The *c-myc* Gene Is a Direct Target of Mammalian SWI/SNF–Related Complexes during Differentiation-Associated Cell Cycle Arrest

Norman G. Nagl, Jr.,[1] Daniel R. Zweitzig,[1] Bayar Thimmapaya,[2] George R. Beck, Jr.,[3] and Elizabeth Moran[1]

[1]Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, Pennsylvania; [2]Department of Microbiology-Immunology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois; and [3]Division of Endocrinology, Metabolism and Lipids, Emory University School of Medicine, Atlanta Georgia

Abstract

The activity of mammalian SWI/SNF–related chromatin remodeling complexes is crucial for differentiation, development, and tumor suppression. Cell cycle–regulating activities dependent on the complexes include induction of the $p21^{WAF1/CIP1}$ kinase inhibitor and repression of E2F-responsive promoters. These responses are linked through effects on pRb phosphorylation, but the direct role of the SWI/SNF–related complexes in their regulation is not fully understood. Results presented here reveal that the complexes are required for regulation of a distinct pathway of proliferation control involving repression of c-*myc* expression in differentiating cells. This involves direct promoter targeting of the *c-myc* gene by the complexes. Induction of $p21^{WAF1/CIP1}$ is specifically dependent on prior repression of c-*myc*, but repression of E2F-responsive genes is dissociable from the regulation of c-*myc* and $p21^{WAF1/CIP1}$. (Cancer Res 2006; 66(3): 1289-93)

Introduction

The activity of mammalian SWI/SNF–related chromatin remodeling complexes is crucial for proper differentiation and development. The complexes are required for activation of tissue-specific genes and are also essential for repression of proliferation. Subunits critical for proliferation control are recognized as important tumor suppressors (reviewed in refs. 1, 2). The complexes contain a core ATPase (either BRG1 or BRM) plus seven or more noncatalytic subunits containing various DNA-binding and protein-binding motifs that modulate the targeting and activity of the ATPase. The stably associated noncatalytic components of the complex include the ARID1A subunit (synonyms: p270, BAF250a, hOSA1, SMARCF1), which is a member of the ARID family of DNA-binding proteins (3, 4). ARID1A is frequently deficient in tumor tissue samples and is thus implicated in the tumor suppression function of the complexes (5, 6). Depletion by small interfering RNA (siRNA) reveals that ARID1A is required for proper differentiation-associated cell cycle arrest, whereas a broadly expressed, independently encoded, alternative subunit ARID1B (7) is not required for this function (8). Monitoring the ARID1A subunit thus enables analysis of a specific subset of complexes linked with cell cycle arrest, permitting a more direct analysis of this function than previously possible. A proper understanding of the role of the complexes in tumor suppression requires identification of the critical target promoters through which the complexes restrict proliferation. This question has been addressed mostly through study of the BRG1 and BRM ATPases and of the noncatalytic subunit INI1 (synonyms: SMARCB1, hSNF5, BAF47), deficiency of each of which is linked with loss of cell cycle control and tumorigenesis.

Efforts to identify direct targets of the cell cycle arrest function of the mammalian SWI/SNF–related complexes have focused on repression of cyclins and activation of cyclin-dependent kinase inhibitors, particularly $p21^{WAF1/CIP1}$ (e.g., refs. 9–11; reviewed in ref. 2). Chromatin immunoprecipitation experiments have identified one or more of the INI1, BRG1, or BRM subunits at the cyclin D, cyclin E, and cyclin A promoters (12–15). Induction of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ occurs upon ectopic expression of BRG1 and is also dependent on INI1 expression (9, 10). Chromatin immunoprecipitation experiments have shown the presence of BRG1 at the $p21^{WAF1/CIP1}$ promoter during activation (10, 16). ARID1A depletion in differentiating cells likewise results in impaired induction of $p21^{WAF1/CIP1}$ and failed repression of E2F-responsive promoters (8). Studies described here add cdc2 and cyclin B2 to the list of E2F-responsive promoters identified as direct targets of the complexes and show that the ARID1A subunit is present on these promoters specifically upon differentiation. Parallel examination of the $p21^{WAF1/CIP1}$promoter yielded the unexpected result that ARID1A does not target this promoter directly during activation. This selective focus on ARID1A targets, distinct from the more promiscuous range of BRG1 targets, leads here to the realization that the c-*myc* promoter is a critical direct target of mammalian SWI/SNF complexes during differentiation. Repression of c-*myc*, dependent on the complexes, is a required upstream event in the induction of $p21^{WAF1/CIP1}$. Genetic analysis of these pathways shows further that ARID1A-containing complexes are required independently for repression of the E2F-responsive genes.

Materials and Methods

Cell lines, cell culture, DNA, RNA, and protein analysis. Conditions and reagents for culture and differentiation (by addition of ascorbic acid and β-glycerol phosphate to normal culture medium) of low-passage murine calvarial MC3T3-E1 cells, as well as the generation of the p270/ARID1A knockdown lines (series MC.p270.KD) from parental MC3T3-E1 cells, and the integrity of the complexes in the absence of the ARID1A subunit have been described previously (8). The previous work described three independent lines with reproducible phenotypes. Experiments shown here were done with the AA2 and CA6 lines. Preparation of samples for Western blotting and the RNA blots and glyceraldehyde-3-phosphate dehydrogenase probe were described previously, as was the protocol for the ^{3}H-thymidine incorporation (DNA synthesis) assay (8).

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation assays were done with the EZ ChIP system (Upstate Cell Signaling Solutions, Lake Placid, NY), according to manufacturer's directions.

Requests for reprints: Elizabeth Moran, Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA 19140. Phone: 215-707-7313; Fax: 215-707-6989; E-mail: betty@temple.edu.

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Virus infection. The generation and culture of the E1A-inactivated 9S adenovirus (used here as a negative control) and of the c-*myc* expression virus have been described previously (8, 17). MC3T3-E1 cells were infected at a multiplicity of infection of 25 plaque-forming units per cell.

Results and Discussion

Promoter association of ARID1A during differentiation. ARID1A stable knockdown lines were constructed in the MC3T3-E1 preosteoblast cell line. These nontransformed cells were chosen as a model system because upon exposure to differentiation medium, they undergo a tightly regulated and well-characterized progression through cell cycle arrest and into tissue-specific gene expression (e.g., refs. 8, 18). In addition, comprehensive gene expression profiles are available for each stage of differentiation (19). Generation of the ARID1A knockdown lines (the MC.p270.KD series) and their inability to withdraw from the cell cycle normally during differentiation by induction of $p21^{WAF1/CIP1}$ and repression of cell cycle specific products, such as cdc2 and cyclin B2, have been described previously (8). Illustration of ARID1A expression in a representative knockdown line is shown in Fig. 1*A*.

To confirm that the phenotype of the stable knockdown lines is solely a result of targeting ARID1A, the phenotype was tested further here using three different ARID1A-targeted siRNA sequences in a transient assay under puromycin selection. As seen previously, $p21^{WAF1/CIP1}$ is induced in parental cells in response to the differentiation signal (Fig. 1*B*, *lane 2* compared with *lane 1*). Puromycin-selected parental cells behave the same way (*lane 3*). The ARID1A knockdown line fails to induce $p21^{WAF1/CIP1}$ in these conditions (*lanes 4* and *5*). Transient transfection of the knockdown sequence used to generate the stable lines yields the same result: failure to induce $p21^{WAF1/CIP1}$ (*lane 9*). Either of two other independent ARID1A-targeted sequences again yields the same result (*lanes 7* and *8*). Transfection of a scrambled sequence has no effect (*lane 6*). Induction of differentiation is accompanied by repression of cdc2 and cyclin B2. Repression fails in the ARID1A-knockdown line (*lanes 4* and *5*) and in cells transiently transfected with any of the three ARID1A-targeted siRNA sequences (*lanes 7-9*). A probe for ARID1A levels confirms the effectiveness of the knockdown sequences in the transiently transfected cells. The transient transfection assay was also used to verify the DNA synthesis phenotype of the knockdown lines. We have shown previously that induction in parental cells results in a 7-fold decrease in DNA synthesis activity by day 4, whereas ARID1A-depleted cells maintain activity at a high level (8). The same difference is seen here between the parental and knockdown lines assayed at day 3 (Fig. 1*C*, *samples 1* and *2*) and between cells transiently transfected with any of the three knockdown sequences compared with cells transfected with a scrambled sequence (*samples 3-6*). We conclude that the phenotype observed in the stable knockdown lines is a specific result of ARID1A depletion.

ARID1A-specific monoclonal antibodies were used here in chromatin immunoprecipitation assays to monitor the specific presence of this subunit at various promoters in differentiating MC3T3-E1 cells (Fig. 2*A*). Association of ARID1A with the cdc2 or cyclin B2 promoters is not detectable on chromatin prepared from exponentially growing cells (day 0); however, ARID1A is clearly present on both the cdc2 and cyclin B2 promoters in differentiating cells (day 4). These results increase the number of E2F-responsive promoters identified as direct targets of the complexes and show the specific presence of the ARID1A subunit at a time when these genes are repressed.

Parallel analysis of association of ARID1A with the $p21^{WAF1/CIP1}$ promoter (Fig. 2*A*) revealed association in exponentially growing cells (day 0) but not in differentiating cells (day 4). This was entirely unexpected because whereas it suggests that ARID1A-containing complexes may play a direct role in suppression of the $p21^{WAF1/CIP1}$ promoter before differentiation, it indicates no direct role for ARID1A-containing complexes in induction of $p21^{WAF1/CIP1}$, although ARID1A is required for normal induction. This result also stands in potential contrast to detection of ectopically expressed

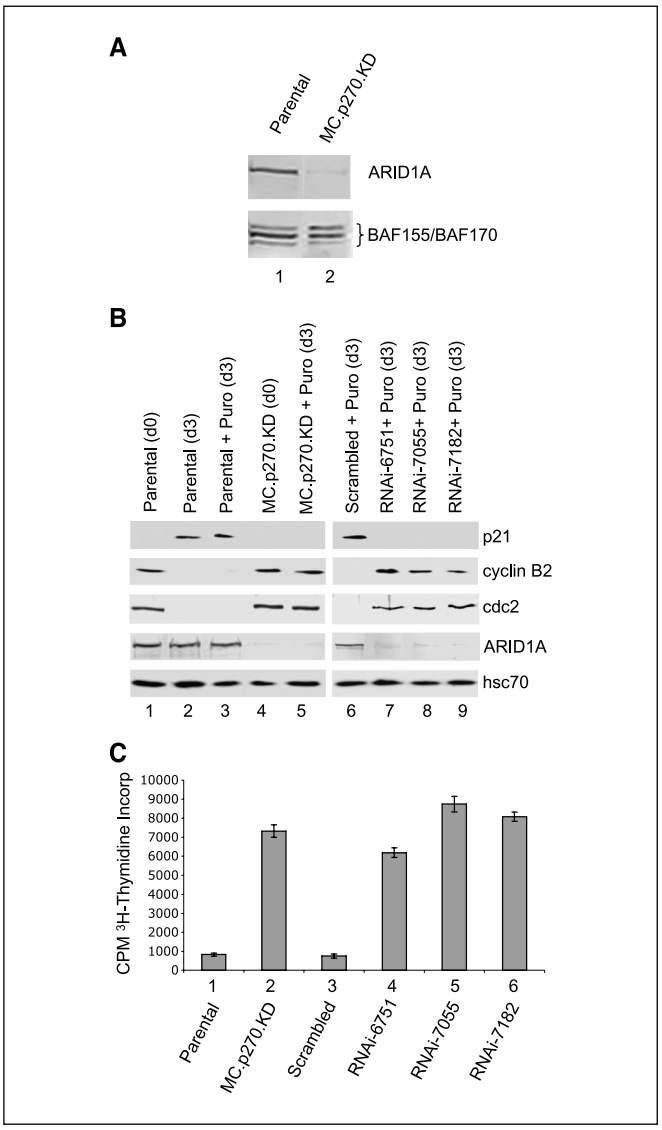

Figure 1. Phenotype of ARID1A/p270 knockdown cells. *A,* depletion of ARID1A levels in the knockdown line compared with parental MC3T3-E1 cells is shown by Western blot. Levels of the BAF155 and BAF170 subunits are shown for comparison. *B,* a stable ARID1A knockdown line (MC.p270.KD), or parental cells transfected with plasmids containing ARID1A-specific siRNA sequences or a scrambled siRNA sequence, were cotransfected with a puromycin selection plasmid (pMSCV). Cells were selected in 4 mg/mL puromycin. Transfected cells were induced to differentiate at 24 hours after transfection and assayed at day 3 after induction. The RNAi-7182 sequence has been reported previously (8). The ARID1A-specific portion of the RNAi-6751 sequence is CTCAGCATCCAGGACAACA, and of the RNAi-7055 sequence is CTAGTGTGGACATGATGCG. The scrambled sequence is CCTCCAATCTTCGCGCGTC. Antibody sources have been described previously (8). *C,* cells, transfected as described in (*B*), were assayed at day 3 after induction for incorporation of ^{3}H-thymidine. All samples were plated in triplicate. *Columns,* average counts per minute (*CPM*) per sample; *bars,* SD.

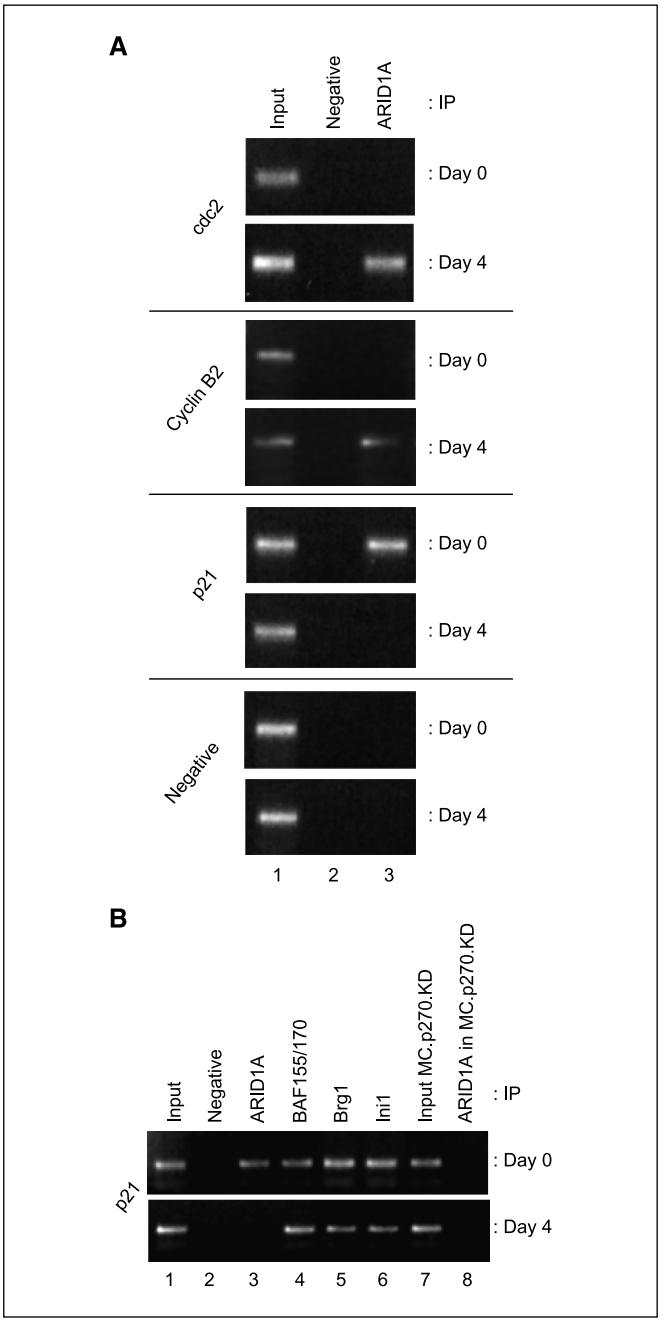

Figure 2. Chromatin immunoprecipitation assays. *A,* chromatin was prepared from cells harvested at day 0 or day 4 of differentiation. The input lane is a positive control done with total genomic DNA. Immune precipitation was done with antibodies of the specificities indicated above the lanes. The negative control antibody is a monoclonal of irrelevant specificity (to SV40 T antigen) described previously (8). PCR was done with primers specific to the promoters indicated left of the lanes. The negative control primer set is specific for the COOH-terminal coding region of p21. *B,* chromatin was prepared as described above from either parental cells (*lanes 1-6*) or an ARID1A/p270 knockdown line (*lanes 7* and *8*) used here as a negative control. Generation of the BAF155/BAF170–reactive monoclonal antibody DXD12 has been described previously (7, 8). The INI1-specific antibody (612110) was obtained from BD Biosciences (San Jose, CA), and the BRG1-specific antibody (H-88) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primer sequences used are cdc2 forward, CACACAGAAAGGTAGCTGGAG; cdc2 reverse, CAATCAGAGCT-GAGCTACGCT; p21 forward, TGCGTGACAAGAGAATAGCCCAG; p21 reverse, TGCAGTTGGCGTCGAGCTGC; cyclin B2 forward, GAAACAA-CAAAGCCTGGTGG; cyclin B2 reverse, ACAGCCACTCCGGTCTGCGA; negative control forward, TTGACTTCGTCACGGAGACG; negative control reverse, GAGTGCAAGACAGCGACAAG.

BRG1 on the promoter during $p21^{WAF1/CIP1}$ activation in human tumor cells (9, 10). To resolve the latter discrepancy, the presence of other SWI/SNF complex subunits on the $p21^{WAF1/CIP1}$ promoter was probed before and during activation. An antibody reactive with the ubiquitous subunits BAF155 and BAF170 indicates the presence of a version of the complex at both times (Fig. 2*B, lane 4*). Probing with antibodies individually specific for BRG1 and INI1 indicates the presence of these subunits as well (*lanes 5* and *6*). Thus, the pattern of ARID1A association does not conflict with results indicating the presence of the BRG1 ATPase at an active $p21^{WAF1/CIP1}$ promoter. However, the dynamics of association specifically by the ARID1A subunit raise an essential question about the required role of ARID1A-containing complex assemblies in the activation of $p21^{WAF1/CIP1}$. If their role in activation is not direct, perhaps their direct function is to repress a repressor of $p21^{WAF1/CIP1}$ expression.

c-*myc* promoter is a direct target of mammalian SWI/SNF complexes during differentiation. In recent years, c-myc has been identified as a repressor of $p21^{WAF1/CIP1}$ expression in several cell types, apparently acting by interfering directly with the Sp1/3 transcription factors (reviewed in ref. 20). Gene array analysis indicates that c-myc is sharply down-regulated in MC3T3-E1 cells between day 0 and day 3 of differentiation (19), and ectopic expression of BRG1 in human tumor cells can cause c-myc down-regulation (9). We therefore investigated the possibility that a direct role of ARID1A-containing complexes in activation of $p21^{WAF1/CIP1}$ is repression of c-*myc*. Western blots confirm the gene array results at the protein level, showing that c-myc levels fall sharply in the parental MC3T3-E1 cells upon differentiation (day 4 and later); this response is greatly impaired in ARID1A-depleted cells (MC.p270.KD; Fig. 3*A*). The inability of ARID1A-depleted cells to repress c-myc levels was confirmed in a transient assay with the three different RNA interference sequences described above (Fig. 3*B, lanes 7-9*). We also verified that the effect of ARID1A depletion on c-*myc* is manifested at the RNA level (Fig. 3*C*). These results indicate that repression of c-*myc* is indeed dependent on the activity of SWI/SNF–related complexes and specifically on complexes that contain ARID1A. Chromatin immunoprecipitation assays were done to determine whether the c-*myc* promoter is a direct target of the complexes. The results support the prediction that ARID1A-containing complexes act directly to repress c-*myc* during differentiation: association with the c-*myc* promoter is not detected in exponentially growing cells but is clearly apparent in differentiating cells (Fig. 3*D, lane 3*, compare days 0 and 4). Probes with antibodies reactive with the BAF155/170, BRG1, or INI1 subunits (Fig. 3*D, lanes 4-6*) indicate the consistent presence of a version of the complex on the c-*myc* promoter, much as was seen on the $p21^{WAF1/CIP1}$ promoter. This is not surprising because the remodeling activity of the complexes is believed to contribute to both activation and repression of highly regulated promoters. The overall results suggest, however, that probing for ubiquitous complex components may not always be sufficiently discriminating to reveal functional aspects of promoter association.

Repression of c-*myc* is critical for $p21^{WAF1/CIP1}$ activation, whereas regulation of E2F-responsive gene products is independent of c-myc and $p21^{WAF1/CIP1}$ levels. The simplest interpretation of these results is that ARID1A-containing complexes are required directly for repression of c-*myc* during differentiation, and that repression of c-*myc* is, in turn, required for activation of $p21^{WAF1/CIP1}$. However, none of these observations proves that repression of c-*myc* is an actual prerequisite for $p21^{WAF1/CIP1}$ activation in this system. To address this question, c-myc was

expressed in the MC3T3-E1 cells using a constitutively active c-*myc* construct encoded in an adenovirus vector, Ad-cMyc (17). Parental MC3T3-E1 cells infected with Ad-cMyc or a negative control adenovirus, Ad-9S, were harvested at days 0, 2, 4, and 6 after induction and assayed by Western blot for expression of c-myc and $p21^{WAF1/CIP1}$ (Fig. 4*A*). The Western blots verify the maintenance of high levels of c-myc in induced cells infected with Ad-cMyc, compared with the decreasing level seen in cells infected only with the control virus Ad-9S (*1*). Probing the same lysates for $p21^{WAF1/CIP1}$ shows that maintenance of a high level of c-myc is sufficient to block completely the induction of $p21^{WAF1/CIP1}$ (Fig. 4*A*, *2*). Thus, repression of c-*myc* is indeed a necessary event in $p21^{WAF1/CIP1}$ activation. These results do not exclude a conclusion that the

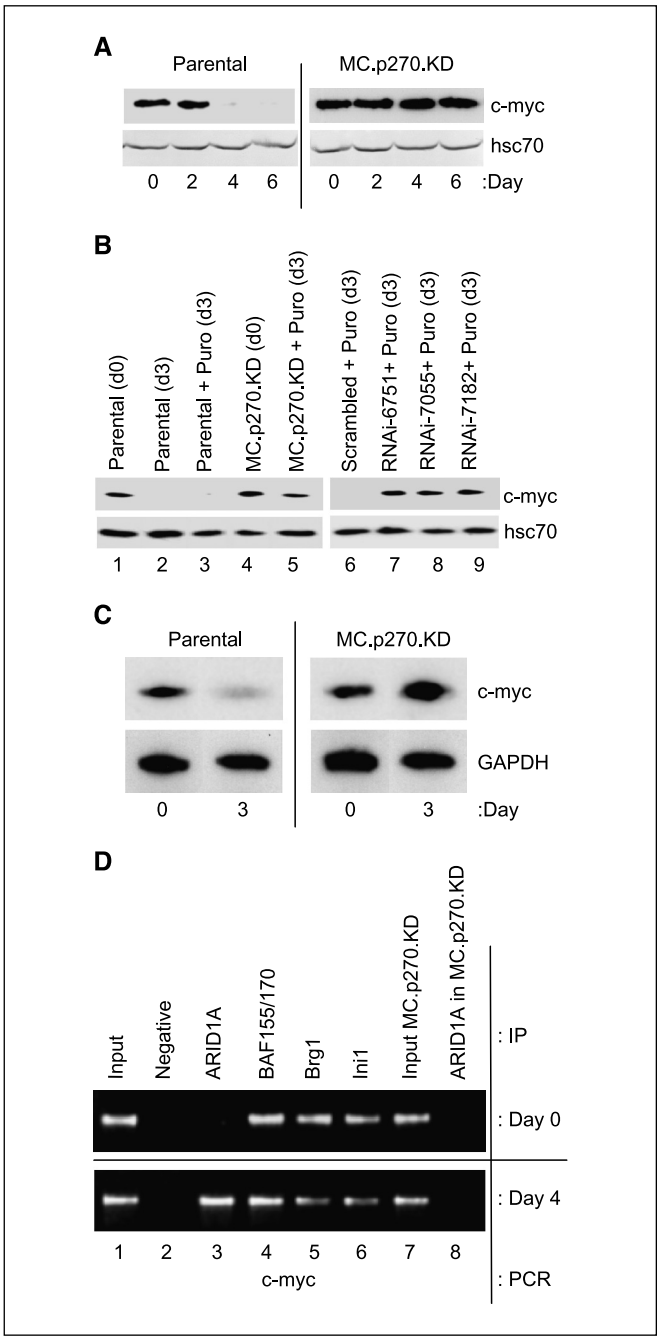

Figure 3. c-*myc* is a direct target of SWI/SNF complex–dependent promoter repression. *A,* Western blot. c-myc was probed in parental cells or ARID1A-depleted cells (MC.p270.KD) harvested at days 0, 2, 4, and 6 of differentiation. The c-myc–specific antibody (9E10) was obtained from Santa Cruz Biotechnology. *B,* the puromycin-selected transient transfection assay was done as described in Fig. 1*B*. *C,* RNA blots were prepared from differentiating cells at day 0 and day 3 as described previously (8). The ^{32}P-labeled c-myc probe was constructed by random priming from a reverse transcription-PCR fragment generated from MC3T3-E1 cell RNA using c-*myc*–specific primers (forward, AGAGACAGAGGGAGTGAGCG) and (reverse GCTGCAATGGG-CAAAGTTTG). *D,* chromatin immunoprecipitation assay. Chromatin preparation and controls are as described in Fig. 2. The c-*myc*–specific primers used for the chromatin immunoprecipitation assay are c-myc forward, AGCAAGA-GAAAATGGTCGGG; c-myc reverse, GCTGCAATGGGCAAAGTTTG.

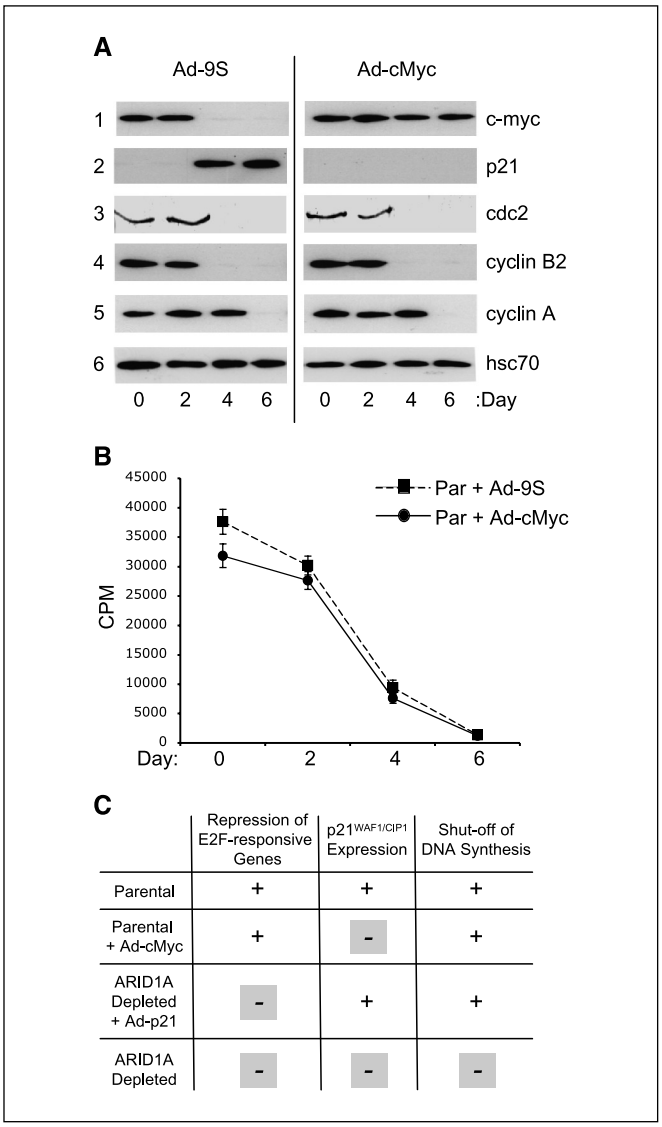

Figure 4. Exogenous expression of c-myc. *A,* MC3T3-E1 cells were infected 24 hours before the time of ascorbic acid induction with an adenovirus vector expressing c-*myc* (Ad-cMyc) or with a negative control virus containing an inactivated E1A gene (Ad-9S). Cells were harvested for assay at days 2, 4, and 6 after induction. Levels of c-myc, p21, cdc2, cyclins B2 and A, and hsc70 (included as a loading control) were probed by Western blotting. Ectopic expression of c-myc does not result in higher expression levels at early times, presumably because autoregulation represses endogenous levels. *B,* cells infected as described in Fig. 3 were assessed for DNA synthesis activity monitored by ^{3}H-thymidine incorporation. *Points,* averages of results obtained with three independent platings of each set of virus-infected cells; *bars,* SD. *C,* summary of relative independence of p21 activation and E2F repression in shutting down DNA synthesis, as revealed by ectopic expression of c-myc or p21 in the appropriate cell line (either parental or ARID1A depleted). Data for ARID1A-depleted cells infected with Ad-p21 were reported in ref. (8).

$p21^{WAF1/CIP1}$ promoter is also a direct target of the complexes during induction. The positive signals seen with other subunits (refs. 9, 10; Fig. 2*B*) confirm that it is. However, the results described here identify direct targeting of the c-*myc* promoter by the SWI/SNF–related complexes, specifically by the ARID1A-containing subset of complexes, as a critical upstream event in the pathway leading to induction $p21^{WAF1/CIP1}$ in differentiating cells.

Another question raised by these observations is whether ectopic expression of c-myc is sufficient to prevent repression of E2F-responsive promoters. To address this question, additional aliquots of the virus-infected cell lysates were probed for expression levels of proteins produced from characteristic E2F-responsive genes. For each protein analyzed (cdc2, cyclin B2, and cyclin A), the result was the same. Ectopic expression of c-myc and the concomitant failure of $p21^{WAF1/CIP1}$ induction does not affect repression of the E2F-responsive promoters (Fig. 4*A, 3, 4*, and *5*). Repression of the E2F-responsive gene products is not a downstream event dependent on c-*myc* repression nor on $p21^{WAF1/CIP1}$ activation. Previous results showing that constitutive expression of $p21^{WAF1/CIP1}$ is not sufficient for repression of E2F-responsive promoters in the absence of ARID1A (8) emphasize further that induction of $p21^{WAF1/CIP1}$ is neither necessary nor sufficient for repression of the E2F-responsive promoters.

The degree of regulation remaining in the induced cells in the presence of constitutively high levels of c-myc is sufficient not just for repression of E2F-responsive gene products but also for shutdown of DNA synthesis, as shown by the kinetics of ^{3}H-thymidine incorporation (Fig. 4*B*). Previous results showed that ectopic expression of $p21^{WAF1/CIP1}$ in the absence of ARID1A (which can now be understood as bypassing the need for c-*myc* repression) is not sufficient for repression of E2F-responsive genes but is sufficient to reduce DNA synthesis to <10% of proliferating cell levels; the same cells infected with a negative control virus maintain incorporation at >85% of proliferating levels (8). Together, these results illustrate a dual level of control present in normal cells (summarized in Fig. 4*C*). The phenotype of the ARID1A-depleted cells, by revealing the dependence of c-*myc* repression on the complexes, sheds further light on the extent to which proliferation controls are dependent on the functions of SWI/SNF complexes.

Rapid down-regulation of c-myc has been seen previously following exposure to differentiation inducers (reviewed in ref. 20), and constitutive expression of c-myc is sufficient to block induction of $p21^{WAF1/CIP1}$ in other cell types (e.g., ref. 17; reviewed in ref. 20). However, the finding that the SWI/SNF complexes are required for repression of c-*myc* during differentiation illuminates an important new aspect of their role in tumor suppression. This study also highlights functional differences between subsets of the SWI/SNF–related complexes. It is important to recognize that the entity typically designated the mammalian SWI/SNF complex, sometimes called the BAF complex, is actually a small series of related complexes of variable composition resulting from the presence in most cells of alternative versions of the ATPase subunits and of the ARID family subunits. The ATPase subunits are not interchangeable, but they do show considerable overlap of function (reviewed in refs. 1, 2). The ARID family subunits confer very specific functional differences on the complexes. In particular, the ARID1A subunit is a determinant of a specific subset that is required for differentiation-associated cell cycle arrest and which directly targets cell cycle–regulated genes, including *c-myc*, *cdc2*, and *cyclin B2*, specifically at the time of repression. The significance of loss of ARID1A function to proliferation control in normal cells during differentiation is profound, impairing each of two major pathways of growth regulation.

Acknowledgments

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