and Brm and Dbf4 (D). Input and immunoprecipitated DNA were amplified by PCR with primers specific for the RNRII, TS, and DHFR promoters.

FIGURE 2. SWI/SNF is required for RB-mediated repression of RNRII, TS, and DHFR expression. A, SW13 and TSUPr-1 cells were cotransfected with cytomegalovirus β-galactosidase and RNRII-Luc, TS-Luc, and DHFR-Luc reporter plasmids and either vector or p16ink4a expression plasmids. Relative luciferase activity was normalized to β-galactosidase activity for transfection efficiency, and the vector control was set to 100. B, SW13 cells were cotransfected with cytomegalovirus β-galactosidase, RNRII-Luc, TS-Luc, or DHFR-Luc reporter plasmids and either vector, p16ink4a, or BRG-1 expression plasmids. The relative luciferase activity was normalized to β-galactosidase activity for transfection efficiency, and vector control was set to 100. Error bars represent average relative luciferase activity and S.D. Results are representative of three independent experiments. C, SW13 cells were infected with either GFP (lane 1), p16ink4a (lane 2), BRG-1 (lane 3), or p16ink4a and BRG-1 (lane 4)-encoding adenoviruses. Total RNA was extracted 36 h post-infection and subjected to linear reverse transcriptase PCR amplification with primers specific for the indicated genes. D, SW13 cells were infected as in panel C. Cells were harvested 36 h post-infection, total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting.
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**Figure 4.** RB-induced deacetylation at RNRII, TS, and DHFR promoters is mediated by HDACs and dependent on SWI/SNF. A, SW13 and TSUPr-1 cells were infected with GFP or p16ink4a-encoding adenoviruses. ChIP assays were performed with antibody specific for acetylated histone H4. Real-time PCR was performed as described under “Experimental Procedures,” and the GFP control was set to 100. Error bars represent the mean ± S.D.; n = 3. B, TSUPr-1 cells were infected with GFP (lane 1), GFP + TSA (lane 2), p16ink4a (lane 3), or p16ink4a and TSA (lane 4) for 18-24 h. Total RNA was extracted and reversed-transcribed into cDNA. This cDNA was subjected to linear PCR amplification with specific primers for the indicated genes. C, TSUPr-1 cells were infected and treated with TSA as indicated in panel B. Total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting.

In summary, these data demonstrate that SWI/SNF activity is required for RB-mediated repression of RNRII, TS, and DHFR promoters and attenuation of its RNA and protein levels.

**SWI/SNF Is Dispensable for E2F4 and RB-related Pocket Protein Association at RNRII, TS, and DHFR Promoters.**—The inability of the RB pathway to repress RNRII, TS, and DHFR promoters in the absence of SWI/SNF activity could be a consequence of multiple levels of dysfunction in repression. Initially, the requirement of SWI/SNF for E2F family members to access the respective promoters was determined. Specifically, the ability of E2F proteins to bind the RNRII, TS, and DHFR promoters was evaluated by chromatin immunoprecipitation assays (38). The E2F family of transcription factors consists of at least seven members (E2F1-7), of which E2F4 and E2F5 are transcriptional repressors (39-41). SW13 and TSUPr-1 cells were infected with either GFP or p16ink4a-encoding adenoviruses, and protein-DNA complexes were cross-linked with formaldehyde and immunoprecipitated with antibodies specific for E2F4 and Dbf4 (nonspecific control). Approximately equal recruitment of E2F4 at RNRII, TS, and DHFR promoters was observed in the presence or absence of SWI/SNF (Fig. 3A). Thus, SWI/SNF activity is apparently not required for the recruitment of E2F4 to these promoters.

As the subsequent step in transcriptional repression, pocket proteins are believed to be recruited to promoters to facilitate the transcriptional repression. For these analyses, SW13 and TSUPr-1 cells were infected with either GFP or p16ink4a to activate endogenous pocket proteins, and recruitment of p107 and p130 to the promoters was then determined by chromatin immunoprecipitation. We observed differential recruitment of p107 and p130 to the respective promoters. Specifically, there was a general trend toward loss of p107 with p16ink4a expression (Fig. 3B), which is countered by enhanced p130 recruitment to the promoters (Fig. 3C). These findings are consistent with the differential involvement of p107 and p130 in promoter regulation in quiescent versus proliferative cells (42, 43). Importantly, this differential recruitment occurred in both SWI/SNF-proficient and -deficient cells, indicating that p107/p130 recruitment to chromatin is SWI/SNF-independent.

Lastly, SWI/SNF ATPase recruitment to the promoters was determined. As shown in Fig. 3D, Brm p16ink4a and BRG-1 in SWI/SNF-deficient SW13 cells. In summary, these data demonstrate that SWI/SNF activity is required for RB-mediated repression of RNRII, TS, and DHFR promoters and attenuation of its RNA and protein levels.

**RB-induced Deacetylation at RNRII, TS, and DHFR Promoters Is Mediated by HDACs in the Presence of SWI/SNF.** Pocket proteins are believed to repress transcription by facilitating alterations in chromatin structure that are mediated via the recruitment of additional co-repressors (10, 44-46). However, the extent to which these multiple chromatin-modifying enzymes act in concert to mediate transcription is not well documented. To initially investigate chromatin modifications and corresponding dependence on SWI/SNF for this process, we examined histone deacetylation. Chromatin immunoprecipitation assays were performed utilizing anti-acetyl histone H4
antibody (Fig. 4A). As expected, infection with p16ink4a resulted in significant histone deacetylation at the RNRII, TS, and DHFR promoters in SWI/SNF-proficient TSUPr-1 cells. In contrast, p16ink4a infection failed to induce histone H4 deacetylation at the promoters in SWI/SNF-deficient SW13 cells. Thus, these data suggest that whereas recruitment of E2F and pocket proteins to these promoters is SWI/SNF-independent, the histone deacetylation of these promoters is SWI/SNF-dependent.

To address the functional role of histone deacetylation, we suppressed histone deacetylase activity using the pharmacological inhibitor of HDAC, TSA. For these analyses, SWI/SNF-proficient TSUPr-1 cells were infected with adenoviruses encoding GFP or p16ink4a in the presence or absence of TSA for 18–24 h. The administration of TSA alone did not have a significant effect on target gene expression. However, TSA was able to partially reverse the transcriptional repression induced by p16ink4a, as observed at both the RNA (Fig. 4B) and protein levels (Fig. 4C). These results indicate that HDAC activity contributes to the SWI/SNF-mediated transcriptional repression of these target promoters.

**Recruitment of HDAC1 and mSin3B at the RNRII, TS, and DHFR Promoters Is Dependent on SWI/SNF Activity**—Multiple HDAC complexes have been implicated in RB-mediated transcriptional repression, many of which contain the Sin3 subunits (47–50). Therefore, we investigated the recruitment of mSin3B and HDAC1 to target promoters in the presence of p16ink4a. As shown in Fig. 5, both mSin3B (A) and HDAC1 (B) are effectively recruited to the RNRII, TS, and DHFR promoters in TSUPr-1 cells exposed to p16ink4a. Thus, the recruitment of these co-repressors correlates with transcriptional repression and histone deacetylation. Strikingly, we observed little recruitment of mSin3B or HDAC1 to the same promoters in SW13 cells, suggesting that SWI/SNF plays a critical role in the recruitment of these co-repressors to these promoters. 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by the RB pathway is compromised in SWI/SNF-deficient cells. This altered regulation of expression is not a consequence of altered E2F or pocket protein loading at the promoters. Rather, it is specifically associated with a deficit in histone deacetylation. This is dependent on a failure to recruit mSin3B to target promoters. Re-introduction of functional SWI/SNF ATPase subunits into these cells facilitated this recruitment, demonstrating a dependence on full SWI/SNF complex assembly.

A multitude of transcriptional events are dependent on SWI/SNF activity (34, 51–53). Here we specifically focused on the role of SWI/SNF in coordinating the transcriptional repression of dNTP synthetic enzymes RNRII, TS, and DHFR. Together these factors modulate the dNTP pools and are additionally the targets of dNTP synthetic enzymes RNRII, TS, and DHFR. Together these factors modulate the dNTP pools and are additionally the targets of specific agents that have medical applications (e.g. 5-Fluorouracil and methotrexate) (28, 54, 55). While it is well appreciated that activation of the RB pathway can repress the expression of these genes (27, 56), our studies demonstrate that deficiency in SWI/SNF activity precludes the ability to effectively modulate their gene expression. Thus, akin to Cyclin A and Plk1 these targets of the RB/E2F axis are dependent on SWI/SNF activity for transcriptional repression. This finding suggests that such regulated gene expression may be intrinsically dependent on SWI/SNF activity. These results support the concept that loss of SWI/SNF activity is tantamount to the inactivation of pocket proteins. Importantly, it should be noted that none of these targets is observed in a recently described list of genes deregulated upon SNF5 deletion (57–60). Thus, it remains unclear as to how deficiency in SWI/SNF activity influences the basal levels of these genes. However, it is increasingly apparent that SWI/SNF activity is often specifically required in the context of a change in gene expression that is elicited by a physiological signal. For example, multiple studies have demonstrated an important role for SWI/SNF function in differentiation paradigms.

The SWI/SNF chromatin-remodeling enzyme plays a highly complex role in modulating gene expression (61–64). Dependent on the target gene, SWI/SNF can be important for a variety of critical processes involved in gene activation (65–68) or repression (34, 46, 69). Particularly, SWI/SNF activity facilitates the recruitment of either transcription factors or co-activators to facilitate transcriptional activation. Here we find that neither the recruitment of E2F nor the subsequent recruitment of p107 or p130 is dependent on SWI/SNF activity. Importantly, during pocket protein-mediated transcriptional repression there is often a switch between p107 and p130 in the “repressive” complexes (42). This dynamic switch is believed to reflect the relative levels of the proteins as a function of phosphorylation status, and importantly this switching occurs even in the absence of SWI/SNF activity. Thus, SWI/SNF seems to hold little influence upon the proximal events associated with E2F-mediated transcriptional regulation, and Brm recruitment to these promoters is not modulated during the engagement of transcriptional repression. However, the repression of many E2F-regulated genes is not solely dependent on pocket protein status but requires the co-recruitment of co-repressors (34, 45, 46). These co-repressors can have a myriad of activities, including histone deacetylation (34, 46) or histone methylation (70–72). Here we specifically interrogated histone deacetylation and observed that HDAC activity was important for transcriptional repression. Additionally, this facet of gene regulation was exquisitely dependent on the presence of SWI/SNF activity. Such a finding is consistent with the observation that SWI/SNF can play an important role in facilitating transcriptional repression via histone deacetylation (46, 73). Here we further demonstrate that this lack of deacetylation occurs in concert with a failure to recruit mSin3B and HDAC1 to the RNRII, TS, and DHFR promoters in the absence of SWI/SNF. Thus, these findings strongly argue that the role of SWI/SNF in the process of pocket protein-mediated repression is to facilitate mSin3B recruitment to promoters in a manner that enforces transcriptional repression. These studies further reinforce the concept that SWI/SNF activity is critical for the stable assembly of transcription modulatory complexes that impinge upon chromatin to mediate effects on gene expression.

Acknowledgments—We thank Dr. Emily Bosco, Dr. Kathleen McClendon, Dr. Chris Mayhew, and Seetha Srinivasan for critical reading of the manuscript and the members of Knudsen laboratories for thought-provoking discussion. Critical reagents for this study were provided by Dr. Rod Bremner (University of Toronto).

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