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**14. ABSTRACT**

Mammalian SWI/SNF complexes are ATPase-powered nucleosome remodeling assemblies crucial for proper development and tissue specific gene expression. The ATPase activity of the complexes is critical for tumor suppression in mice and humans. The complexes also contain seven or more noncatalytic subunits, only one of which, hSNF5, has been individually identified as a tumor suppressor thus far. The noncatalytic subunits include p270, which is of particular interest because results from a cDNA tissue array analysis indicate p270 may be deficient in as many as 10% of breast cancers and 30% of kidney cancers. The complexes can also include the alternate ARID1B subunit, which is very closely related to p270, but the product of an independent gene. The respective importance of these proteins in the control of cell proliferation was explored here using an siRNA approach and a cell system that permits analysis of differentiation-associated cell cycle arrest. p270-depleted cells fail to undergo normal cell cycle arrest, and show other characteristics of cells that have failed to undergo arrest, such as upregulation of cyclins, increased cdk activity, and failure to induce cdk inhibitors. In contrast, cells depleted of ARID1B behave like parental cells. These results show that p270-containing complexes are functionally distinct from ARID1B-containing complexes.

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SWI/SNF, chromatin remodeling, cell cycle, breast cancer

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**Introduction:**

The ATPase-powered SWI/SNF chromatin remodeling complex in yeast regulates the mating type switch and other areas of specialized gene expression. Mammalian SWI/SNF-related complexes likewise contain an ATPase-driven nucleosome remodeling activity asspciated with transcriptional regulation. The activity of these complexes is crucial for proper tissue specific gene expression, development, and hormone responsiveness. Recently, it has become apparent that these complexes also play crucial roles in suppression of tumorigenesis in mice and humans.

The complexes contain seven or more noncatalytic subunits that presumably help to modulate the targeting and activity of the core ATPase. Mammalian complexes have variable compositions because some subunits occur as sets of related proteins. The catalytic ATPases BRG1 and BRM are closely related proteins, but in mouse studies only BRG1 proved essential for embryonic development and tumor suppression (Bultman et al., 2000; Reyes et al., 1998). Among the noncatalytic subunits, hSNF5 is recognized as a tumor suppressor in mice (Guidi et al., 2001). In humans, hSNF5 is deficient in malignant rhabdoid tumors (Biegel et al., 2002), and carriers of germ line mutations are predisposed to malignant rhabdoid tumors and tumors of the central nervous system (Sevenet et al., 1999).

Subunits required for the tumor suppression activity of the complexes have great potential as diagnostic and prognostic markers. A major question is the distinction of which additional noncatalytic subunits are required for the cell cycle arrest functions of the complexes. These subunits include p270 (also designated ARID1A or BAF250) and the closely related protein ARID1B (also designated KIAA1235). These proteins are members of the ARID family of DNA binding proteins, and share 60% identity across their open reading frames. Despite this remarkable degree of identity, all of the functional motifs of ARID1B are modified with respect to p270. Work performed over the course of this pre-doctoral training fellowship has shown that p270 and ARID1B contribute different functions to mammalian SWI/SNF-related complexes.

**Body:**

**Key Accomplishments:**

My proposal had three objectives:

1. To determine the relative levels of expression of ARID1B and p270 in a panel of breast cancer cell lines, and the proportion of hSWI/SNF complexes bound by each protein in representative lines.
2. To identify binding partners of the LXXLL motif-containing region of ARID1B.
3. To determine the effect of overexpressing ARID1B in breast cancer cells.

*Objective 1:* The first part of this objective was to develop a hybridoma line from mice that were serum-positive for ARID1B-specific antibodies. I have successfully accomplished this. The monoclonal, which was raised against a ARID1B-GST fusion peptide, is selective for ARID1B and does not cross-react with p270. I have demonstrated this *in vivo* and *in vitro*, using both native and denatured proteins. I have also confirmed that p270 and ARID1B are mutually exclusive, alternative members of SWI/SNF-related complexes. In addition, we have shown that both p270 and ARID1B associate with either BRG1- or hBRM-containing SWI/SNF complexes, therefore both have the potential to influence the tumor suppressor function of the complex. These results have been published (Wang et al., 2004a), which is included in the appendix. As mentioned previously, p270 and ARID1B have specific alterations to functional motifs, and SWI/SNF complexes containing either protein are likely to have different functions. I therefore proposed to screen a panel of breast cancer cell lines to assay for relative levels of p270 and ARID1B. We have screened a panel of breast cancer cell lines, and the ratio of p270 to ARID1B in typical cells is approximately 3.5 to 1, with BRG1 being distributed equally between the two ARID subunits. My screen confirmed loss of expression of p270 in T47D cells, while levels of ARID1B were generally low to undetectable in all lines examined. In addition to screening by western blot, we have screened a commercially available cancer profiling array for levels of p270 RNA in panels of matched tumor versus normal tissue. Levels of p270 RNA were sharply downregulated in 10 percent of

breast cancer tissue examined. Strikingly, p270 expression was also sharply reduced in 30 percent of primary kidney tumors. All of the outcomes above have been reported in Wang et al., 2004b, included in the appendix). p270 and ARID1B probes were also used in a collaborative study to show that p270 and ARID1B are expressed in a complimentary manner in developing rhesus monkey embryos (Zheng et al., 2004, included in the appendix).

*Objective 2:* A major portion of this aim was to complete the sequence of the ARID1B cDNA, in order to know how many potential nuclear hormone binding motifs were present. I have successfully used RT-PCR to extend our coding region of ARID1B an additional 198 amino acids to 1817 residues. While this expression clone is nearly complete, I was unable to extend this coding region further. While this work was in progress, Inoue et al. (2002) reported that both p270 and ARID1B lacking their N-terminal LXXLL motifs are able to stimulate transcription by the glucocorticoid, estrogen, and androgen receptors in reporter assays. p270 has been previously reported to stimulate a glucocorticoid response element in the presence of dexamethasone (Nie et al., 2000). These data suggest that both p270 and ARID1B enhance expression of a similar panel of hormone responsive genes. As these assays were performed by cotransfection of reporter constructs, they may not be reflective of true physiological regulation of hormone-responsive genes, but they do suggest that two-hybrid binding assays will be likely to give false, non-physiological positives. In light of these data, I believed it would be more productive to focus first on Objective 3 to obtain more physiologically relevant data.

*Objective 3:* The purpose of my third objective was to determine the biological effects of overexpressing ARID1B in breast cancer cells. Obtaining a full-length expression clone of ARID1B proved to be particularly challenging due to high GC content. To accommodate the goal of determining the biological function of ARID1B in comparison to p270, I pursued an alternate approach. As p270 and ARID1B are independent members of SWI/SNF complexes, it was likely that altering the level of expression of either ARID protein would change the proportion of complexes associated with either p270 or ARID1B, and thus change their function. To best compare the physiological function of ARID1B and p270 without full-length expression constructs, I generated stable cell lines that stably express siRNAs specific for either p270 or ARID1B (see Brummelkamp et al., 2002 for methodology). Studies by other groups to determine the role of p270 and ARID1B involvement in hormone responsiveness have produced relatively homogeneous results, failing to delineate a difference in function between p270 and ARID1B. Subsets of SWI/SNF complexes are also implicated in control of the cell cycle, regulation of cell growth, and control of tissue specific gene expression. These complexes are widely regarded as having a tumor suppressor function dependent upon the core ATPase. Generation of cell lines with stable knock down of p270 and ARID1B have proven a powerful tool to study the contribution of these noncatalytic subunits to the tumor suppressor function of SWI/SNF complexes. I have ablated expression of p270 or ARID1B in MC3T3-E1 cells. When stimulated with ascorbic acid and betalycerol phosphate, these cells will undergo osteoblastic differentiation, mineralizing over 28 days. The switch from proliferation to tissue specific gene expression and cell cycle exit is very tightly regulated and well characterized in these cells (Quarles et al., 1992). Data included in previous progress reports show that cells depleted of either p270 or ARID1B show defects in tissue specific gene expression, while only p270 is critical for cell cycle arrest. We have shown that p270 and pRb (the retinoblastoma tumor suppressor protein) function in the same pathway with respect to differentiation and expression of tissue specific genes (shown in my second annual report). Additional data reported in Nagl et al. (in revision, included in the appendix) confirms that the functional distinction between p270 loss and ARID1B loss is very marked. Cells depleted of p270 fail to undergo normal cell cycle arrest in comparison to controls, as measured by increased cdc2 kinase activity and protein expression, as well as increased incorporation of tritiated thymidine in a DNA synthesis assay. Also, p270 deficient cells show disregulation of cyclin A, cyclin B2, and Cyclin C. These cyclins are expressed at high levels in cells depleted of p270, suggesting that p270-containing SWI/SNF complexes are crucial for the repression of E2F responsive genes that regulate the switch from proliferation to cell cycle arrest and tissue specific gene expression. In addition, cells deficient for p270 fail to induce the cyclin

dependent kinase inhibitor $p21^{wafl/cip1}$. We have confirmed that induction of $p21^{wafl/cip1}$ and repression of E2F-responsive promoters are independent events that each require p270 by exogenously overexpressing $p21^{wafl/cip1}$. Exogenous expression of $p21^{wafl/cip1}$ caused cells depleted of p270 to shut down DNA synthesis with the same rapid kinetics seen in control wild type cells. Interestingly, western blotting showed that E2F-responsive products such as cyclins A, B2, and C, as well as cdc2 remain highly expressed in cells deficient for p270 despite exogenous expression of $p21^{wafl/cip1}$, indicating that regulation at the $p21^{wafl/cip1}$ promoter and at the E2F-responsive promoters each independently requires the function of a SWI/SNF chromatin remodeling complex, and of p270 specifically, during differentiation-associated cell cycle arrest. While cells depleted of p270 show a marked change in phenotype, ARID1B deficient lines exhibit a phenotype that is similar to that of parental cells in all assays performed.

**Key Research Accomplishments:**

- Established that ARID1B and p270 independently associate with BRG1- and hBRM-containing hSWI/SNF complexes.
- Determined that p270 is down regulated in approximately 10% of breast cancer cell lines examined, as well as 10% of primary breast and 30% of primary kidney tumors.
- Developed a series of vector-based RNAi oligos against ARID1B and p270, and generated several stable ARID1B and p270 knock down cell lines.
- Determined that ARID1B and p270 are required for induction of the tissue specific gene alkaline phosphatase in differentiating MC3T3-E1 cells.
- Complementation assays suggest that p270 and pRb act in the same pathway in this differentiation model system.
- Loss of p270 alters the cell cycle profile of differentiating MC3T3-E1 cells, driving the cells to remain in the cell cycle.
- Regulation of the $p21^{wafl/cip1}$ promoter and E2F-responsive promoters each independently require p270-containing SWI/SNF complexes during differentiation-associated cell cycle arrest.
- ARID1B and p270 have distinctly separate biological functions and are determinants that distinguish key divisions among the multiple, distinct SWI/SNF complexes that exist in mammalian cells.

**Reportable Outcomes:**

- Several stable cell lines expressing reduced levels of p270 and ARID1B were generated as part of this work.
- Work performed over the course of this fellowship allowed the award recipient to complete his doctoral degree.

**Publications:**


- Wang X, Nagl NG Jr, Van Scoy M, Pacchione S, Yaciuk P, Dallas PB, Moran E. *Two related ARID family proteins are alternative subunits of human SWI/SNF complexes.* Biochemical Journal 2004a; 383:319-325.
- Wang X, Nagl NG Jr, Flowers S, Zweitzig DR, Dallas PB, Moran E. *Expression of p270 (ARID1A), a component of human SWI/SNF complexes, in human tumors.* Int J Cancer 2004b; 112:636-642.
- Zheng P, Patel B, McMenamin M, Paprocki AM, Schramm RD, Nagl NG Jr, Wilsker D, Wang X, Moran E, Latham KE. *Expression of genes encoding chromatin regulatory factors in developing Rhesus monkey oocytes and preimplantation stage embryos: Possible roles in genomic activation.* Biol Reprod. 2004 May; 70(5): 1219-27.
- Nagl NG Jr, Patsialou A, Haines DS, Dallas PB, Beck GR Jr, Moran E. *The p270 (ARID1A/SMARCF1) subunit of mammalian SWI/SNF-related complexes is essential for normal cell cycle arrest.* Cancer Res. *In revision.*


**Abstracts:**


- Norman G. Nagl, Jr., Antonia Patsialou, Dale S. Haines, Peter B. Dallas, George R. Beck, Jr., and Elizabeth Moran. *The p270 (ARID1A/SMARCF1) subunit of mammalian SWI/SNF-related complexes is essential for normal cell*

*cycle arrest.* Presented at the 2005 Edward A. Smuckler Memorial Workshop on the Pathobiology of Cancer, Snowmass, CO (Awarded Honorable Mention in Basic Science Research).

- Norman G. Nagl, Jr., Xiomei Wang, Deborah Wilsker, Stephen Flowers, Daniel Zwietzig, Antonia Patsialou, and Elizabeth Moran. *Expression of p270 (ARID1A) a Component of Human SWI/SNF Clomplexes, in Human Tumors.* Presented at the 2005 Annual Era of Hope Meeting, Philadelphia, PA.
- Norman G. Nagl, Jr., Antonia Patsialou, Stephen Flowers, George R. Beck, Jr., Peter B. Dallas, and Elizabeth Moran. *Functional Complementation Between Adenovirus E1A Targets and the p270 Subunit of SWI/SNF-related Complexes.* Selected for oral presentation at the meeting on Small DNA Tumor Viruses and Cell Cycle Control, July 2004, University of Wisconsin, Madison. (NCI Travel Award to attend ($750)).
- Norman G. Nagl, Jr., Xiaomei Wang, Deborah Wilsker, Michael Van Scoy, Peter Dallas, Peter Yaciuk, and Elizabeth Moran. *Functional Characterization of a Novel Paralogue of Human SWI/SNF Member p270.* Presented at the 10th Annual Meeting of the Federation of Asian and Oceanic Biochemists and Molecular Biologists, December 2003, Bangalore, India.
- Norman G. Nagl, Jr., Xiaomei Wang, Deborah Wilsker, Michael Van Scoy, Peter Dallas, Peter Yaciuk, and Elizabeth Moran. *Characterization of a Novel Paralogue of Human SWI/SNF Member p270.* Presented at the 2003 Annual Meeting of the AACR, Washington DC. (University Travel Award to attend the 2003 Annual Meeting of the AACR ($350).)
- Norman G. Nagl Jr., Xiaomei Wang, Deborah Wilsker, Antonia Patsialou, Michael van Scoy, Peter B. Dallas, Peter Yaciuk and Elizabeth Moran. *p270 DNA Binding in Relation to ARID Protein Family Structure.* Selected for oral presentation at the meeting on Small DNA Tumor Viruses and Cell Cycle Control, July 2002, University of Wisconsin, Madison.
- Norman G. Nagl, Jr., Xiaomei Wang, Deborah Wilsker, Michael Van Scoy, Takahiro Nagase, Peter Dallas, Peter Yaciuk, and Elizabeth Moran. *Characterization of a Novel Homologue of Human SWI/SNF Member p270.* Presented at the 2002 Fels Institute Annual Research Day, First Place, platform presentation.
- Norman G. Nagl, Jr., Xiaomei Wang, Deborah Wilsker, Michael Van Scoy, Takahiro Nagase, Peter Dallas, Peter Yaciuk, and Elizabeth Moran. *Characterization of a Novel Paralogue of Human SWI/SNF Member p270.* Presented at the 2001 Meeting on Small DNA Tumor Viruses and Cell Cycle Control, Cambridge University, Cambridge, UK. (NCI Travel Award to attend the 2001 Small DNA Tumor Virus Meeting ($1000))

**Conclusion:**

The data that I have gathered over the course of this fellowship are particularly significant because previous studies concerning the roles of SWI/SNF complex components in regulating expression of cell cycle markers have largely relied on reintroduction of BRG1 or hSNF5 into tumor cell lines where they were lacking. This is disadvantageous from monitoring the role of complex components in cells undergoing physiological progression from a proliferative state to cell cycle arrest as seen here. The identification of p270 as a subunit required for cell cycle arrest *in vivo* is additionally significant, because unlike BRG1 and hSNF5, p270 is not among the those subunits considered to form the "functional core" of the complex(es) (Phelan et al., 1999). The *in vivo* requirement for p270 shows that it plays an essential role in the physiological functions of the complex(es), regardless of whether it contributes directly to the overall enzymatic activity. Additionally, this work provides a further distinction among the various sub-groups of SWI/SNF complexes. The ARID family components are determinants that distinguish key divisions among the multiple, distinct SWI/SNF complexes that exist in mammalian cells. We have shown that SWI/SNF complexes encompass at least four different entities because p270 and ARID1B can each associate with mammalian BRG1 and BRM, in all four possible combinations (Wang et al., 2004a, included in appendix). Like the core ATPases BRG1 and BRM, the ARID family components exist as mutually exclusive, alternate members of SWI/SNF complexes (Wang et al., 2004a, included in appendix). This work clearly ascribes a critical cell cycle exit regulatory function to complexes that contain p270. This is a distinct demarcation of function from complexes

containing ARID1B, further separating the types of complexes observed in mammalian cells. It is particularly interesting that p270 and ARID1B are functionally very different and exist in alternate complexes. This is a characteristic that is also observed with respect to BRG1 and BRM. Mouse knockout studies have shown that only BRG1 proved essential for embryonic development and tumor suppression (Bultman et al., 2000; Reyes et al., 1998). The work that I have performed over the course of this predoctoral training fellowship has contributed a significant amount of novel data to the field of SWI/SNF complexes and the regulation of transcription and cell cycle control. Although I have completed my doctoral training, work will continue in this lab to further characterize the function of these ARID proteins and SWI/SNF complexes in which they are members.

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# Two related ARID family proteins are alternative subunits of human SWI/SNF complexes

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p270 (ARID1A) is a member of the ARID family of DNA-binding proteins and a subunit of human SWI/SNF-related complexes, which use the energy generated by an integral ATPase subunit to remodel chromatin. ARID1B is an independent gene product with an open reading frame that is more than 60 % identical with p270. We have generated monoclonal antibodies specific for either p270 or ARID1B to facilitate the investigation of ARID1B and its potential interaction with human SWI/SNF complexes *in vivo*. Immunocomplex analysis provides direct evidence that endogenous ARID1B is associated with SWI/SNF-related complexes and indicates that p270 and ARID1B, similar to the ATPase subunits BRG1 and hBRM, are alternative, mutually exclusive subunits of the complexes. The ARID-containing subunits are not specific to the ATPases. Each associates with both BRG1 and hBRM, thus increasing the number of distinct subunit combinations known to be present in cells. Analysis of the panels of cell lines indicates that ARID1B, similar to p270, has a broad tissue distribution. The ratio of p270/ARID1B in typical cells is approx. 3.5:1, and BRG1 is distributed proportionally between the two ARID subunits. Analysis of DNA-binding behaviour indicates that ARID1B binds DNA in a non-sequence-specific manner similar to p270.

Key words: ARID family, ARID1B, BRG1, p270, SWI/SNF.

## INTRODUCTION

SWI/SNF-related complexes are chromatin-remodelling complexes that play fundamental roles in the regulation of gene expression during cell growth and development in all eukaryotes. Individual human SWI/SNF complexes contain at least eight identified proteins, but these complexes are not completely defined and they can exist in multiple forms (see [1–4] for recent reviews). However, an essential component of the complexes is an ATPase of the SWI2/SNF2 family. Human complexes can contain either of the two related but distinct ATPases, BRG1 and hBRM [5,6].

Human SWI/SNF complexes also include a large subunit that contains a DNA-binding domain of the ARID family. This 270 kDa protein, p270, shares antigenic specificity with the chromatin-modifying histone acetyltransferases p300 and CBP [7,8], although p270-containing complexes do not show histone acetyltransferase activity [8]. Analysis of p270-associated proteins revealed that p270 is a component of human SWI/SNF complexes, and cloning of the p270 cDNA suggested that p270 is an orthologue of yeast SWI1 [8,9]. Cloning of a BRG1-associated factor, designated BAF250, with a cDNA sequence co-linear with p270 independently confirmed the presence of p270 in the complexes [10].

p270 is expressed in all the human tissues examined [9,10]. The most prominent feature seen in the p270 open reading frame is the presence of an ARID DNA-binding domain. This is a recently defined helix–turn–helix-based domain, typical of a family that includes at least 15 distinct human proteins suggested to play a role in the regulation of development, tissue-specific gene expression and/or cell proliferation (reviewed in [11–13]). p270 binds linear duplex DNA depending on the integrity of the ARID consensus sequence [9]. Some ARID family members bind selectively to AT-rich sequences, a behaviour that prompted the acronym ARID (AT-rich interactive domain); in contrast, p270 and its closest *Drosophila* counterpart, Osa, do not select oligonucleotides of any preferred sequence from a random pool [9,13,14]. Other recognizable features in p270 include glutamine-rich regions and multiple LXXLL motifs (where L stands for leucine and X for any amino acid), which are generally indicative of a potential for association with liganded nuclear hormone receptors [15,16]. The p270 (synonym: BAF250) subunit is one of many components of SWI/SNF complexes that appear to interact directly with the glucocorticoid receptor [10,17], and the exogenously introduced expression of p270 can stimulate expression from a glucocorticoid receptor-dependent reporter construct [10,18].

A specific search for large open reading frames expressed in the human brain revealed a partial cDNA, designated KIAA1235, which is closely related to p270 [19]. This cDNA has been isolated and its characterization has begun in several laboratories under various names (p250R, hELD/OSA1, hOSA2 or BAF250B; [18,20–22]). The Human Genome Organization Gene Nomenclature Committee and the Mouse Genomic Nomenclature Committee have recently recommended that ARID family members carry gene designations that reflect their relationship. According to this scheme, the p270 and KIAA1235 genes, which map to the chromosomal loci 1p35.3 and 6q25.1 respectively, are designated *ARID1A* and *ARID1B* respectively. Since the *ARID1B* gene product does not have a widely accepted common name, we adopt the latter designation in the present study for both the gene and the protein.

Abbreviations used: Dri, *Drosophila* deadringer; CMV, cytomegalovirus; DTT, dithiothreitol; GST, glutathione S-transferase; mAb, monoclonal antibody; NP40, Nonidet P40; poly(A)$^+$, polyadenylated.

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The apparent full-length sequence of ARID1B is described in Nie et al. [22]. ARID1B and p270 are more than 60 % identical across their entire lengths. The ARID consensus is intact in ARID1B, but the exons encoding the glutamine-rich regions are shorter, essentially eliminating the pattern of glutamine enrichment. The pattern of LXXLL motifs is also somewhat different. Regardless of these differences, ARID1B can activate the expression from androgen, oestrogen and glucocorticoid receptor-dependent reporter constructs in co-transfection assays [18].

Although p270 and ARID1B behave similarly in transient transfection assays, their patterns of expression differ during early primate development, suggesting that the proteins have different functions *in vivo* [23]. A central question in the composition of the complexes is whether p270 and ARID1B are present together, similar to the closely related proteins BAF170 and BAF155, or whether they are mutually exclusive, similar to the alternative ATPases, BRG1 and hBRM. This issue has been examined, but not resolved. One report [18] concluded from *in vitro* association assays and immunocomplex analysis that both ATPases associate with ARID1B (synonym: hOSA2). However, this study did not establish whether p270 and ARID1B are in separate complexes. Another study, using immunocomplex analysis, concluded that p270 and ARID1B (synonym: BAF250b) are in separate complexes, but MS analysis of the associated proteins led these investigators to conclude that ARID1B associates only with BRG1 and not with hBRM [22].

We have generated mAbs (monoclonal antibodies) specific for either p270 or ARID1B to facilitate comparative analysis of these proteins *in vivo*. The immunocomplexes demonstrate directly that endogenous ARID1B is associated with SWI/SNF complexes and indicate that p270 and ARID1B, similar to BRG1 and hBRM, are alternative, mutually exclusive subunits of the complexes. The ARID-containing subunits are not specific to the ATPases. Each associates with both BRG1 and hBRM, thus increasing the number of distinct subunit combinations known to be present in cells. Analysis of panels of cell lines indicates that ARID1B, similar to p270, has a broad tissue distribution. The ratio of p270 to ARID1B in typical cells is approx. 3.5:1, and BRG1 is distributed proportionally between the two ARID subunits. Analysis of DNA-binding behaviour indicates that ARID1B binds DNA in a non-sequence-specific manner similar to p270.

## MATERIALS AND METHODS

### Immunoprecipitation

Cells were harvested in PBS, pelleted and lysed in Tris lysis buffer [250 mM NaCl, 0.1 % NP40 (Nonidet P40), 40 mM Tris (pH 7.4) and 1 mM DTT (dithiothreitol), supplemented with the following protease inhibitors at a final concentration as indicated: aprotinin (1.0 $\mu$g/ml), leupeptin (1.0 $\mu$g/ml) and pepstatin (1.0 $\mu$g/ml)]. Immunoprecipitation was performed as described previously [8].

### Immunoblotting

Preconfluent cells were harvested in PBS. Cells were lysed, and proteins were separated on 8 % gels, transferred on to an Immobilon-P membrane (Millipore) and visualized as described previously [8] or by using a chemiluminescence system (NEL602; NEN).

### Northern blots

Poly(A)$^+$ (polyadenylated)-selected RNA was prepared from appropriate cell cultures using TRI Reagent (Sigma), and PolyATract mRNA Isolation System (Promega) according to the supplier's instructions. Then, 18 $\mu$g of RNA was loaded per lane and fractionated by electrophoresis on a 0.7 % formaldehyde–agarose gel. The RNA was transferred on to a Hybond-N nylon membrane (Amersham Biosciences, Arlington Heights, IL, U.S.A.) and cross-linked by UV irradiation and baking at 80 °C. $^{32}$P-labelled probes were prepared using a random-primed labelling kit (Boehringer Mannheim). Between successive probes, blots were stripped by boiling in 0.1 % SDS. The specificity of the probes was verified by hybridization with plasmid DNA under the same conditions.

### Probes

A p270 probe hybridizing to the middle region of the 270 kDa protein was generated by PCR from the plasmid pNHXSS98, which contains the p270 cDNA sequence reported in [9], using the primer sequences TACCAGCAGAACTCCATGGGGAGCTAT and TTTCTTGGGTTTTCCGGTTCATGC. An ARID1B probe hybridizing to the corresponding region of ARID1B was generated by PCR from the plasmid pfh08704 (a gift from Dr T. Nagase, Kazusa DNA Research Institute, Kisarazu, Chiba, Japan), which contains the ARID1B cDNA sequence from residues 1 to 5834 (according to accession number AB033061) in a pBluescript II vector, using the primer sequences TTCAGCAGAGTAACTCAAGTGGGAC and TTACGGTTCACAGTTGGCATT. The $\beta$-actin probe was described previously [24].

### Cell lines

HeLa, C33A, SW13, MCF7 and MDA-MB-435s cells were obtained from the A.T.C.C. PC-3, DU-145 and TSU-Pr1 cells were gifts from B. Lokeshwar (University of Miami School of Medicine, Miami, FL, U.S.A.). Saos-2, U2-OS, MG 63, and OHS 50 cells were gifts from M. F. Hansen (Center for Molecular Medicine, University of Connecticut Health Center Graduate School, Farmington, CT, U.S.A.). All cell lines were cultured according to A.T.C.C. recommendations.

### Antibodies

The p270 mAb, PSG3, was raised against the pNDX2 GST (glutathione S-transferase)-fusion protein described previously [9]. The fusion protein contains 419 residues from the mid-portion of p270 and also contains the ARID. The BRG1-specific mAb, mAb 320.7, was raised against an N-terminal peptide sequence, STPDPPLGGTPRPG(T), corresponding to residues 2–15 of human BRG1. The BAF155-specific mAb, DXD7, was raised against a peptide sequence, EKPVDLQNFGLRTDIYSK(C), corresponding to residues 591–608 in the BAF155 sequence. The ARID1B mAb, KMN1, was raised against a GST-fusion protein product containing a portion of ARID1B roughly analogous to the p270 antigen. The fusion protein includes an optional additional ARID1B exon sequence noted in some cDNA versions; the amino acid sequence corresponds to residues 1–422 of accession number BAA86549, which is a middle region of ARID1B containing the ARID consensus. All hybridoma isolation work was performed at the St. Louis University Hybridoma Facility. A BRM-derived antibody was purchased from Transduction Laboratories (B36320).

### Expression plasmids

The *in vitro* translated constructs used in the antibody tests are pNHXSS98 (which expresses p270 residues 471–2285 according to accession number NM_006015) and pKM5 (which expresses the C-terminal portion of ARID1B corresponding to residues 1–1485 in the partial-sequence accession number BAA86549

described above). The p270 *in vitro* translation plasmid NE9-B2 used for the DNA-binding assay has been described previously [13]; it expresses amino acid residues 901–1187, including the ARID consensus, which extends from residue 1013 to 1107. The ARID1B *in vitro* translation plasmid KM20 used for the DNA-binding assay contains an insert derived by PCR from the KM15 plasmid described previously [13] in the pCR2.1-TOPO vector (Invitrogen). The KM20 insert extends from 2003 to 2864 bp and expresses amino acids 658–944, including the ARID consensus, which extends from residue 768 to 864 (accession number AF253515). The GST-fusion protein constructs are p410 [*Drosophila* deadringer (Dri)] [25], pNDX (p270) [9] and pKM19. Plasmid KM19 contains an ARID1B insert in a pGEX-4T-1 vector (Amersham Biosciences); the insert extends from 2174 to 3292 bp and expresses residues 715–1087, according to the same accession numbers cited above. The CMV (cytomegalovirus)-promoted mammalian expression constructs used were pNXCMV, which expresses p270 residues 471–2285 (according to accession number NM_006015), and pKM12. The pKM12 plasmid was generated by ligating a 3530-bp *SalI–NotI* restriction fragment of plasmid KIAA1235 (accession number AB033061; a gift from Dr T. Nagase) into the backbone of *SalI–NotI*-digested plasmid HRC04412 (accession number AK025945, a gift from Dr S. Sugano, Institute of Medical Science, The University of Tokyo). The resulting pKM12 plasmid expresses a 1706-residue C-terminal portion of ARID1B, and includes the optional additional 52-residue sequence described above.

### *In vitro* expression

p270 and ARID1B cDNA fragments in appropriate plasmid vectors were used to generate [$^{35}$S]methionine-labelled polypeptides using the TNT-coupled reticulocyte system (Promega).

### Sequence-specific selection of DNA

GST-fusion protein pull-down assays were performed as described previously [13,14]. Restriction fragments were filled in with [$\alpha$-$^{32}$P]dATP. Labelled DNA (0.8 $\mu$g) was incubated with 100 ng of GST-fusion protein bound to glutathione–agarose beads for 1 h at 4 °C in Lambda DNA-binding buffer [20 mM Hepes, pH 7.6, 1 mM EDTA, pH 8, 10 mM $(NH_4)_2SO_4$, 0.2 % Tween 20, 1 mM DTT, 25 $\mu$g/ml BSA and 25 $\mu$g/ml poly(dI-dC)·(dI-dC)] containing varying amounts of KCl, as indicated in the text. The beads were washed three times with Lambda DNA-binding buffer without DTT, BSA and poly(dI-dC)·(dI-dC). Bound DNA was eluted by boiling in formamide loading buffer (90 % formamide, 1 × TBE, 0.04 % Bromophenol Blue and 0.04 % xylene cyanol), separated on a 6 % sequencing gel and visualized by autoradiography.

### DNA–cellulose chromatography

*In vitro* translated proteins were diluted in 1 bed volume (0.5 ml) of column loading buffer [10 mM potassium phosphate, pH 6.2, 0.5 % NP40, 10 % glycerol, 1 mM DTT, 1 mg/ml aprotinin, 1 mg/ml pepstatin and 1 mg/ml leupeptin], and applied to native DNA–cellulose columns (Amersham Biosciences). The protein sample was passed through the column four times. Unbound material is designated as flow-through. The columns were then washed several times with 1.0 bed volume of column loading buffer containing 50 mM NaCl (these are the 50 mM wash fractions) and then eluted stepwise with column loading buffer adjusted to contain increasing concentrations of NaCl from 100 to 800 mM, as indicated in the text. Fractions were analysed by SDS/PAGE. The signal on the dried gel was quantified using a PhosphoImager (Fuji) and associated software. The signal in each fraction was plotted as a percentage of the total recovered.

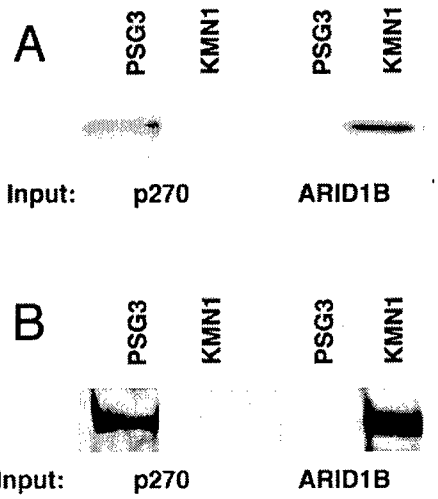


**Figure 1 Specificity of mAb raised against p270 and the ARID1B protein**

(**A**) *In vitro* translated peptides containing the antigenic regions used to generate each of the mAbs were immunoprecipitated with either mAb PSG3 or mAb KMN1 as indicated. The origin of the *in vitro* translated product, either p270 or ARID1B, is shown as 'input' below each lane. (**B**) CMV-promoted expression vectors for portions of p270 and ARID1B, containing the antigenic regions used to generate each of the mAbs, were transiently expressed in HEK-293 cells (human embronic kidney 293 cells). Cell lysates were collected and immunoprecipitated with mAb PSG3 or KMN1, as indicated above each lane. The immunocomplexes were separated by SDS/PAGE and transferred for Western blotting. The specificity of the mAb used in the Western blot matches the origin of the mammalian expression product, shown as 'input' below each lane.

## RESULTS

### Generation of mAbs that distinguish the p270 and ARID1B proteins

The high degree of identity (over 60 % at the amino acid level) between p270 and ARID1B means that polyclonal antibodies may not distinguish these products clearly *in vivo*. Therefore we raised mAbs to each using GST-fusion proteins as antigens. After preliminary characterization of the resulting hybridoma cell lines, a p270-specific antibody-secreting line designated PSG3 and an ARID1B-specific antibody-secreting line designated KMN1 were selected. Immunoprecipitation and Western blotting of the respective products expressed *in vivo* and *in vitro* verified that the antibodies do not cross-react (Figure 1).

### Expressions of p270 and ARID1B

Antibodies were used to screen the expression of p270 and ARID1B in a panel of common laboratory cell lines (Figure 2). These included HeLa (cervical carcinoma), SW13 (adenocarcinoma), PC-3 and DU145 (prostate cancer), Saos-2, MG 63, U2OS and OHS 50 (osteosarcomas), MCF-7 and MDA-MB-435s (breast carcinomas) and TSU-Pr1 (formerly considered to be a prostate carcinoma cell line; recently identified by Van Bokhoven et al. [26] as a derivative of the T24 bladder carcinoma cell line). ARID1B is detectable in each of these cell lines, but the signal is weaker than the p270 signal. Reaction of each of the antibodies against the respective purified GST-fusion proteins (not shown) indicates that the antibody signals are proportional to the respective amounts of proteins. The Western-blot signals, therefore, indicate that expression levels of ARID1B are generally

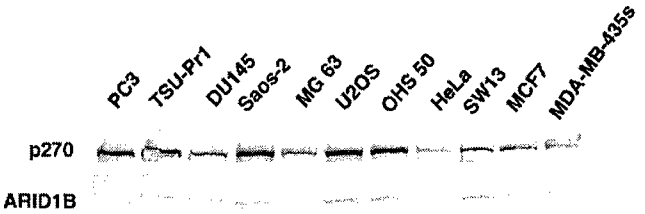


**Figure 2 Western-blot analysis of total cell lysates of selected tumour cell lines: distribution of ARID1B expression**

Aliquots (150 $\mu$g) of each of the various cell lysates were separated by SDS/PAGE, transferred on to a PVDF membrane and probed with antibodies specific to either p270 or to the ARID1B protein, as indicated in the Figure.

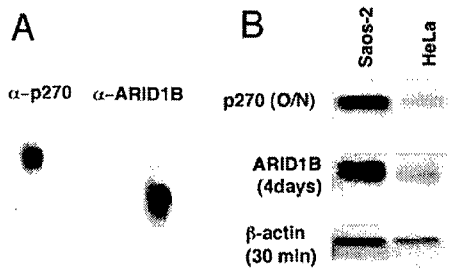


**Figure 3 Northern-blot analysis of ARID1B expression**

(**A**) p270 plasmid NHXSS98 (100 ng; lane 1) and ARID1B plasmid pKM10_v1 (lane 2) were separated in duplicate by agarose-gel electrophoresis, transferred on to a Hybond-N nylon membrane and hybridized separately with either the p270- or the ARID1B-specific probes, as indicated, to verify the specificity of the probes. (**B**). Aliquots of poly(A)$^+$-selected RNA were separated by electrophoresis, transferred on to membranes and hybridized with the p270- or ARID1B-specific probes, as indicated in the Figure.

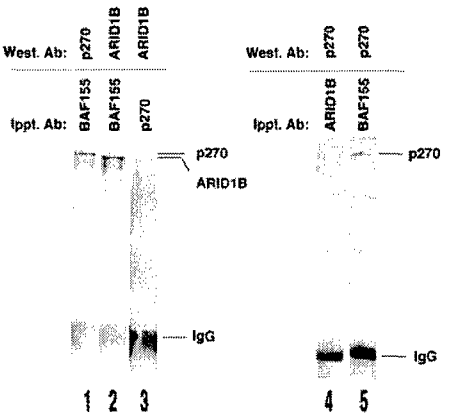


**Figure 4 ARID1B and p270 are mutually exclusive subunits of human SWI/SNF complexes**

Saos-2 cell lysate (2 mg/lane) was precipitated with antibodies of specificity indicated in the 'Ippt. Ab' lanes. Immunocomplexes were separated by SDS/PAGE and transferred on to a PVDF membrane. The membrane was cut to separate the individual lanes, which were then probed in Western blots with antibodies of specificity indicated as 'West. Ab'. Since the samples were immunoprecipitated before Western blotting, a prominent signal corresponding to the IgG heavy chain is apparent in each lane.

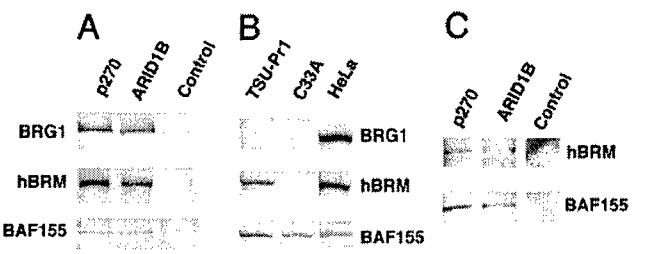


**Figure 5 The ARID subunits are not specific to the ATPase subunits**

p270- or ARID1B-specific immunocomplexes were isolated from Saos-2 cells (**A**) or TSU-Pr1 cells (**C**), separated by SDS/PAGE, transferred on to membranes and analysed by Western blotting for the presence of BRG1, hBRM or BAF155, as indicated. Control immune reactions were performed with a monoclonal antibody specific to an irrelevant viral protein. Direct Western blots shown in (**B**) confirm that TSU-Pr1 cells lack BRG1 expression, but express relatively normal levels of hBRM. Western-blot results are also shown in this panel for C33A cells (which express low levels of BRG1 and undetectable hBRM), as well as HeLa cells, which express both proteins.

lower than p270, although tissue distribution is widespread. Expression of ARID1B is generally strongest in the osteosarcoma cell lines, but there is variability.

To obtain a more quantitative comparison, expression levels in representative cell lines were also examined by Northern blotting (Figure 3). Probes of comparable size from directly comparable regions of each cDNA were used to maximize the utility of the comparison. The specificity of the Northern-blot probes was verified against isolated cDNA under the same conditions as the Northern blot (Figure 3A). RNA was prepared by poly(A)$^+$ selection to optimize the clarity of the signal. The same blot was probed successively for p270, ARID1B and $\beta$-actin. To obtain a comparable signal, the ARID1B probe had to be exposed approx. four times longer than the p270 probe (Figure 3B). The RNA signals were roughly proportional to the protein signals, with expression consistently higher in Saos-2 cells when compared with, for example, HeLa cells. From quantification of these and other cell lines (not shown), we estimate the message level of p270 to be 3–4-fold higher than that of ARID1B. The Northern blots indicate a message size for ARID1B approx. 1 kb shorter than that of p270.

## ARID1B and p270 are mutually exclusive components of human SWI/SNF-related complexes

Saos-2 cells were used to probe directly whether the endogenous ARID1B protein is associated with human SWI/SNF complexes. An antibody to BAF155, a ubiquitous component of the SWI/SNF family of complexes (composition reviewed in [4]), was used to pull down the complexes from Saos-2 cells where ARID1B is well expressed. The isolated immunocomplexes were reactive with both the p270-specific and ARID1B-specific antibodies (Figure 4, lanes 1 and 2). In contrast, when the complexes were isolated using the p270-specific antibodies, ARID1B was not detected (lane 3). Similarly, when the complex was immunoprecipitated with ARID1B-specific antibodies, no p270 could be detected (lane 4), although a p270 signal is present in complexes isolated in parallel with the BAF155-specific antibody (lane 5). Additional controls confirm the presence of other major complex components in the ARID1B and p270-specific lanes (see e.g. Figure 5). The immunocomplex analysis demonstrates, first, that endogenous ARID1B associates with human SWI/SNF complexes and, secondly, ARID1B is a mutually exclusive alternative to p270 in the complexes. The assay shown in Figure 4 also permits a direct comparison of the relative migration rates of p270 and ARID1B (lanes 1 and 2). ARID1B migrates ahead of p270, consistent with their deduced amino acid lengths (accession numbers AF253515 for ARID1B and NM_006015 for p270). We estimate the relative molecular mass of ARID1B to be approx. 240 kDa.

### p270 and ARID1B can partner with either ATPase

Human SWI/SNF complexes can contain either of two closely related ATPases, BRG1 and hBRM. Despite their close structural relationship, mouse genetics indicates that BRG1 and mammalian BRM are functionally distinct. *Brm*-null mice are viable and fertile, and heterozygotes are not prone to tumorigenesis [27]. In contrast, *Brg1*-null mice die at a pre- or peri-implantation stage, and the corresponding heterozygotes have heightened tumour susceptibility [28]. Expression of *BRG1* and *hBRM* is activated at different times during early primate embryogenesis [23], consistent with the evidence that they play different roles during development. Recent evidence indicates that BRG1 and hBRM associate with different promoters during cellular proliferation and differentiation, and interact preferentially with distinct classes of transcription factors [29]. The mutual exclusion of p270 and ARID1B raises the possibility that each ATPase has a specific associated ARID family subunit. To explore this question, endogenous p270-specific or ARID1B-specific complexes were immunoprecipitated from Saos-2 cell lysates and probed with antibodies targeted to either BRG1 or hBRM. The results show that each of the ARID-containing subunits partner with both ATPases *in vivo* (Figure 5A). The BRG1-specific antibody used in the present study is an mAb raised against an N-terminal peptide sequence that is not present in hBRM and is not cross-reactive. The hBRM-targeted antibody is a commercially prepared mAb, which may be cross-reactive *in vivo* [30]. To ensure that the antibody is detecting authentic hBRM, immunocomplexes were isolated from the TSU-Pr1 cell line. This line lacks BRG1 expression as a result of bi-allelic mutation [31], but has relatively normal levels of hBRM (Figure 5B) as well as p270 and ARID1B (as shown in Figure 2). The antibody signal in TSU-Pr1 cells (Figure 5C) confirms that hBRM is associated *in vivo* with both the ARID proteins. The ability of each ARID protein to partner with both classes of ATPase-containing complexes expands the diversity of subunit combinations known to be present in cells.

### Ratio of BRG1 in p270 versus ARID1B complexes

As discussed above, BRG1 is essential for tumour suppression and embryonic viability, whereas BRM is not [27,28]. Therefore it is of particular interest to know how BRG1 complexes are specifically apportioned with respect to the p270 and ARID1B subunits. To address this question, saturating levels of antibody were used to immunoprecipitate either ARID1B complexes or p270 complexes from aliquots of Saos-2 cell lysate. The complexes were assayed by Western blotting using a chemiluminescence method for the presence of BRG1. A range of lysate amounts and a range of exposure times were included to ensure that signal would be obtained in a linear range (Figure 6). Quantification of the signal obtained in the 1 mg of lysate series at 15, 30 and 60 s exposure yielded ratios of BRG1 in p270 versus ARID1B complexes of 3.3, 3.7 and 2.8 respectively. By 3 min exposure, the signal from this series was no longer within the linear range. From this titration, we estimate that approx. 3.3-fold more BRG1 is associated with p270 compared with ARID1B in these cells. This is consistent with the relative amounts of p270 and ARID1B present in the cells, implying that the ARID subunits compete equally well for the complexes.

### DNA-binding activity of ARID1B

The role of the ARID DNA-binding region in p270 and ARID1B is not clearly established. p270 binds DNA in a non-sequence-specific manner [9,13]. Deletion of the ARID region of p270 moderately reduces its ability to enhance glucocorticoid receptor-

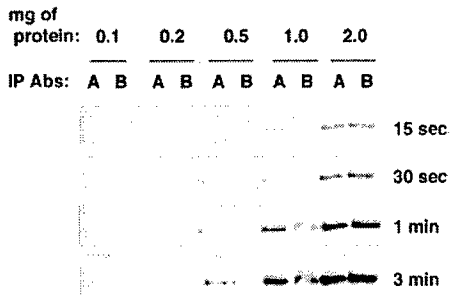


**Figure 6 Ratio of BRG1 in p270 versus ARID1B complexes**

The indicated amounts of Saos-2 cell lysates were immunoprecipitated with saturating amounts of p270-specific mAb PSG3 (lanes A) and ARID1B-specific mAb KMN-1 (lanes B). Immunocomplexes were separated by SDS/PAGE (8 % gel), transferred on to a PVDF membrane, Western-blotted with anti-BRG1 mAb and visualized by chemiluminescence assay. Different exposures were obtained at the indicated time points, and the ratio of the amount of BRG1 in the p270 complex to that in the ARID1B complex was quantified by densitometry.

mediated transcription in a co-transfection reporter assay [10]. Deletion of the ARID-containing region from ARID1B abrogates its activity in a similar assay [18]. It is not clear whether differences in the severity of the effect result merely from assay conditions as opposed to true physiological differences in the role of the domain in the context of protein function. ARID1B binds DNA with an affinity comparable with that of p270 or the prototypical sequence-specific ARID family member Dri [13], but experiments addressing the potential for sequence-specific binding by ARID1B have not been reported. Within the 94-residue ARID consensus, p270 and ARID1B differ at 17 amino acid positions; several of these are non-conservative changes. The potential of ARID1B for sequence-specific DNA-binding behaviour was evaluated in the present study in a DNA pull-down assay. Lambda phage DNA was cut with restriction enzymes to produce a large pool of DNA fragments of different sizes and a wide range of sequences. GST-fusion proteins were used to probe for preferential binding within the lambda DNA restriction fragment pool. The control ARID family protein Dri shows selectivity in this assay, as in other approaches [13,25]. Increasing the stringency of the interaction by adjusting the salt concentration results in increasingly more specific preference for selected fragments (Figure 7A, lanes 2–4). In contrast, a p270 fusion protein binds the fragments with no obvious selectivity (lanes 5–7). Increasing stringency does not reveal a preference for specific fragments, except for the eventual selection of longer fragments over shorter ones, probably because there are more binding surfaces on longer pieces of DNA. An ARID1B fusion protein, identical in length with the p270 fusion protein, behaves like p270 in this assay, showing no selectivity for specific fragments except for increasing the selection of longer DNA pieces (Figure 7B, lanes 2–4).

In the salt titration shown in Figure 7, relatively little DNA remains bound to the ARID1B protein after the 200 mM salt wash, raising the possibility that ARID1B has a weaker DNA-binding affinity when compared with p270. However, a previous analysis on DNA–cellulose affinity columns showed indistinguishable elution profiles for p270 and ARID1B [13]. The *in vitro* translated ARID1B peptide used in that assay was longer than the p270 protein with which it was compared and also longer than the respective GST-fusion protein segment analysed here. To control the possibility that sequences outside the ARID region contribute to DNA-binding affinity, a smaller expression vector, designed to be identical in length with p270, was constructed. The $^{35}$S-labelled *in vitro* translated peptides were applied to native DNA–cellulose

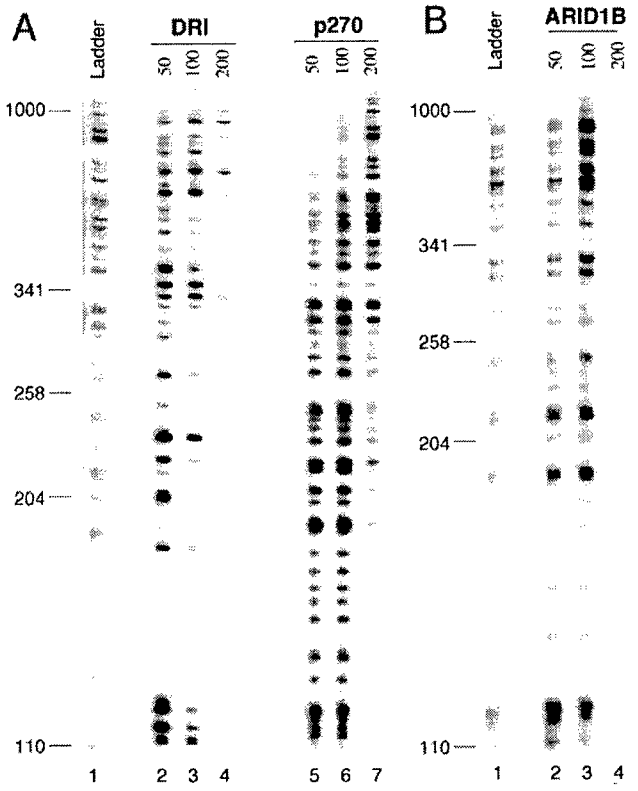


**Figure 7 ARID1B binds DNA non-sequence-specifically**

Lambda phage DNA was digested with *Eco*RI, *Hin*dIII and Sau3A1 to generate a large DNA oligonucleotide pool predicted to contain 128 fragments ranging in size from 12 to 2225 bp. The fragments were filled in with [$^{32}$P]dATP, incubated with GST-fusion proteins containing the ARID regions of p270, Dri or ARID1B as indicated, pulled-down with glutathione beads and analysed by PAGE. Lane 1 in each panel shows the unselected pool of DNA fragments. The remaining lanes show the fragments selected in Lambda DNA-binding buffer with increasing KCl concentrations as indicated.

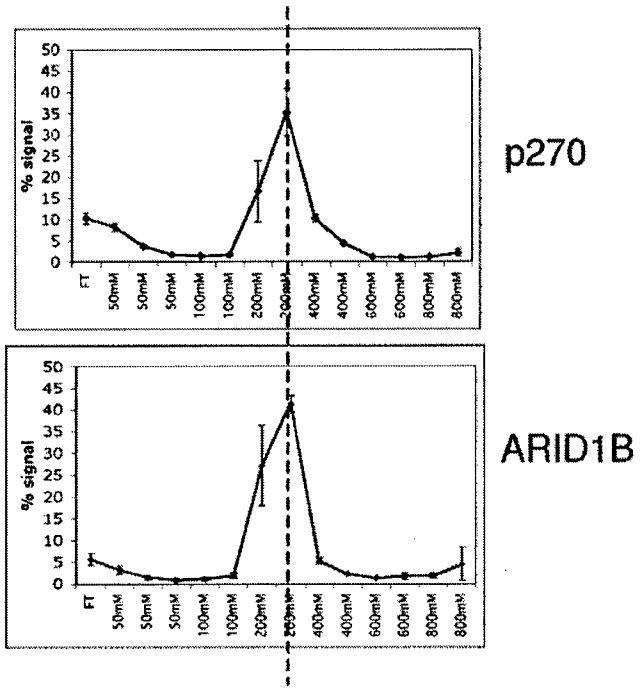


**Figure 8 DNA-binding affinity of p270 and ARID1B**

*In vitro* translated [$^{35}$S]methionine-labelled peptides were applied to native DNA–cellulose columns as described in the Materials and methods section. Bound protein was eluted stepwise with loading buffer adjusted to contain increasing concentrations of NaCl from 100 to 800 mM, as indicated in the Figure. Fractions were separated by SDS/PAGE and the p270 signal in each fraction was quantified by phosphoimaging. The results are plotted as the percentage of signal in each fraction relative to the entire signal recovered. Error bars represent S.D. Graphs are aligned for ease of comparison.

columns and washed with increasing salt concentrations. The p270 and ARID1B proteins again show similar elution profiles (Figure 8), reaching a peak at the same fraction determined previously. Both the proteins begin to be eluted at the 200 mM salt concentration; therefore, the difference seen in the pull-down assay might reflect slightly different sensitivities over this range.

## DISCUSSION

Generation of mAbs that react selectively with either p270 or ARID1B has allowed us to probe the expression of ARID1B and its potential interaction with human SWI/SNF complexes *in vivo*. These screens indicate that ARID1B levels are normally low relative to p270. A survey of tumour cell lines of various tissue origins, including cervix, adrenal cortex, breast, prostate, bladder and bone, indicates that ARID1B is widely expressed and that its expression level relative to p270 is fairly constant. Analysis with mAbs whose signal equivalence was checked by reaction against purified p270 and ARID1B GST-fusion proteins, combined with Northern-blot results using directly comparable probes, suggest that the ratio of p270 to ARID1B in most cells is approx. 3.5:1. The immunocomplexes confirm that endogenous ARID1B is associated with SWI/SNF complexes and show that p270 and ARID1B, similar to BRG1 and hBRM, are alternative, mutually exclusive subunits of the complexes. The ARID-containing subunits are not specific to the ATPases. Each associates with both BRG1- and hBRM-containing complexes, thus increasing the number of distinct subunit combinations known to be present in cells. Titration of the ARID subunit-associated BRG1 signal in Saos-2 cells indicates that BRG1 is distributed proportionally between the two ARID subunits.

The mAbs described here are unique reagents. Previous reports were unable to clarify whether p270 and ARID1B are present in distinct complexes [18,20,21] or conflicted in their conclusions about the associations between the two ARID proteins and the two ATPases [18,22]. All of the interactions discussed in the present study were probed with endogenous proteins under normal physiological conditions, under conditions permitting a relatively quantitative analysis not possible previously. The *in vivo* analysis does not address the question whether the association of the ARID proteins and ATPases is direct, but *in vitro* association data indicate that the interaction is direct and requires the C-terminal portion of the ARID proteins [18].

The functional distinction between p270 and ARID1B-containing complexes is not yet known. The expression profiles of p270 and ARID1B are distinguishable during early primate development, similar to BRG1 and hBRM [23], implying that the ARID-containing subunits also have distinct functions. Expression patterns of p270 (synonym: Osa1) and ARID1B (synonym: BAF250b) have been examined during mouse development, but have not been compared directly, although differences

do appear to exist [22,32]. Transient transfection assays reveal similar abilities to enhance expression from steroid hormone-responsive promoters; however, it is clear that more physiologically relevant assays are needed to distinguish the roles of each protein. Knockdown studies are in progress in our laboratory and they should offer an indication of the respective biological roles of p270 and ARID1B in differentiating cells.

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# EXPRESSION OF p270 (ARID1A), A COMPONENT OF HUMAN SWI/SNF COMPLEXES, IN HUMAN TUMORS

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**Human SWI/SNF complexes use the energy of ATP hydrolysis to remodel chromosomes and alter gene expression patterns. The activity of the complexes generally promotes tissue-specific gene expression and restricts cell proliferation. The ATPase that drives the complexes, BRG1, is essential for tumor suppression in mice and deficient in a variety of established human tumor cell lines. The complex contains at least 7 other core components, one of which is a large subunit designated p270. p270 RNA is expressed in all normal human tissues examined, but protein expression is severely reduced in at least 2 human tumor lines, C33A and T47D. We show here that loss of p270 in the C33A and T47D cell lines is evident at the RNA level as well as the protein level. The implication that p270 can be informatively screened at the RNA level made a high-efficiency cancer profiling array approach to screening human tumors feasible. Expression was screened in an array containing RNA-derived cDNA from 241 tumor and corresponding matched normal tissues from individual patients. p270 deficiency was observed at a higher overall frequency than BRG1 deficiency, but all tissues were not equally affected. Deficiency of p270 was observed most frequently in carcinomas of the breast and kidney. The results were most striking in kidney, where p270 expression was deficient in 30% of carcinoma samples screened. Screening of a panel of established human renal carcinoma–derived cell lines supports the frequency observed in the primary tumor tissue samples.**


**Key words:** *p270; SWI/SNF; kidney cancer; breast cancer; tumor suppression*

Human SWI/SNF complexes are transcription-regulating complexes that are essential to normal differentiation and development. They also play a vital role in the control of cell proliferation and suppression of carcinogenesis (reviewed by Klochendler-Yeivin and Yaniv[1] and by Muchardt and Yaniv[2]). The complexes can vary in composition, but all are able to remodel chromatin structure through ATP-dependent mechanisms.[3–7] ATPase activity, which is DNA-dependent, resides in the SWI2/SNF2 enzyme in yeast and in the SWI2/SNF2-related proteins BRG1 and hBRM in humans. Shortly after BRG1 was cloned on the basis of its homology to the *Drosophila* and yeast ATPases,[8] it became apparent that BRG1 is occasionally found at low or undetectable levels in common tumor-derived laboratory lines.[2,9,10] This suggestion that loss of BRG1 may be a causative factor in human tumorigenesis is strongly supported by the enhanced tumor susceptibility of BRG1 heterozygous mice; tumors of epithelial origin were observed in the head and inguinal regions of these mice.[11] Additional components of the complex are also likely to be vital for tumor suppression. The hSNF5 complex component (also designated INI1 or BAF47) is lost with high frequency in specific types of human tumors, primarily pediatric rhabdoid tumors,[12–15] and Snf5-deficient mice also develop tumors at an early age.[16,17] BRG1-associated factor-155 (BAF155) is occasionally deficient in tumor cell lines,[10] and BAF155-null mouse embryos die at an early implantation stage, indicating the gene is required for normal development although neoplasia in BAF155 heterozygotes has not been examined.[18] Despite this background, there is very little information on expression of SWI/SNF complex components other than hSNF5 in primary human tumor tissue.

Human SWI/SNF complex components include a large subunit designated p270[19,20] or BAF-250[21] (HUGO Gene Nomenclature Committee, http://www.gene.ucl.ac.uk/nomenclature/, and the Mouse Genomic Nomenclature Committee, http://www.informatics.jax.org/mgihome/nomen/index.shtml, recommend the official gene designations *ARID1A* in humans and *Arid1a* in mice). p270 contains a DNA-binding domain (the ARID) that distinguishes a family of at least 14 human proteins, all of which are implicated in the regulation of differentiation and tumorigenesis.[22] p270 RNA is expressed in all normal human tissues examined,[20] but p270 protein expression is undetectable or severely reduced in some human tumor lines, including the cervical carcinoma–derived line C33A and the breast cancer–derived line T47D.[10,21]

We show here that loss of p270 in the C33A and T47D cell lines is evident at the RNA level as well as the protein level. The implication that p270 can be informatively screened in human tumors at the RNA level makes possible a high-efficiency cancer profiling array approach to screening primary tumor samples. Expression was screened in a cancer array containing RNA-derived cDNA from 241 tumor and corresponding matched normal tissues from individual patients. We detected p270 deficiency at a higher overall frequency than BRG1 deficiency. p270 expression was diminished more than 2-fold relative to normal expression in tumor samples of distinct tissue origins, including breast, uterus, colon, lung and kidney, with breast and kidney carcinomas being the most frequently affected. The results were most striking in cancers of the kidney, where p270 expression was reduced by more than 2-fold in 30% of carcinoma samples.

## MATERIAL AND METHODS

### *Immunoblotting*

The immunoblotting procedure was described previously.[19] Preconfluent cells were harvested in PBS. Cells were lysed, and 100 μg of total cell protein per lane were separated on 8% SDS-PAGE gels, transferred to an Immobilon-P membrane (Millipore, Bedford, MA) and visualized using alkaline phosphatase–conjugated secondary antibody.

### *Northern blots*

PolyA-selected RNA was prepared from appropriate cell cultures using Tri-Reagent (Sigma, St. Louis, MO) and the PolyATract

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mRNA Isolation System (Promega, Madison, WI) according to the supplier's recommendations. RNA (18 μg/lane) was loaded and fractionated by electrophoresis through a 0.7% formaldehyde-agarose gel. (This generous amount of polyA-selected RNA permitted quantitative assessment of the p270 signal in p270-deficient cell lines.) RNA was transferred to a Hybond-N nylon membrane (Amersham, Arlington Heights, IL) and crosslinked by UV irradiation and baking at 80°C. $^{32}$P-labeled probes were prepared using a random primed labeling kit (Boehringer-Mannheim, Mannheim, Germany). Between successive probes, blots were stripped by boiling in 0.1% SDS. Signals were obtained by autoradiography. Quantification from appropriate exposures was determined using a Fuji (Tokyo, Japan) phosphoimager.

*Probes*

The p270 2.5 kb 3′ probe includes the untranslated region (UTR) and was described previously.[20] The 1.8 kb C-terminal p270 probe was generated from a p270 cDNA plasmid containing the 2.5 kb probe (p110) using primer sequences AGAGAGGAATTCACCTCTCCTAGCAAGT and ATCGAGCAGGAAGCCCAGGAGGTTGC, which exclude the UTR. A probe hybridizing to the mid-region of 270 was generated by PCR from plasmid pNHXSS98, which contains the p270 cDNA sequence described by Dallas *et al.*,[20] using the primer sequences TACCAGCAGAACTCCATGGGGAGCTAT and GCATGAACCGGAAAACCCAAGA. The BRG1 probe was made by RT-PCR using WI38 cell cDNA as the template, and primer sequences were AAGTGGCAGCGAAGAAGACTGA and TGCGTTTTGTTGTTGGTTTAA. The resulting amplicon was 481 bp and covered the entire BRG1 3′ UTR (461 bp) as well as 20 bp of the coding region. Computer alignments indicate no significant homology to the 3′ UTR of hBRM across this sequence. The β-actin probe was described previously.[23] The ubiquitin probe was supplied by Clontech (Palo Alto, CA) as part of the cancer profiling array package.

*Southern blot*

Genomic DNA was isolated using Tri-Reagent. DNA (10 μg) from each cell line was digested with XbaI and separated by electrophoresis through a 0.7% agarose gel. DNA was transferred to a Hybond-N nylon membrane and crosslinked by UV irradiation and baking at 80°C. The resulting blot was hybridized to the 1.8 kb p270 probe labeled by random priming (Boehringer-Mannheim).

*Cancer profiling array*

The cancer profiling array was obtained from Clontech, BD Biosciences and screened in the hybridization solution provided, according to the supplier's recommendations. The array was probed sequentially with probes corresponding to p270, BRG1 and ubiquitin. p270 and BRG1 signals were normalized to the ubiquitin signal before calculation of relative intensities. Quantification was performed using a Fuji phosphoimager.

*Cell lines*

HeLa, WI-38, C33A, UACC-812 and 293 cells were obtained from the ATCC (Rockville, MD). BT-20, MCF7, ZR-75-30, MDA-MB-435, MDA-MB-36T47D, Caki-1 and Caki-2 were a gift from Dr. S. Coszenza (Fels Institute, Temple University School of Medicine). MDA-MB-435 results were verified with the MDA-MB-435s line obtained fresh from the ATCC. PC-3, DU-145, LNCaP and TSU-Pr1 were a gift from Dr. B. Lokeshwar (University of Miami School of Medicine, Miami, FL). Saos-2, OHS-50, MG-63, U-2OS and TE-85 cells were a gift from Dr. M.F. Hansen (Center for Molecular Medicine, University of Connecticut Health Center, Farmington, CT). A-498, ACHN, 786-P and 769-O cells were a gift from Dr. T. Griffith (Department of Urology, University of Iowa Roy J. and Lucille A. Carver College of Medicine, Iowa City, IA). UO-31 and SN12C cells were a gift from Dr. R. Bernacki (Roswell Park Cancer Institute, Buffalo, NY). All cell lines were cultured according to ATCC recommendations.

*Antibodies*

The p270 monoclonal antibody (MAb) PSG3 was raised against the pNDX2 GST-fusion protein described previously.[20] The BRG1-specific MAb 320.7 was raised against an N-terminal peptide sequence [STPDPPLGGTPRPG(T)] corresponding to residues 2–15 of human BRG1. All hybridoma isolation work was done at the St. Louis University Hybridoma Facility (St. Louis, MO). A BRM-directed antibody was purchased commercially (Transduction Laboratories, Lexington, KY; B36320). The hsc70-specific antibody was also obtained commercially (Stressgen, Vancouver, Canada).

## RESULTS

*Diminished expression of p270 in established tumor cell lines at the RNA level*

Loss or severe reduction of p270 expression has been reported in a total of 4 human carcinoma-derived cell lines: C33A cervical carcinoma cells, MDA-MB-435 and UACC-812 breast carcinoma cells,[10] and T47D breast carcinoma cells.[21] DeCristofaro *et al.*[10] reported results from a series of 40 human tumor cell lines that included 21 breast cancer lines and 19 lines of other tissue origin. They probed at the protein level for each of the major components of human SWI/SNF complexes. Their results suggest that roughly 40% of common laboratory lines lack normal protein levels of at least one component of the complex. BRG1 deficiency has been attributed to truncation or deletion at the DNA level, reduced RNA expression or posttranscriptional mechanisms, depending on the cell line;[9,24] but the level of loss of most other components has not been examined past the protein level.

Western blotting with a p270-specific MAb confirms the reduced expression of p270 in T47D and C33A cells compared to normal levels in the BT-20 breast tumor and HeLa cervical carcinoma–derived lines (Fig. 1). In contrast to the previous report, we saw normal expression of p270 in the MDA-MB-435 breast tumor–derived line (Fig. 1). This was confirmed with MDA-MB-435s cells freshly obtained from the ATCC (not shown) and may reflect a difference in the lines in laboratory culture. The UACC-812 breast tumor–derived line was also obtained fresh from the ATCC but could not be maintained reliably in culture, so no additional data were obtained from this line.

We examined p270 expression in C33A and T47D cells at the RNA level. Northern probes corresponding to 2 different regions of p270 (middle and C-terminal) were used to probe polyA-selected RNA from each line in parallel, with MCF7 breast carcinoma cells and HeLa cervical carcinoma cells included as controls. Northern blots with either probe showed sharply reduced RNA levels in both the C33A and T47D lines. A representative blot with the middle-region probe is shown in Figure 2*a*. Quantification of the signal after normalization to β-actin, averaged for the 2 independent probes, indicated reductions of 3.8-fold in the p270 RNA

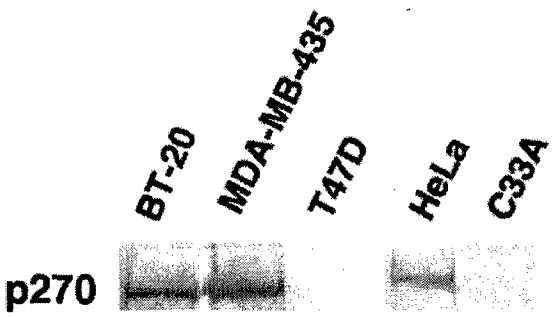


FIGURE 1 – Western blot. Whole-cell lysates of the indicated cell lines were separated on 8% gels, transferred to membrane and probed with a p270-specific MAb.

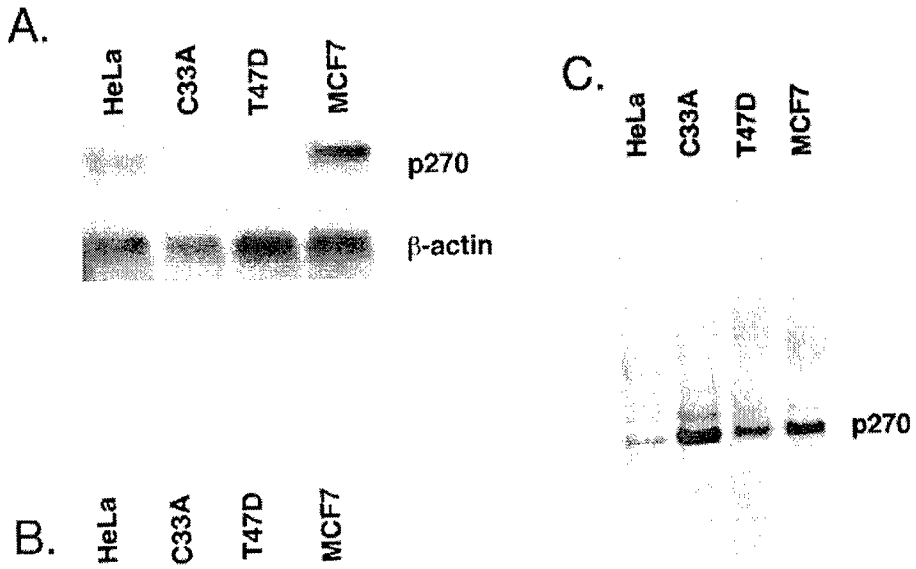


FIGURE 2 – Northern and Southern blots. *(a)* PolyA-selected RNA was prepared from the indicated cell lines, fractionated by electrophoresis through a 0.7% formaldehyde-agarose gel, transferred to membrane and hybridized with a probe corresponding to the mid-region of p270. *(b)* An RNA blot, prepared as described in *(a)*, was hybridized with a C-terminal 1.8 kb p270 probe and overexposed to reveal the signal in lines with low expression. *(c)* Genomic DNA (10 μg) was digested with XbaI, separated by electrophoresis, transferred to membrane and hybridized to the same probe used in *(b)*.

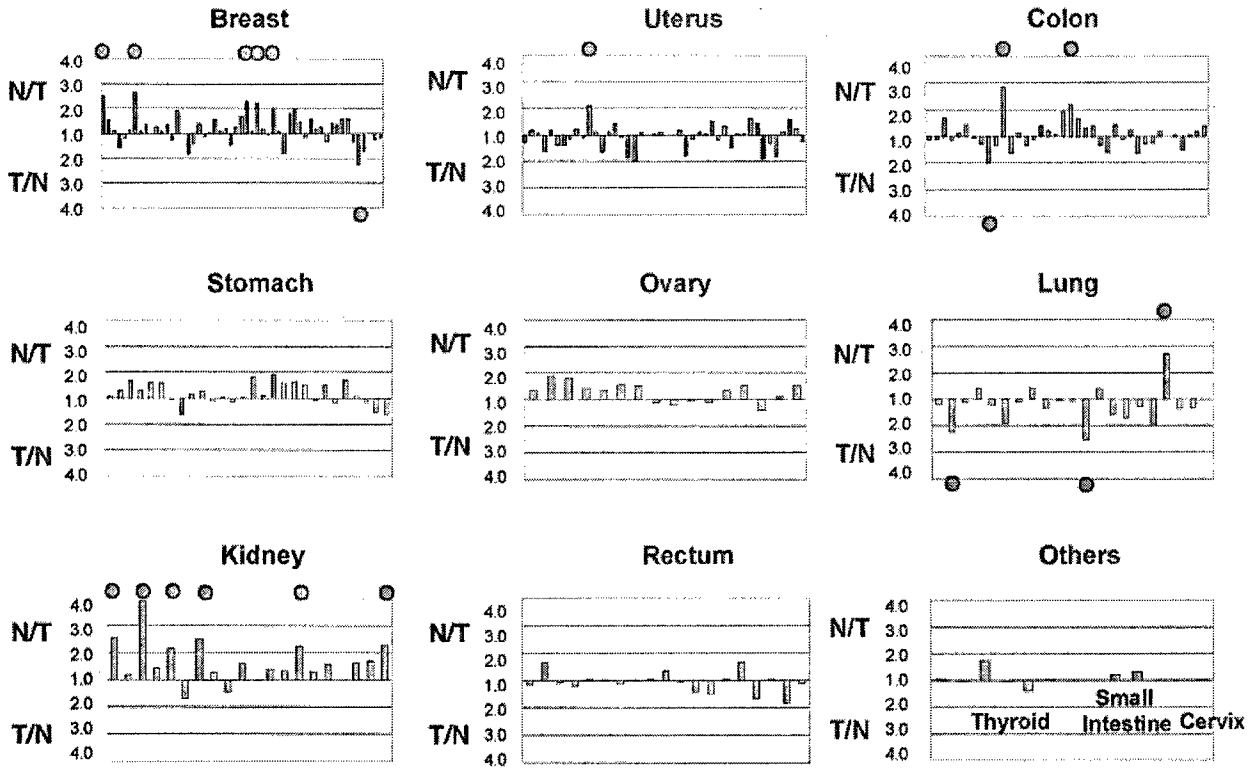


FIGURE 3 – Histogram of p270 signal on the cancer profiling array. The cancer profiling array was hybridized with a p270-specific probe as described in Material and Methods. The same blot was hybridized with a ubiquitin probe for standardization. After normalization of the p270 signal to the ubiquitin signal, the ratio of signal between normal tissue (N) and tumor tissue (T) for each tissue pair was calculated. Pairs where the tumor sample showed a lower signal are plotted above the line. Pairs where the tumor tissue showed a higher signal are plotted below the line. Ratios >2-fold are marked with red (N/T) or green (T/N) dots.

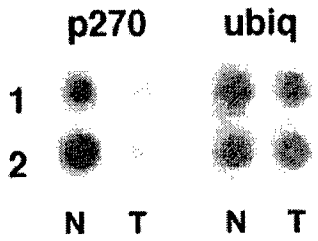


FIGURE 4 – Portion of kidney tumor array. The figure shows 2 pairs of samples from the kidney portion of the array in which p270 expression is reduced in the tumor tissue (T) relative to the matched sample of normal tissue (N). The same blot was stripped and probed with ubiquitin for normalization of the signal. After normalization, the relative intensities showed ratios (N/T) of 2.5 in pair 1 (loci G31 and G32 on the array) and 3.9 in pair 2 (loci C31 and C32 on the array). These spots correspond, respectively, to the seventh and third samples from the left in the kidney panel of the histogram in Figure 3.

signal in C33A cells relative to HeLa cells and of 4.5-fold in T47D cells relative to MCF7 cells. Very long exposures with either probe yielded signals comigrating with the full-length p270 message seen in the control cells, indicating that the message in lines with low expression is not missing or truncated. Long exposure with the C-terminal probe is shown in Figure 2*b*. Consistent with this, Southern blots revealed the presence of a normal (single prominent band) DNA signal (Fig. 2*c*). Together these results indicate that impairment of p270 expression is at the level of RNA expression or stability. While this may not be the only level of p270 impairment in human tumors, the results indicate that p270 expression can be informatively screened at the RNA level. Such a screen should yield at least a minimal estimate of the frequency of p270 loss.

*Expression of p270 in primary tumor samples*

Expression of p270 in tumor tissue samples was screened using a cancer profiling array that contained RNA-derived cDNA from 241 tumor and corresponding matched normal tissues from individual patients. The 241 samples included 13 tissue types: breast (50), uterus (42), colon (34), stomach (28), ovary (14), lung (21), kidney (20), rectum (18), thyroid (6), cervix (1), prostate (4), pancreas (1) and small intestine (2). The blot was screened with a 2.5 kb probe that included the 3′ UTR. After normalization to a control signal from ubiquitin, the results were calculated as the ratio of expression between normal and matched tumor samples and are presented as histograms in Figure 3. A reliable signal from the prostate and pancreas tissue pairs was not obtained in either screen, so the total number of samples considered here is 236.

Northern blots showed an approximate 4-fold difference in p270 RNA levels between normal cells and p270-deficient cells. The dot blot screening method used on the cancer array is not expected to be as sensitive as Northern blots, so for initial consideration we arbitrarily defined apparent p270 deficiency as a >2-fold difference between tumor and corresponding normal samples. More than 90% (91.9% or 217/236) of the tissue pairs showed <2-fold variation. A total of 19 tissue pairs showed a >2-fold difference. Of these, only 4 registered an increased signal in the tumor sample

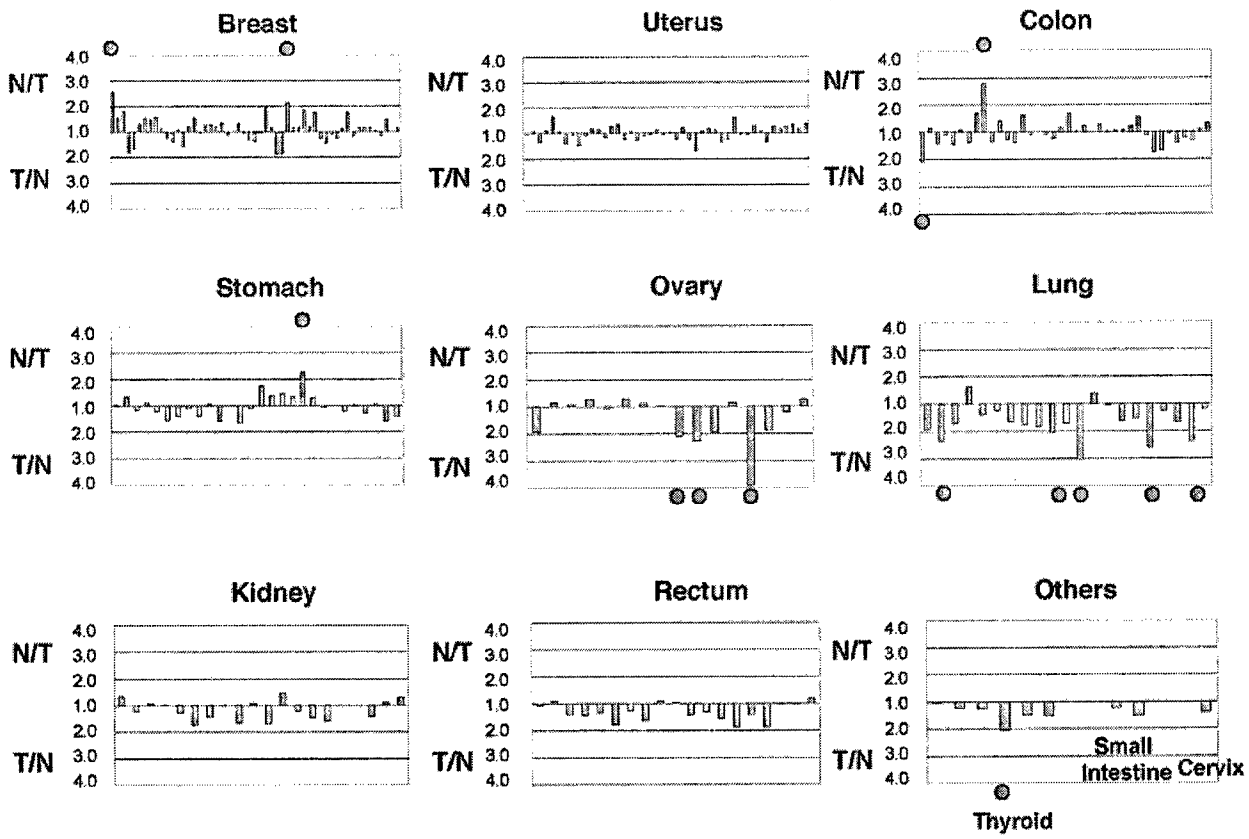


FIGURE 5 – Histogram of BRG1 signal on the cancer profiling array. The cancer profiling array was hybridized with a BRG1-specific probe. After normalization to the ubiquitin signal, as described in Figure 3, the ratio of signal between normal tissue (N) and tumor tissue (T) for each tissue pair was calculated. Pairs where the tumor sample showed a lower signal are plotted above the line. Pairs where the tumor tissue showed a higher signal are plotted below the line. Ratios >2-fold are marked with red (N/T) or green (T/N) dots.

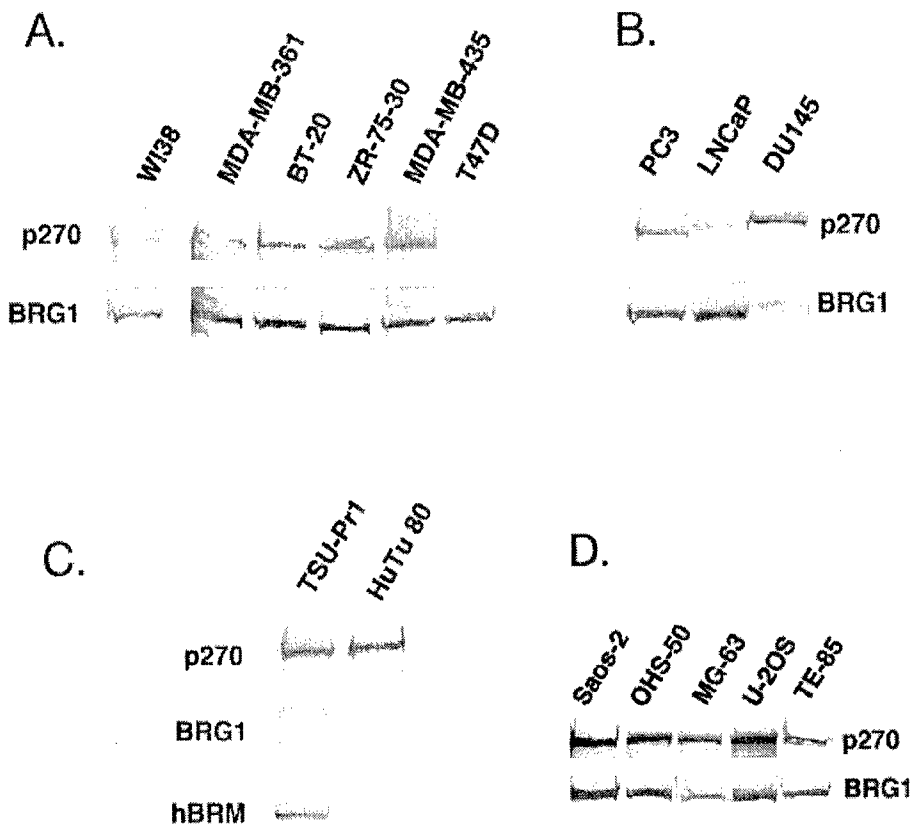


FIGURE 6 – Western blot survey of tumor cell lines. Whole-cell lysates of the indicated cell lines were separated on 8% gels, transferred to membrane and sequentially probed with MAbs specific for p270, BRG1 or hBRM.

with respect to the normal (1 breast, 1 colon and 2 lung). In contrast, 15 samples registered a decrease >2-fold in the tumor sample compared to the matched normal sample. These were not randomly distributed with respect to tissue origin. They included 5 breast, 6 kidney, 2 colon, 1 uterus and 1 lung. The results in the kidney panel were especially striking, with expression reduced >2-fold in fully 30% of samples (6/20). The second highest frequency was the 10% deficiency seen in the breast carcinoma panel. The results indicate that overall about 6% of malignant epithelial tumors are deficient for p270 expression. However, the frequency of loss is higher in tumors of some tissue origins than others, with renal and breast carcinomas being the most frequently affected. The signal from 2 of the kidney tissue pairs on the array is shown in Figure 4.

The same array was also screened with a BRG1 C-terminal probe. The BRG1 signal was deficient in tumor samples less frequently than the p270 signal. Indeed, where the difference exceeded 2-fold, the BRG1 signal was more often higher in the tumor sample (10 cases: 1 colon, 3 ovary, 5 lung, 1 thyroid) than in the normal tissue sample. In only 4 cases was the BRG1 signal higher in normal tissue (2 breast, 1 colon, 1 stomach). Histograms of these results are shown in Figure 5. The signals for the 1 pancreas and 4 prostate pairs were obtainable from the BRG1 blot but showed no significant difference between normal and tumor samples (not shown).

*Screening of established tumor cell lines*

The cancer profiling array suggests that p270 is particularly important to tumor suppression in renal and breast tissue. However, results from this type of approach must be considered carefully. The array offers the advantages of screening large numbers of samples, which can point to unsuspected tissue types where the gene of interest may have particular importance; but any conclusions drawn from the array are necessarily based on an arbitrary definition of loss of expression. The results require independent validation. If deficiency of p270 does indeed heighten susceptibility to tumor formation in kidney and breast cells, we would expect to see comparable frequencies of p270 deficiency in tumor cell lines originating from these tissues. To complement the tissue array, we screened a panel of human tumor cell lines for expression of p270 and BRG1. These included 2 breast tumor lines not previously examined (the carcinoma line ZR-75-30 and the adenocarcinoma line MDA-MB-361) and various lines originating from other types of tissue. It is already known that p270 expression is widespread in normal tissue, with roughly comparable levels in all tissue types probed.[20]

Breast cancer lines ZR-75-30 and MDA-MB-361 showed approximately normal levels of both p270 and BRG1 in comparison with BT-20 breast carcinoma cells and WI-38 cells, which are nonimmortalized human fibroblasts (Fig. 6*a*). The prostate line PC-3 has previously been reported to express normal levels of p270 and BRG1[10] and was screened here in comparison with 2 other prostate lines, DU-145 and LN-CaP (Fig. 6*b*). DU-145 showed a severely reduced level of BRG1, consistent with a previous report,[9] and normal expression of p270. LNCaP showed normal expression of BRG1 but had a slightly reduced level of p270.

Several other carcinoma cell lines were also examined. Two of these are shown in Figure 6*c*. The TSU-Pr1 line has been extensively studied as a prostate line, but the evidence indicates it is a derivative of the T24 bladder carcinoma line.[25] As reported previously,[9] TSU-Pr1 shows no detectable BRG1. The antibody used here is a MAb highly specific for BRG1. Probing with a BRM-reactive antibody shows that hBRM expression in TSU-Pr1 cells is relatively normal. Our screen revealed that the duodenal carcinoma line HuTu-80 is severely deficient in BRG1. Rescreening with the BRM-reactive antibody indicated that hBRM expression is likewise severely decreased in this line. This result adds intestinal tissue to the list of

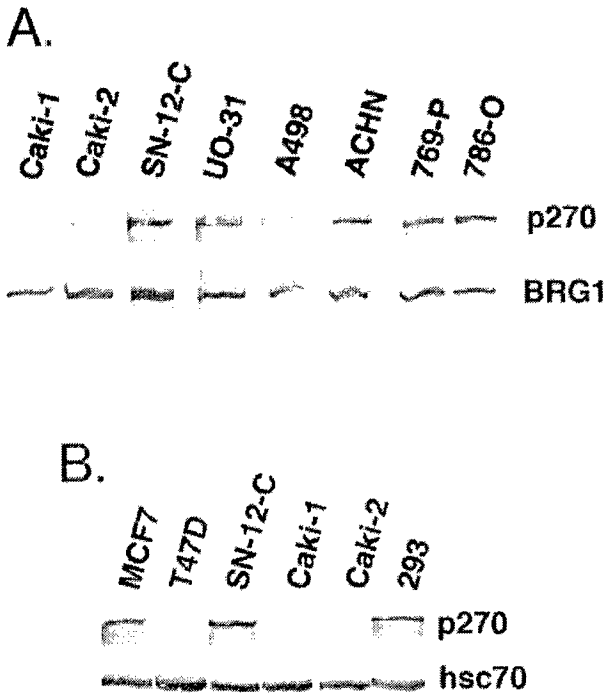


FIGURE 7 – Western blot survey of kidney tumor cell lines. Whole-cell lysates of the indicated cell lines were separated on 8% gels, transferred to membrane and sequentially probed with MAbs specific for p270, BRG1 or hsc70.

tissues that have generated tumor cell lines lacking both BRG1 and hBRM. Simultaneous loss of both BRG1 and hBRM has previously been reported in tumor cell lines originating from lung, breast, cervix, pancreas and adrenal gland.[2,9,24] Expression of p270 in both TSU-Pr1 and HuTu-80 cells is normal. We also examined a panel of human osteosarcoma lines (Fig. 6*d*). The complex components are generally well expressed in these lines. No line showed reduced expression of either p270 or BRG1.

These results combined with the only other reports that have considered p270 expression in tumor cell lines[10,21] yield a total of 24 breast cancer lines screened, with 2 being deficient for p270 expression. This frequency correlates well with the 5/50 breast tumor tissues that showed deficiency of p270 on the array and supports the general validity of the array results. No other type of tumor cell line panel has been examined systematically for p270 expression.

The most striking result from the array is the indication that deficiency of p270 is particularly significant for tumor susceptibility in renal tissue. To explore this further, we screened a panel of 8 independently established kidney tumor lines. Western blots showed normal levels of p270 in SN12C, UO-31, ACHN, 769-P and 786-O cells. The A498 line consistently showed reduced expression of p270, and the Caki-1 and Caki-2 lines showed sharply reduced levels of p270 (Fig. 7*a*). Caki-1 was established from a metastasized renal carcinoma, while Caki-2 was established from a primary renal carcinoma in a different patient.[26] All of the kidney tumor lines showed normal expression of BRG1 (Fig. 7*a*). Reduced expression of p270 was also apparent at the RNA level in the Caki-1 and Caki-2 lines (not shown). Low expression of p270 is not characteristic of normal kidney tissue. As indicated above, a Northern blot indicated similar expression of p270 across an entire panel of normal tissue samples; the panel included kidney tissue.[20] To compare protein expression levels in the kidney tumor lines directly with lines from other tissues, lysates from the breast cancer lines MCF7 and T47D were run on the same gel as selected kidney lines. 293 cells, a virus-transformed derivative of normal embryonic kidney cells, were also included; and the gel was further probed with an antibody to hsc70 as a loading control (Fig. 7*b*). The results indicate that the respective high and low expression levels in kidney lines are similar to what has been noted previously in breast cancer lines. The >25% frequency of p270 deficiency in the kidney tumor cell line panel supports the findings from the tissue array and implies that p270 contributes significantly to tumor suppression in renal tissue.

## DISCUSSION

A cancer profiling array offered an efficient initial means to probe tumors originating from a range of tissue types for expression of p270. The array detected reduced expression of p270 in tumors originating in breast, uterus, colon, lung and kidney tissue. A reduced p270 signal is associated with kidney and breast tumors at higher frequencies than other tissues tested. Parallel screens of established tumor cell lines support this finding. The present study brings to 24 the number of breast tumor lines that have been screened for p270 expression, with 2 of the 24, T47D and UACC-812, identified as p270-deficient.[10,21] This is in rough agreement with the frequency of p270 deficiency in primary breast tissue samples indicated by the array (10%) and supports the validity of the results. The most striking result from the array is the identification of kidney as a tissue where tumorigenesis is linked with deficiency of p270 with particularly high frequency (approx. 30%). A parallel probe of 8 established kidney tumor cell lines supports this percentage. Both of the kidney tumor cell lines that are sharply deficient for p270 expression (Caki-1 and Caki-2) are derived from clear cell carcinomas. p270 deficiency may not be limited to clear cell carcinomas, however, as the Clontech literature that accompanies the array indicates that 2 of the kidney tumor tissue samples that exhibited p270 deficiency were classified as renal cell carcinomas (ICD code M8312/3) but not as clear cell carcinomas (ICD code M8310/3). Additional screens will be required before any link between p270 deficiency and specific subclasses of renal tumors can be determined.

The activity of the complex is dependent on the energy provided by the ATPase, so the expectation is that BRG1 deficiency would be linked with tumor susceptibility in many tissues. The array did not reveal any tissue where tumorigenesis is linked with deficiency of BRG1 at a frequency above the limit of detection of the assay, which is about 5–10%. This is consistent with surveys of tumor cell lines, where BRG1 deficiency has been noted in lines derived from various tissues including cervical, prostate, adrenal, pancreatic, breast and lung[2,9,24] but not at high frequency in any individual tissue type. The present study adds intestinal tissue to this list with the HuTu-80 duodenal carcinoma line. Surveys of a total of 26 human breast tumor lines,[9,10] including the present study, have identified only one line deficient in BRG1 expression, ALAB.[9] The highest frequency noted for BRG1 deficiency thus far is in lung tissue, where a survey of 60 samples of primary human lung tumor tissue by immunohistochemistry found BRG1 to be deficient at the protein level in 10% of tumors, with a correlation between loss of BRG1 and tumor aggressiveness.[27]

In the large majority of cases, deficiency of BRG1 in human tumors is accompanied by deficiency of the alternative ATPase, hBRM,[10,24] as seen here in the HuTu-80 line, even though BRM, in contrast to BRG1, is not required for tumor suppression in mice.[28] Complementation studies in human cells suggest that the total dosage of ATPase activity from the 2 proteins is more important to a transformed phenotype than the presence or absence of one or the other.[24] The functional contribution of p270 to the DNA-dependent ATPase activity of the complex is not clear; however, p270 is a member of the ARID class of DNA-binding proteins,[22,29] and we postulate that the DNA-binding activity of p270 enhances the DNA-dependent ATPase activity of the complex. In this sense, loss of p270 may decrease the effective dosage of ATPase activity. Typically, BRG1 is well expressed in lines

where p270 is sharply reduced, as seen in Figure 6 for the T47D line and in Figure 7 for the Caki-1 and Caki-2 lines. The C33A cervical carcinoma line is the single exception, where p270, BRG1 and hBRM are all poorly expressed (Fig. 1).[10]

The results suggest that p270 plays a particularly important role in signal pathways that restrict proliferation and promote differentiation in the kidney. The most likely mechanism by which p270 contributes in a tissue-selective manner is through its potential as a coactivator for nuclear hormone receptors. p270 is one of several proteins in the complex that contain clusters of LXXLL motifs, which are binding sites through which nuclear hormone receptors can potentially recruit the activity of the complex. Presumably, the role of any individual component of the complex other than the ATPase is more critical in some tissues than in others. Thus far, only the glucocorticoid receptor has been identified in physical association with p270.[21] However, the physiologic role of this and other potential interactions is not yet known, and we are investigating these mechanisms.

In all of the p270-deficient tumor cell lines we have examined, the signal corresponding to the p270 message is present and of full length but at low intensity. Most likely, expression is silenced by methylation of p270 genomic sequences. The *p270* gene contains regions with classic features of CpG islands, which generally span the 5′ region, including the promoter, untranslated region and first exon.[30] One such island, extending from −1060 to −6 with respect to the predicted p270 translation start site, is 80.5% G+C and contains 115 CpG sites. A second island is present in exon 1, extending from +3 to +1124 with respect to the translation start site. This region has a G+C content of 79.5% and contains 146 CpG sites. Hypermethylation of CpG islands in tumor-suppressor genes occurs frequently in cancer, with profiles of tumor-suppressor gene inactivation specific to particular cancers.[31] Analysis of the presence of methylation in the p270 promoter and of potential methylation sites is in progress.

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# Expression of Genes Encoding Chromatin Regulatory Factors in Developing Rhesus Monkey Oocytes and Preimplantation Stage Embryos: Possible Roles in Genome Activation[1]

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## ABSTRACT

**One of the most critical events of preimplantation development is the successful activation of gene transcription. Both the timing and the array of genes activated must be controlled. The ability to regulate gene transcription appears to be reduced just prior to the time of the major genome activation event, and changes in chromatin structure appear essential for establishing this ability. Major molecules that modulate chromatin structure are the linker and core histones, enzymes that modify histones, and a wide variety of other factors that associate with DNA and mediate either repressive or activating changes. Among the latter are chromatin accessibility complexes, SWI/SNF complexes, and the YY1 protein and its associated factors. Detailed information about the expression and regulation of these factors in preimplantation stage embryos has not been published for any species. In order to ascertain which of these factors may participate in chromatin remodeling, genome activation, and DNA replication during early primate embryogenesis, we determined the temporal expression patterns of mRNA encoding these factors. Our data identify the predominant members of these different functional classes of factors expressed in oocytes and embryos, and reveal patterns of expression distinct from those patterns seen in somatic cells. Among each of four classes of mRNAs examined, some mRNAs were expressed predominantly in the oocyte, with these largely giving way to others expressed stage specifically in the embryo. This transition may be part of a global mechanism underlying the transition from maternal to embryonic control of development, wherein the oocyte program is silenced and an embryonic pattern of gene expression becomes established. Possible roles for these mRNAs in chromatin remodeling, genome activation, DNA replication, cell lineage determination, and nuclear reprogramming are discussed.**

*embryo, gene regulation*

## INTRODUCTION

Perhaps the most crucial, and yet least understood, events in the early embryo are those related to transcriptional activation of the embryonic genome. Both the timing and the array of genes activated in the early embryo need to be regulated correctly to ensure proper execution of the developmental program [1–3]. Precocious activation could be associated with abnormal epigenetic modifications [3], while a delay in transcriptional activation could lead to developmental arrest.

Numerous studies in the mouse and other species have documented changes in chromatin structure, and specifically changes in histone content and posttranslational modification [4–7], which are accompanied by overall changes in transcriptional permissiveness [8–13]. Other notable observations include changes in promoter preference and differences in transcription between maternal and paternal pronuclei [5, 14, 15]. The current view is that the balance between histone deacetylation and acetylation becomes shifted in favor of greater histone acetylation around the time of genome activation [2]. Along with changes in histone acetylation, transitions in linker histone content [16–18], changes in histone methylation [19], and global DNA demethylation [19–21] could also participate in genome activation. Paradoxically, the transcription of exogenous target genes appears to be quite precocious during the period preceding genome activation, with transcriptional enhancers dispensable for this process [22–26], indicating that, along with transcriptional activating events, come other changes that impose a transcriptionally repressive chromatin environment. This paradox is explicable by a model wherein the establishment of a repressive chromatin environment that permits gene-specific regulation is necessary to allow transcriptionally activating changes to be targeted to the correct array of genes [2].

The molecular mechanisms that underlie such changes and that provide correct temporal coordination between transcriptional activation and other key events such as DNA replication have been only partly illuminated. Some studies revealed that progression through S phase is required for major changes in transcriptional activity in the mouse embryo [15, 22, 27]. Other studies have revealed that protein synthesis is required for transcriptional activation [28] and that recruitment of essential maternal mRNAs likely accounts for this requirement [29]. Because the recruitment of masked maternal mRNAs can be regulated by cell cycle regulators [30, 31], temporally regulated recruitment of maternal mRNAs could provide a mechanism for coordinating acquisition of transcriptional capacity with other events, such as cell cycle progression [1, 2]. Products encoded by the recruited maternal mRNAs may then in turn direct the transcription and production of other important regulatory genes as development progresses. Thus, temporal patterns of synthesis of key transcription factors throughout the preimplantation period may regulate the timing of important

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events during early development that prepare the embryo for implantation [29].

The identities of those transcription factors that participate in the early chromatin remodeling process and in the subsequent transcriptional activation events are largely unknown. In order to understand how the developmental program is executed, it is essential to determine the temporal patterns of expression of genes encoding key transcription factors and to determine which of these genes are expressed maternally, which are expressed at later stages and which display the appropriate stage-specificity of expression to participate in the above processes. Additionally, it is important to understand to what degree such regulatory mechanisms are conserved among different species, and in particular how such mechanisms may contribute to normal development in human and nonhuman primate embryos. Many of the relevant observations to date have been made in species such as the mouse, rabbit, and cow, but few data are available for the human embryo, and a clear paucity of data exist for nonhuman primates. Because primate embryos may differ in fundamental ways from embryos of other species [32, 33], it will be important to acquire data for primate species and to relate those data to data obtained in other species. Knowledge of the factors that exist in healthy primate oocytes and embryos could provide new criteria by which to evaluate oocyte and embryo quality, and thus facilitate improvements in embryo culture and fertility treatments. Last, an understanding of those factors that regulate genome function in fertilized oocytes would provide an understanding of how the oocyte can direct nuclear reprogramming during cloning procedures and how cloning might be made more efficient.

To understand better the molecular controls that regulate preimplantation development during primate embryogenesis, we have developed a new resource, the Non-Human Primate Embryo Gene Expression Resource (PREGER), to permit detailed quantitative gene expression studies in a nonhuman primate species, the rhesus monkey (see accompanying paper [33]). We have used this novel resource to examine the expression of mRNAs encoding a variety of transcription factors in rhesus monkey oocytes and preimplantation stage embryos. Specifically, we have examined the temporal coordination between the expression of these mRNAs and key events such as transcriptional activation and cellular differentiation of the trophectoderm, with particular emphasis on mRNAs encoding transcription factors that play a role in modulating chromatin structure. These include histone acetyltransferases, histone deacetylases, components of the chromatin accessibility complex, members of SWI/SNF transcription regulatory complexes, and other factors that recruit modifiers of chromatin structure, such as YY1. The expression data reveal that the mRNAs encoding certain of these factors are expressed at the appropriate times to play key roles in regulating processes such as genome activation, nuclear reprogramming, DNA replication and repair, and lineage determination.

## MATERIALS AND METHODS

### *Oocytes and Embryos*

The Wisconsin National Primate Research Center is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and animal protocols and experiments were approved by the Graduate School Animal Care and Use Committee. The animals were maintained according to recommendations of the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act with its subsequent amendments.

The isolation and culture of rhesus monkey oocytes and embryos is described in detail in the accompanying paper [33]. Briefly, over 160 samples of oocytes and embryos of various stages and produced by various protocols were obtained. From among this collection, we employed samples of germinal vesicle stage oocytes, in vivo-matured metaphase II oocytes, and in vitro fertilization (IVF)-derived embryos cultured in vitro in HECM9 sequential media [34]. Between 3 and 13 samples of 1–4 oocytes/embryos were obtained for each stage. It should be noted that, because the entire mRNA population is uniformly amplified during the PCR procedure, the amount of input mRNA (i.e., the range of 1–4 embryos) does not affect the quantitative representation of sequences within the amplified material. As noted in the accompanying paper [33], the embryos collected for inclusion in the PREGER sample set were of high quality and healthy in appearance, with blastomeres displaying uniform granularity. Fragmented embryos were avoided. A minimum of three females were employed to obtain samples for each stage, with the exception of the two-cell stage, for which two females were employed.

### *cDNA Probes and Hybridization*

The cDNA probes employed in these studies are described in Table 1. The cDNA probes were obtained by reverse transcription-polymerase chain reaction (RT-PCR) or from other sources as indicated. The identities of amplified cDNAs were confirmed either by using diagnostic restriction digests or DNA sequencing. Blot preparation, probe preparation, hybridization, and quantitative analyses were performed as described in the accompanying paper [33] and elsewhere [35, 36]. Data were expressed as the mean (±SEM) cpm bound value for each stage/condition of oocytes and embryos included in the analysis. The significance of differences between stages and conditions was evaluated using a *t*-test.

## RESULTS

### *Expression of mRNAs Encoding Regulators of Histone Acetylation*

Histone acetylation status is a key regulator of chromatin structure and gene transcription. Numerous changes in histone acetylation have been observed in the early embryo and correlated with transcriptional activity, but the identities of the factors responsible have not been examined in any species. To identify those factors present in oocytes and preimplantation stage rhesus monkey embryos, we examined the temporal expression patterns of mRNAs encoding five histone acetyltransferases and five histone deacetylases (Fig. 1). Among the histone acetyltransferases, striking stage-specific differences in expression were observed. The PCAF mRNA displayed two discrete periods of abundant expression, the first being at the eight-cell stage, and the second corresponding to the hatched blastocyst stage. Expression of PCAF mRNA at the eight-cell stage was variable, being elevated in only 5 of 13 samples and thus did not differ significantly from the α-amanitin-treated embryos. This may reflect the transient nature of its expression at this stage. The GCN5 mRNA was expressed first as a maternal mRNA of somewhat low abundance, judging from the strength of the hybridization signal. GCN5 mRNA expression remained at a low level, and this expression was α-amanitin insensitive through the eight-cell stage, indicating persistence of the maternal supply. The HAT1 mRNA expression was significantly increased in early blastocysts relative to the small amount of maternal mRNA remaining in α-amanitin-treated embryos ($P < 0.01$). The expression patterns observed for HAT1 and GCN5 are quite similar to those reported previously for bovine embryos [37], although our data reveal induction of mRNA at the morula stage, a stage not analyzed in the bovine study. The CBP mRNA was expressed abundantly as a maternal mRNA in oocytes that persisted at least through the eight-cell stage. Based on the strength of the hybridization signals, it appeared that CBP was the predominant member of the class

TABLE 1. Primers and probes used in the study to examine expression of the four gene classes.

| Genes | GeneBank accession no. | Primers | Product size (base pairs [bp]) | Product bp range |
|---|---|---|---|---|
| HDAC1 | NM_004964 | Up5′-AGACCCAGAGGAGAAGAAAG-3′<br>Low5′-TTCCAAAGCTACTAAGCAGG-3′ | 692 | 1416–1827 |
| HDAC2 | NM_001527 | Up5′-TGCAGTTGCCCTTGATTGTG-3′<br>Low5′-CAGCTCAGAAAGGCCAATTAC-3′ | 813 | 1155–1967 |
| HDAC3 | AF039703 | Up5′-CCCGCATCGAGAATCA-3′<br>Low5′-CCTTGTCTACCCGTTCATCC-3′ | 603 | 1053–1655 |
| HDAC4 | NM_006037 | Up5′-GCCTTGTGACGGTTTAT-3′<br>Low5′-GTGAAATCTTAGCCATACATC-3′ | 115 | 8189–8303 |
| HDAC6 | NM_006044 | Up5′-TGATCCAAACTCCTCTAGCCT-3′<br>Low5′-AGAGCCTGGTGGTGGACATAG-3′ | 532 | 3146–3677 |
| HAT1 | NM_003642 | Up5′-GCTCTTTGCGACCGTAGGCTA-3′<br>Low5′-AACACGCCGGTAATCT-3′ | 595 | 678–1272 |
| GCN5 | AF029777 | Up5′-GAGCGTTCCTGGCATTCGAGA-3′<br>Low5′-AGCTGAGTCGGGTCCGT-3′ | 495 | 2142–2636 |
| PCAF | NM_003884 | Up5′-AAAGATGGCCGTGTTATTGGT-3′<br>Low5′-CTCTCAGCGGCGTTGTACTCT-3′ | 773 | 2106–2878 |
| p300 | UO1877 | N/A | 2394 | 5912–8306 |
| CBP | NM_004380 | Up5′-TGTGATGTTTCGGGAAG-3′<br>Low5′-ACATTCTTACAGGGGATCTT-3′ | 638 | 7994–8631 |
| SMARCA5 (SNF2H) | BC023144 | Up5′-AATTGGGCAGACTAAGACA-3′<br>Low5′-CTGAGGAGAGTTGCGAATAC-3′ | 1162 | 1998–3159 |
| SMARCA1 (SNF2L1) | BL051825 | Up5′-GAGAGGCTTTGCGTGTC-3′<br>Low5′-AATCAGAGTGTTACAGCGTC-3′ | 770 | 2067–2836 |
| ACF1 | AF213467 | Up5′-GCGAAAAAGTCACGAATCTG-3′<br>Low5′-GGAAAAAGGGAGGGTGTTAC-3′ | 712 | 4817–5528 |
| CHRAC1 | NM_017444 | Up5′-GCCACCATGCCCAACCTAT-3′<br>Low5′-TTACCTGGCTGCTTTCGTGC-3′ | 682 | 1640–2321 |
| CHRAC17 | BC004170 | Up5′-TCTCCCAGACTTGTGACTAT-3′<br>Low5′-GAGAGTTAGATTCCTGAGCA-3′ | 506 | 1358–1863 |
| BR140 | M91585 | Up5′-CCCTCCTGCCCTAAGTGC-3′<br>Low5′-CCCGGAAACGCAGTTAC-3′ | 358 | 3815–4172 |
| SMARCA4 (BRG1) | NM_003072 | Up5′-AAGTGGCAGCGAAGAAGACTGA-3′<br>Low5′-TGCGTTTTGTTGTTGGTTTAA-3′ | 481 | 5199–5681 |
| SMARCA2 (BRM) | NM_139045 | Up5′-AGTGCCAGACAAACATATGA-3′<br>Low5′-AGGGAGAAGGAGACGATACA-3′ | 614 | 4940–5553 |
| SMARCE1 (BAF57) | AF035262 | Up5′-ATGTCAAAAAGACCATCTTATGCCCC-3′<br>Low5′-GAACACACAAAACAAGGCAACAC-3′ | 1258 | 90–1348 |
| SMARCB1 (BAF47, INI1, SNFS) | NM_003073 | Up5′-CCCGCTGACGTTTGTGC-3′<br>Low5′-AGACTGGGGAGGGGTAGGGA-3′ | 693 | 834–1526 |
| SMARCC1 (BAF155) | NM_003074 | Up5′-AGCCACAAAAATGCCTTAAT-3′<br>Low5′-TTCTCCATTCTTGCCAAAC-3′ | 952 | 4741–5692 |
| SMARCD1 (BAF60A) | NM_003076 | Up5′-CCTGTGGGCACTCTATAAGC-3′<br>Low5′-TGTGTTGAGTCTGCGAGCCT-3′ | 824 | 2100–2923 |
| ARID1A (p270, SMARCF1) | AF265208 | N/A | 2522 | 3678–6200 |
| ARID1B (KIAA1235) | AF253515 | N/A | 1446 | 5384–6830 |
| FANCA | NM_000135 | Up5′-GCCCAGCTCCCGTGTAA-3′<br>Low5′-CACATTGTCATCGTCGTCCC-3′ | 845 | 4422–5266 |
| RYBP | NM_012234 | Up5′-ACCAAGGAATTTCGCACCC-3′<br>Low5′-TCAAAGCCCTGCCCAACTAA-3′ | 667 | 1030–1696 |
| YAF2 | BC037777 | Up5′-GAACCTTTGCCAATGT-3′<br>Low5′-CCACCAAATATCACAACTGT-3′ | 873 | 1211–2083 |
| YY1 | NM_003403 | Up5′-CCGGAGACAGGCCCTATG-3′<br>Low5′-GATGCTTCCCGTGGTCGAGA-3′ | 152 | 1613–1764 |

of mRNAs encoding proteins with histone acetyltransferase activity in the oocyte and early embryo. The CBP mRNA declined in abundance between oocyte maturation and the eight-cell stage, and then increased again in abundance with development to the blastocyst stage ($P < 0.01$). A very faint hybridization signal was obtained using a probe for p300, and this also displayed maternal expression early. The abundance of the p300 mRNA decreased during oocyte maturation ($P < 0.001$).

Among the histone deacetylases, the predominant mRNAs expressed were those encoding HDAC1 and HDAC2. The HDAC2 mRNA was expressed abundantly in the geminal vesicle (GV) stage oocyte, and diminished in abundance upon maturation ($P < 0.01$). Expression was sensitive to α-amanitin at the eight-cell stage. The HDAC2 mRNA appeared to be the most abundantly expressed member of the HDAC family. The HDAC1 mRNA was expressed at near background levels in oocytes and pronucleate through two-cell stage embryos and was then variably induced at the eight-cell stage, with only five samples displaying elevated rates of synthesis compared with the α-amanitin-treated embryos. Thus, the mean expression levels were not significantly different between treated and untreated embryos at the eight-cell stage ($P = 0.19$). Expression was significantly greater at the blastocyst stage compared with the treated embryos, indicating transcription of the

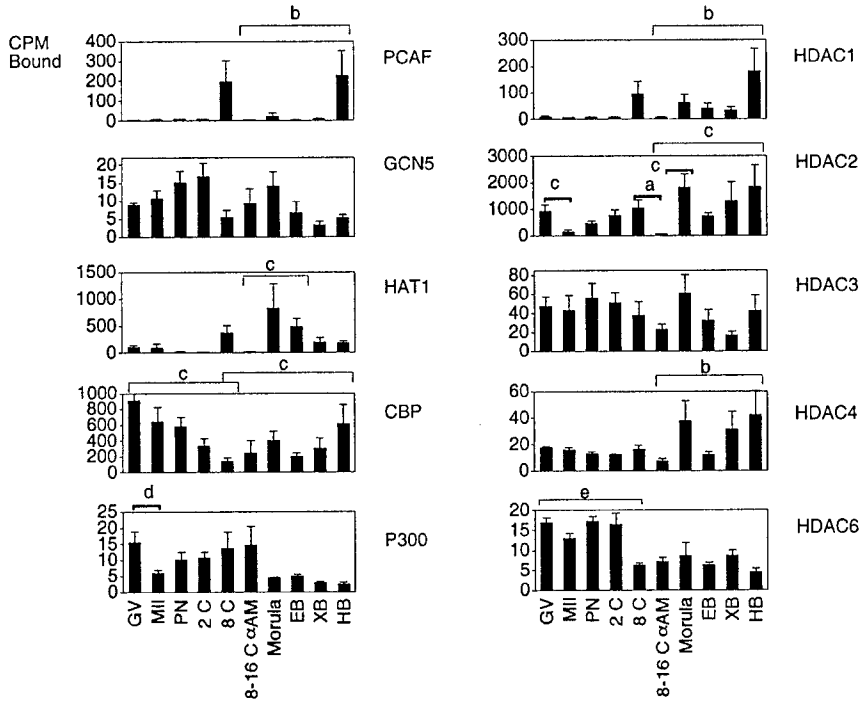


FIG. 1. Temporal expression patterns of mRNAs encoding regulators of histone acetylation. Graphs show the relative levels of expression for GV and MII stage oocytes and pronucleate through hatched blastocyst stage embryos produced by in vitro fertilization of oocytes from hCG-stimulated females and then cultured in vitro in HECM9. GV, Germinal vesicle stage oocyte; MII, MII-stage oocyte; PN, pronucleate one-cell stage embryo; 2C, two-cell stage; 8C, eight-cell stage; 8–16C αAm, 8- to 16-cell stage cultured in α-amanitin; EB, early blastocyst; XB, expanded blastocyst; HB, hatched blastocyst. Data are expressed as the mean CPM bound ± the SEM. Statistically significant differences in gene expression corresponding to some of the major increases or decreases in expression are denoted by the brackets (for comparisons between stages at the ends of the brackets). Letters a through e indicate $P < 0.05$, 0.02, 0.01, 0.001, and 0.0001, respectively.

HDAC1 gene after the eight-cell stage. The predominance of HDAC1 and HDAC2, and the temporal patterns shown here are similar to those reported for bovine embryos, although an apparent species difference exists in that the HDAC1 mRNA was more prevalent than HDAC2 mRNA in the bovine embryo [37]. The HDAC3, HDAC4, and HDAC6 mRNAs were expressed at low levels. HDAC3 mRNA expression displayed no statistically significant changes in expression. The HDAC4 mRNA was poorly represented in oocytes and during early cleavage stages, and a slight but significant increase occurred comparing blastocysts with the α-amanitin-treated embryos, indicating a low level of transcription initiating by the blastocyst stage. The HDAC4 mRNA expression pattern thus resembled the pattern seen for HDAC1. The HDAC6 mRNA was expressed most prevalently in the oocyte and early cleavage stage embryos, being downregulated in abundance by the eight-cell stage ($P < 2 \times 10^{-7}$).

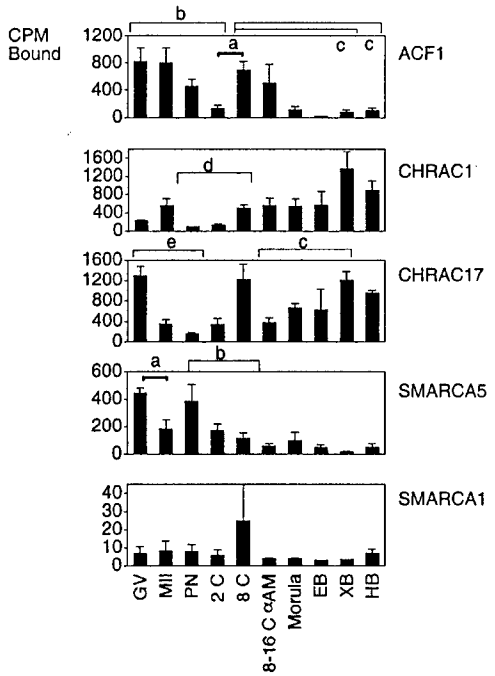


FIG. 2. Temporal expression patterns of mRNAs encoding components of the chromatin accessibility complex. Data and abbreviations are as described in Figure 1.

### *Expression of mRNAs Encoding Regulators of Chromatin Accessibility*

The chromatin accessibility complexes (CHRAC) facilitate the entry of transcription factors into chromatin and also are important for allowing DNA replication [38, 39]. The expression of these complexes, therefore, could be important for both gene transcription and DNA replication during cleavage. We examined the expression of mRNAs encoding five components of the CHRAC (Fig. 2). The ACF1 protein (also known as BAZ1A) appears critical for proper ATP-dependent modification of chromatin structure [38–40]. The mRNA encoding ACF1 was expressed abundantly in oocytes. Its apparent abundance declined ($P < 0.02$) and then increased again at the eight-cell stage ($P < 0.05$) in an α-amanitin-independent manner, possibly indicating regulation at the level of polyadenylation. The ACF1 mRNA then declined significantly in abundance after the eight-cell stage ($P < 0.01$). The ACF1 protein interacts with CHRAC1 and CHRAC17 to form functional complexes that participate in chromatin remodeling [38–41]. The CHRAC1 mRNA was expressed at a low level in oocytes and early-stage embryos and then at an increased level from the eight-cell stage onward ($P < 0.001$). Expression at the eight-cell stage was α-amanitin insensitive, as seen for ACF1, indicating possible coordinate recruitment of these mRNAs. The CHRAC17 mRNA was expressed abundantly in the GV stage oocyte, diminished in abundance during maturation and fertilization ($P < 0.0001$), and then

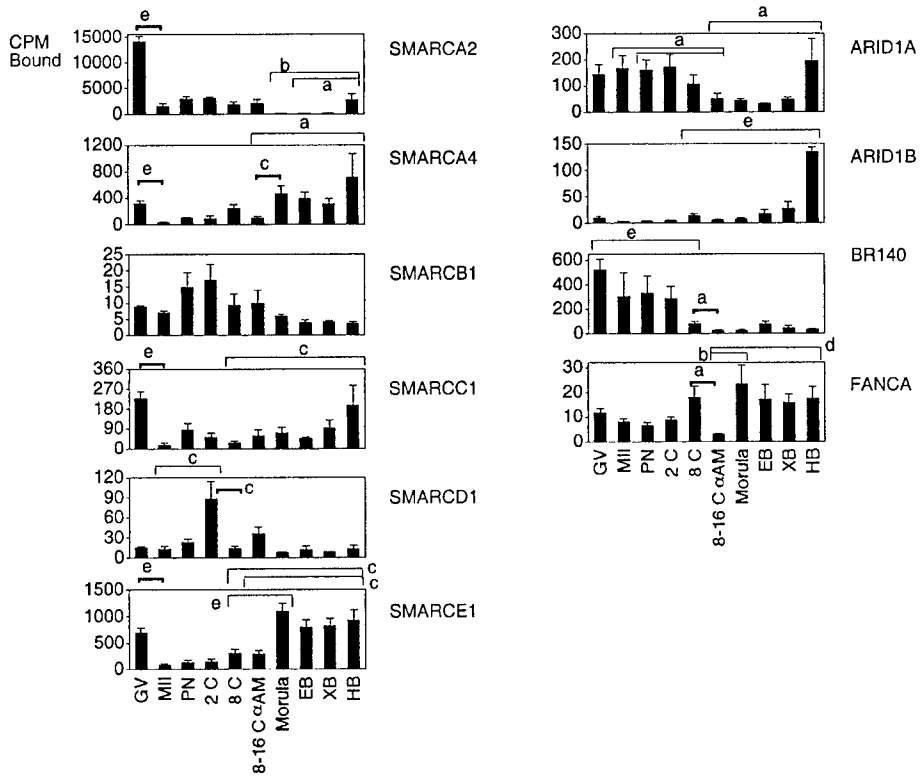


FIG. 3. Temporal expression patterns of mRNAs encoding SWI/SNF-related transcription regulators and associated factors. Data and abbreviations are as described in Figure 1.

increased in apparent expression between the pronucleate and eight-cell stages ($P < 0.02$). This increase appeared to be α-amanitin sensitive, but this was variable and not statistically significant ($P = 0.0635$). Expression at later stages was significantly greater than seen in the α-amanitin-treated embryos ($P < 0.01$), indicating transcription. Two other factors that interact with ACF1, CHRAC1 and CHRAC17 are the ISWI homologues, SMARCA5 (SNF2H) and SMARCA1 (SNF2L1) [40]. These proteins possess ATPase activity and participate in regulating chromatin access. The SMARCA5 mRNA was expressed predominantly as a maternal mRNA, which diminished in abundance during oocyte maturation ($P < 0.05$) and then largely disappeared by the eight-cell stage ($P < 0.02$). Hybridization for the SMARCA1 mRNA was near background throughout development (only one sample of eight-cell embryos produced a significant hybridization signal).

### *Expression of mRNAs Encoding SWI/SNF-Related Transcriptional Regulators*

In addition to the ISWI-related class of ATPases (SMARCA1 and SMARCA5), there exist ATPases of the SWI2/SNF2 class. These ATPases, together with their associated factors (i.e., the SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin class of transcription factors) encompass a diverse group of proteins with homology to the components of the *Drosophila* brahma complex [42, 43]. The complexes formed by these proteins mediate either activating or repressing changes in gene transcription, through alterations in chromatin structure mediated by ATP-dependent movement of nucleosomes or DNA bending and looping [44–54]. To learn which of these proteins might participate in genome activation and subsequent gene regulation in the early embryo, we analyzed their patterns of expression (Fig. 3). Functionally and structurally related members of the family displayed opposing and complementary patterns of expression. For example, of the mRNAs encoding SMARCA2 and SMARCA4 (also known as BRM and BRG1), the two alternative ATPases of SWI/SNF complexes, SMARCA2 mRNA was expressed exclusively as a maternal transcript until the hatched blastocyst stage ($P < 0.05$), whereas SMARCA4 was transcriptionally induced by the morula stage ($P < 0.01$). Both mRNAs displayed significant declines in apparent abundance during oocyte maturation ($P < 0.0001$). The FANCA mRNA, which encodes a BRG-1 interacting protein possibly involved in recruiting SWI/SNF complexes to DNA [55], was expressed at a low level, with α-amanitin-sensitive gene transcription being evident at the eight-cell stage ($P < 0.05$) and beyond ($P < 0.02$). Complementarity in expression was also seen for the mRNAs encoding p270 (SMARCF1/ARID1A) and ARID1B (originally named KIAA1235 [56]), two closely related DNA binding proteins of the ARID class that are alternative components of the SWI/SNF complexes and that are typically coexpressed in somatic cells (X. Wang et al., unpublished data). SMARCC1 (BAF155) mRNA was expressed in GV stage oocytes, was then nearly eliminated ($P < 0.0001$), and was later induced at the hatched blastocyst stage ($P < 0.01$) when ARID1B was also induced. Another contributor to SWI/SNF complex function, SMARCE1 (BAF57), was down-regulated during oocyte maturation ($P < 0.0001$)) and then up-regulated at the morula stage ($P < 0.0001$). In contrast, BR140, a bromo-domain protein, was expressed predominantly as a maternal mRNA, down-regulated between the GV and eight-cell stages ($P < 1 \times 10^{-6}$), but with a low level of transcription evident at the eight-cell stage ($P < 0.05$). The SMARCD1 (BAF60A) mRNA displayed increased expression at the two-cell stage ($P < 0.001$), but otherwise was barely detectable. Thus, among

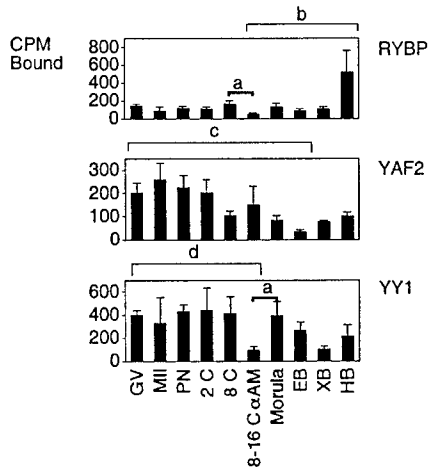


FIG. 4. Temporal patterns of expression of YY1 and associated factors. Data and abbreviations are as described in Figure 1.

this group of genes, some were expressed predominantly as maternal mRNAs, while others were expressed primarily as embryonic transcripts, with functionally related members often showing opposing patterns of expression.

### *Expression of mRNAs Encoding YY1 and Associated Factors*

The YY1 transcription factor is ubiquitously expressed and exerts either activating or repressing effects on transcription by associating with YAF2 or RYBP, respectively [57, 58]. YY1 association with proteins such as YAF2 and RYBP can lead to the recruitment of histone acetylases, histone deacetylases, and histone methylases to DNA to alter chromatin structure [59, 60]. Thus, YY1 and its partners constitute another important group of proteins that can affect chromatin structure. The YY1 mRNA was expressed throughout development (Fig. 4). By the morula stage, transcription was α-amanitin sensitive, indicating active expression from the embryonic genome. The RYBP mRNA was expressed at a low level until the hatched blastocyst stage, when it was upregulated ($P < 0.02$). Expression of RYBP mRNA at the eight-cell stage was α-amanitin sensitive ($P < 0.05$). The YAF2 mRNA, by contrast, was expressed as a maternal mRNA that declined in abundance with development to the early blastocyst stage ($P < 0.01$). Thus, the YAF2 and RYBP mRNAs display complementary patterns of expression.

## DISCUSSION

The data presented here reveal for the first time in any primate species the predominant members of four key classes of chromatin regulatory transcription factors. With the exception of the HDACs, which were examined in bovine embryos [37, 61], this study is the first to examine the expression of these classes of transcription factors in any species of mammalian embryo. Our observations thus provide novel information about which factors are expressed at the appropriate stages to play key roles in processes such as genome reprogramming, genome activation, DNA replication, DNA repair, and cell lineage commitment in the early preimplantation embryo, and what regulatory activities are likely lacking during this period. Because the data presented are for a nonhuman primate species, they provide significant new insight into how early embryogenesis is likely regulated in the human.

One of the most striking trends apparent in the data presented here is that, although the transcription factors analyzed are widely expressed and often considered to be constitutively expressed in somatic cells, some of the mRNAs are poorly expressed or undetectable at all of the stages analyzed, and many others show stage-specific expression. Moreover, the stage specificity of expression divides many of the expressed mRNAs into either of two reciprocal categories, displaying predominantly maternal expression or strong induction in the embryo. These patterns of expression appear for each of the four groups of mRNAs analyzed and are consistent with discrete functions in important processes (Table 2). The division of the mRNAs of all four classes into the two temporally distinct groups suggests a possible mechanism for mediating the switch from maternal control to embryonic control of development. Those factors that are expressed in the oocyte may represent a legacy remaining from the oocyte differentiation and development pathway, but may also mediate early events that could support genome activation. Conversely, those factors that are upregulated during development are likely to play critical roles in establishing an embryonic pattern of gene regulation.

Each of the four classes of mRNAs examined display members that are induced at the time of embryonic genome activation [62] and at the blastocyst stage. Induction of a number of these mRNAs at the eight-cell stage suggests a possible role in promoting or propagating the major embryonic genome activation event, which occurs between the six-cell and eight-cell stages. Interestingly, the ACF1 and CHRAC1 mRNAs display α-amanitin-insensitive increases in apparent abundance, consistent with possible polyadenylation and recruitment at that stage [36]. Such recruitment could provide for a stage-specific increase in the availability of these proteins, thereby facilitating opening of chromatin for transcription. The induction of the CHRAC17 gene by the eight-cell stage could provide further capacity for CHRAC function, thereby facilitating both gene transcription and continued DNA replication and cell cycle progression. The expression of CHRAC complexes in the oocyte could also contribute to the oocytes' ability to reprogram nuclei after somatic cell nuclear transfer.

Our results also point to specific members of the SWI/SNF family of transcriptional regulators as possible key players in genome activation. The induction of the SMARCA4 (BRG1) ATPase-bearing protein at the morula stage and the induction of the SMARCE1 (BAF57) accessory

TABLE 2. Distribution of some analyzed mRNAs between the predominantly maternal class and the embryonically induced class of message.

| Group | Maternal | Embryonic induction |
|---|---|---|
| Histone Acetylation/deacetylation | *GCN5, HDAC6 CBP, p300* | *PCAF, HAT1, HDAC1, HDAC2, CBP* |
| CHRAC | *ACF1, SMARCA5* | *CHRAC1, CRAC17* |
| SWI/SNF-related factors | *SMARCA2, SMARCB1 SMARCD1, BR140 ARID1A* | *SMARCA4, SMARCC1 SMARCE1, ARID1B* |
| YY1 complex | *YAF2, YY1* | *RYBP, YY1* |

protein may be especially relevant for further transcriptional activation of many genes, by permitting ATP-dependent chromatin remodeling. These proteins may also contribute to nuclear remodeling during cloning. Interestingly, homozygous SMARCA4 knockout mouse embryos arrest during preimplantation development [63], consistent with an essential function during cleavage. The up-regulation of SMARCA4 contrasts with the loss of the maternal SMARCA2 mRNA encoding the alternative ATPase for the SWI/SNF complex. Homozygous deficiency for SMARCA2 in mice, however, is not lethal, but homozygous mutants display growth defects, possibly related to altered regulation of the cell cycle [64]. Thus, SMARCA4 may play a predominant role during preimplantation development, whereas SMARCA2 may be important as a maternal factor in the oocyte and early embryo for regulating the cell cycle. Because SMARCA2 may also regulate association of cohesin with chromatin [49], its expression as a maternal factor in the oocyte may be important for regulating chromosome pairing. It was suggested that SMARCA4 expression may compensate for an absence of embryonic SMARCA2 expression [64]. To date, however, it is not known whether a maternal effect phenotype exists for BRM homozygous null females. It is also worth noting that the transitions from SMARCA2 predominance in the oocyte and early embryo, to SMARCA4 predominance after fertilization and through the early blastocyst stage, and finally to coexpression of these two mRNAs in hatched blastocysts may constitute key steps, first in the transition from maternal to embryonic control of development and second during the differentiation of the blastocyst cell lineages. This is because complexes formed with SMARCA4 may activate a different array of genes than that activated by SMARCA2 in the embryo, as they do in other cells [48].

The induction of HAT1 and PCAF at the eight-cell stage, though variable, may also serve to promote gene transcription by increasing the available supply of histone acetylating activities. This increase appears to be balanced by an increase in HDAC1 and HDAC2 mRNA expression. This balance may be needed in order to limit transcriptional activation to the correct array of genes.

The upregulation of p270, ARID1B, SMARCA2, SMARCC1, PCAF, HDAC1, and RYBP by the blastocyst stage likely establishes within the embryonic cells at that stage an array of expression of mRNAs more typical of somatic cells and may be related to cell lineage commitment within the developing blastocyst. In this context, it is interesting that SMARCC1 (BAF155) deficiency in mice is associated with preimplantation lethality and a defect in the formation of the inner cell mass lineage [65]. Additionally, the up-regulation of SMARCE1 (BAF57) at the morula stage could also contribute to cell lineage specification, as this protein participates in lineage bifurcation and gene switching in other cell types [44, 66]. The induction of SMARCA2 at the blastocyst stage may also contribute to lineage-specific changes in gene expression. Interestingly, the noticeably low level of expression of SMARCA5 and SMARCA1 indicates that the widely expressed ISWI components, which play important roles in promoting cellular proliferation and differentiation, respectively, may not play a prominent role during blastocyst formation and may only become key factors during postimplantation life.

Other mRNAs, such as YY1, CBP, and HDAC3, do not readily fit into either maternal or embryonic transcript category. Their constitutive presence as members of both categories may reflect roles for the corresponding proteins during both periods of development. The abundant expression of YY1 in the oocyte may signify a function in promoting gene transcription, but could also signify other functions, such as contributions to DNA repair during the postfertilization period [67, 68]. HDAC3 interacts with a variety of proteins, with which it represses transcription [e.g., 69, 70], including RB, with which it operates to control cell proliferation via PPAR-gamma [71], a function that could also contribute to cell cycle control and DNA repair.

The data presented here clearly reveal certain members of each class of transcription factors as prominent during either the maternally controlled or the embryonically controlled portions of preimplantation development. This is the first detailed study addressing in a common set of samples the expression patterns of each of these classes of factors. As such, these observations provide the necessary foundation for designing further studies to understand the specific roles played by these factors in the early embryo. Of particular interest will be the examination of the functions of maternally expressed mRNAs on processes such as nuclear reprogramming and initial genome activation, and the effects of early embryonically expressed transcripts on continued genome expression and regulation. Alterations in the expression of some of these mRNAs in oocytes of different developmental potentials (unpublished) suggest that the expression of such mRNAs could provide useful markers of oocyte quality. The data presented here provide the basis for undertaking key functional studies in oocytes and embryos of both human and nonhuman primate species.

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# The p270 (*ARID1A/SMARCF1*) subunit of mammalian SWI/SNF-related complexes is essential for normal cell cycle arrest.

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## ABSTRACT

Mammalian SWI/SNF-related complexes are ATPase-powered nucleosome remodeling assemblies crucial for proper development and tissue specific gene expression. The ATPase activity of the complexes is critical for tumor suppression in mice and humans. The complexes also contain seven or more noncatalytic subunits, only one of which, hSNF5/Ini1/BAF47, has been individually identified as a tumor suppressor thus far. The noncatalytic subunits include p270/ARID1A, which is of particular interest because recent results from a cDNA tissue array analysis and corroborating screens of panels of tumor cell lines indicate p270 may be deficient in as many as 30% of renal carcinomas and 10% of breast carcinomas. The complexes can also include an alternative ARID1B subunit, which is closely related to p270, but the product of an independent gene. The respective importance of p270 and ARID1B in the control of cell proliferation was explored here using an siRNA approach and a cell system that permits analysis of differentiation-associated cell cycle arrest. The p270-depleted cells fail to undergo normal cell cycle arrest upon induction, as evidenced by continued synthesis of DNA. These lines fail to show other characteristics typical of arrested cells, including up-regulation of p21, down-regulation of cyclins, and decreases in histone H4 expression and cdc2-specific kinase activity. The requirement for p270 is evident separately in both the up-regulation of p21 and the down-regulation of E2F-responsive products. In contrast, the ARID1B-depleted lines behaved like the parental cells in each of these assays. These results show that p270-containing complexes are functionally distinct from ARID1B-containing complexes. They provide a direct biological basis to support the implication from tumor tissue screens that deficiency of p270 plays a causative role in carcinogenesis.

## INTRODUCTION

The ATPase-powered SWI/SNF chromatin remodeling complex in yeast regulates the mating type switch and other areas of specialized gene expression (reviewed in 1). Mammalian SWI/SNF-related complexes likewise contain an ATPase-powered nucleosome remodeling activity associated with transcriptional regulation. The activity of the complexes is crucial for proper tissue specific gene expression, development, and hormone responsiveness (reviewed in 1). More recently it has become apparent that these complexes also play critical roles in suppression of tumorigenesis in mice and humans (reviewed in 2).

The complexes contain seven or more noncatalytic subunits that presumably help to modulate the targeting and activity of the ATPase. Mammalian complexes have variable compositions because some subunits occur as sets of related proteins. For example, there are two alternative ATPase subunits: mammalian BRM and BRG1. These are closely related proteins, but in mouse knockout studies only BRG1 proved essential for embryonic development and tumor suppression (3; 4). The ATPases are mutated in multiple human tumor cell lines and their loss correlates with poor prognosis of non-small cell lung cancers (5; 6; 7). Noncatalytic components of the complex may be important for tumor suppression as well; however, their individual roles are less well understood.

Among the noncatalytic subunits, hSNF5 (syns: INI1; BAF47) is recognized as a tumor suppressor in mice (8; 9; 10). In humans, hSNF5 is deficient in a high proportion of pediatric malignant rhabdoid tumors (e. g. 11; 12; 13). Germ-line mutations have been identified, and carriers are pre-disposed to malignant rhabdoid tumors and tumors of the central nervous system (14; 15; 16).

Expression of functional BRG1 or hSNF5 is associated with specific aspects of cell cycle regulation. Expression of the cell cycle inhibitor $p21^{CIP1/WAF1}$ has been repeatedly identified as BRG1-responsive, and several studies indicate that BRG1-dependent or hSNF5-dependent cell cycle arrest is enacted through a pRb-dependent or overlapping pathway (e. g. 17; 18; 19; 20; 21; 22; 23; 24; 25). However, these effects have only been seen in the context of re-introduction of exogenous complex components into tumor cell lines where they were deficient. The significance of the complexes in the expression of these biological targets has yet to be demonstrated during differentiation-associated cell cycle arrest, when the effects of complex dysfunction on carcinogenesis would be most significant.

Subunits required for the tumor suppression activity of the complexes have great potential as diagnostic and prognostic markers, and as targets for drug therapy. Thus, a major question now is the distinction of which additional noncatalytic subunits are required for the cell cycle arrest functions of the complexes. The noncatalytic components of the complex include the p270 subunit (26; 27; 28) (syns.: ARID1A, SMARCF1, BAF250a, hOSA1), which is a member of the ARID family of DNA binding proteins (reviewed in 29, 30). The role of p270 in cell cycle regulation is of particular interest because recent results from a cDNA tissue array analysis and corroborating screens of panels of tumor cell lines, indicate p270 may be deficient in as many as 30% of renal carcinomas and 10% of breast carcinomas (28; 31; 32). A mutually exclusive alternative to p270 in the complexes is the ARID1B (syns: hOSA2, BAF250b) subunit, which is approximately 50% identical to p270 across its entire length, but is the product of an independent gene. The ARID family proteins are determinants that distinguish key divisions among the multiple, distinct SWI/SNF-related complexes that exist in mammalian cells. A major distinction is between the complex first identified as the BRG1-associated factors (BAF) complex (also called the human SWI/SNF or hSWI/SNF complex) and a distinct complex designated PBAF. The BAF complex contains at least BRG1 (or BRM), p270 (or ARID1B), BAF170, BAF155, BAF60, BAF57, BAF53, actin, and hSNF5. The PBAF complex is characterized by the absence of both p270 and

ARID1B and the presence of a 180 kDa protein designated Polybromo (syn: BAF180). Thus, p270 and ARID1B distinguish between the BAF and PBAF complexes, while BRG1 and hSNF5 do not (subunit composition of SWI/SNF-related complexes is reviewed in 1). In addition to the BAF and PBAF division, the BAF series of complexes itself encompasses at least four different entities because p270 and ARID1B can each associate with mammalian BRM and BRG, in all four possible combinations (28; 33; 34), so that p270 and ARID1B each define a specific limited set among the various combinational permutations of SWI/SNF-related complexes that exist in mammalian cells.

The importance of p270 and ARID1B in proliferation control was explored here using an siRNA approach. The knockdowns were constructed in the MC3T3-E1 pre-osteoblast line because these non-transformed cells undergo a tightly regulated and well-characterized progression through cell cycle arrest and into tissue-specific gene expression (e.g. 26; 35; 36; 37; 38; 39). This is an important model system because it permits an examination of the normal roles of the complex subunits during differentiation-associated cell cycle arrest. Parental MC3T3-E1 cells arrest by day 4 post-induction with the differentiation signal. The results described here show in parallel conditions that p270-depleted cells fail to arrest normally. This is evidenced by continued synthesis of DNA, and by a lack of other characteristics typical of arrested cells, including up-regulation of p21, down-regulation of cyclins, and decreases in histone H4 expression and cdc2-specific kinase activity. The ARID1B-depleted lines behaved like the parental cells in each of these assays. The analysis of the respective roles of p270 (ARID1A) and ARID1B establishes a new paradigm that the choice of ARID-containing subunits confers specificity of function on the complexes. The specific requirement for p270 is evident separately in both the up-regulation of p21 and the down-regulation of E2F-responsive products. The clinical findings suggested indirectly that deficiency of p270 plays a causative role in carcinogenesis. The identification of specific proliferation control steps dependent on the presence of p270 now provides a direct molecular basis to support the clinical findings. Moreover, the demonstration

that the complexes are required separately for regulation of at least two distinct steps in proliferation control underscores the carcinogenic potential of cells that have lost function of a required subunit.

## MATERIALS AND METHODS

**Materials.** FBS was purchased from Summit Biotech (Fort Collins, CO), a-MEM from Irvine Scientific (Santa Ana, CA), and penicillin and streptomycin from Mediatech (Herndon, VA). Histone H1, Ascorbic acid, β-glycerol phosphate, and protease inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO), and G418 from Gibco BRL (Grand Island, NY). Radiochemicals were obtained from NEN.

**siRNA and isolation of stable p270 knockdown lines.** The siRNA sequences were tested in a pSUPER vector constructed as described in (40). Test oligonucleotides were synthesized as complementary pairs, each 64 bases long, containing two inversely repeated copies of a 19 base pair target sequence separated by a 9 base pair spacer region. Six different target sequences were tested in transient expression assays in 293T cells against an exogenously introduced p270 partial expression construct. In a similar manner, four different target sequences were tested against an ARID1B partial expression construct. Expression was monitored by Western Blot. The most effective sequences were chosen for construction of the stable knockdown lines. The pSUPER-derived vectors containing the respective knockdown sequences (pSUPER.p270.7182 and pSUPER.ARID1B.5400) were introduced into MC-3T3-E1 cells by lipofection together with a selectable neo marker. G418-resistant clones were amplified and screened by Western blot for p270 expression. Aliquots of low passage depleted lines were frozen as stocks. The target sequences for each construct were designed against nucleotide stretches that are identical between the mouse and human genes so that they can be used in cells of either species origin.

**Cell Culture.** Low passage MC3T3-E1 cells were a gift from Roland Baron (Yale University, New Haven, CT). Cells were maintained in α-MEM plus 10% fetal bovine serum supplemented with 50 U per ml penicillin and 50 μg per ml streptomycin. For differentiation assays, cells were plated at an approximate initial density of 5 X $10^4$ cells per $cm^2$. Differentiation was induced by addition

of 50 μg per ml final concentration ascorbic acid and 10 mM final concentration β-glycerol phosphate to standard growth medium. The medium was changed every 3-4 days and the inducing agents were replaced with each media change.

**Immunoblotting.** Cells were washed and harvested in PBS and lysed in p300 lysis buffer [0.1% Nonidet P-40, 250 mM sodium chloride, 20 mM sodium phosphate (pH 7.0), 30 mM sodium pyrophosphate, 5 mM dithiothreitol and protease and phosphatase inhibitors: 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl flouride (PMSF), 100 kIU aprotinin, 1 ug/ml leupeptin, and 1 ug/ml pepstatin]. Proteins were separated by polyacrylamide gel electrophoresis, transferred to Immobilon-P membrane (Millipore), and visualized as described previously (41).

**Radiolabeling and immunoprecipitation**. Cells were washed with methionine-free and serum-free α-MEM and incubated with this medium for one hour. 200 μCi of [$^{35}$S]-methionine (Perkin-Elmer, Boston MA or Amersham, Piscataway NJ) was added to each 10 cm monolayer, and the plates were incubated for a further three hours. Cells were washed and harvested in PBS and lysed in p300 lysis buffer. 3 mg of total cell lystate were precleared with 3% protein A-sepharose beads and immunoprecipitated as described previously (41).

**Alkaline phosphatase assay.** Cell monolayers were rinsed in PBS, fixed in 100% methanol, rinsed with PBS, then overlaid with 1.5 ml of 0.15 mg/ml BCIP (Sigma) plus 0.3 mg/ml NBT (Promega, Madison, WI) for thirty minutes and rinsed again with PBS.

**RNA analysis and Probes.** Total cell RNA was prepared at designated times post-induction using Trizol Reagent (GibcoBRL, Grand Island, NY) according to manufacturer's recommendations. RNA in serial 10-fold dilutions (10 μg, 1 μg, 0.1 μg) was applied to nitrocellulose BA85 in a slot-blotting apparatus (Schleicher and Schuell), and crosslinked by UV

irradiation. $^{32}$P-labelled probes were prepared using a random primed labeling kit (Boehringer-Mannheim). The histone H4 probe was described previously (35). Plasmid pGB.GAPDH was constructed from MC3T3-E1 cell RNA by generating an RT-PCR fragment using primers (5'ACTTTGTCAAGCTCATTTCC-3') and (5'-TGCAGCGAACTTTATTGATG-3') corresponding to the murine glyceraldehyde-3-phosphate dehydrogenase cDNA sequence, and subcloning the resulting PCR fragment into the TA cloning vector, pCR2.1 (Invitrogen, Carlsbad, CA).

**Antibodies.** The p270-specific monoclonal antibody PSG3 and the ARID1B-specific monoclonal antibody KMN1 have been described previously (34). A peptide used to generate a BAF155-specific monoclonal antibody, DXD7, (34) also gave rise to a distinct BAF155-reactive monoclonal antibody, designated DXD12, which cross-reacts with BAF170, and was used here. An SV40 Tag-specific monoclonal antibody, mAb 419 (obtained from Ed Harlow), was used as a negative control. Commercially purchased antibodies include the p21$^{CIP1/WAF1/SDI}$-specific antibody (BD Biosciences, San Jose, CA) and the hsc70-specific antibody (Stressgen, San Diego, CA), as well as antibodies of the following specificities obtained from Santa Cruz: cyclin A (C-19, sc-596), cyclin B2 (N-20, sc-5235), and cyclin C (H-184, sc-5610). Rabbit polyclonal serum was raised against the cdc2-G6 peptide sequence CDNQIKKM.

**Kinase assays.** The cdc2-dependent kinase assays were performed as described previously (42), using cdc2-specific immunoprecipitation complexes from 1 mg of total cell lysate and histone H1 as exogenous substrate.

**DNA synthesis assay.** Induced cells were labeled with $^{3}$H-thymidine (Perkin-Elmer) (5 μCi/ml of culture medium) in one hour pulses at the times post-induction indicated in the text , lysed in 0.3 M NaOH and assayed for trichloracetic acid (TCA)-precipitable counts as described previously (43).

**Virus Infection.** The generation and culture of the E1A-inactivated 9S adenovirus (used here as a negative control) has been described previously (44). A stock of p21 expression virus (Ad5CMVp21) (45) was provided by Judit Garriga, (Fels Institute, Temple University School of Medicine, Philadelphia PA). MC3T3-E1 cells were infected at a multiplicity of infection of 25 plaque forming units per cell.

## RESULTS

### Generation of p270-deficient and ARID1B-deficient MC3T3-E1-derived cell lines.

Potential interfering oligonucleotide sequences were tested in a pSUPER-derived system by standard protocols. Effective sequences were identified and introduced by stable integration from the plasmid vector into the MC3T3-E1 line. Depletion was monitored by Western blotting with p270-specific and ARID1B-specific monoclonal antibodies. Ten independent lines with reduced expression of each target were selected, amplified and stored. As a control, vector-only lines were selected and amplified in parallel. In each transfection, colonies appeared at similar frequencies and showed essentially the same doubling time in normal growth medium as parental cells. A representative p270 knockdown line (MC.p270.KD.AA2) and ARID1B knockdown line (MC.1B.KD.CA6B) are each shown in **Fig. 1A**. The depleted lines (lane 3 and lane 6) show weak p270 or ARID1B signals respectively in comparison with the parental line or a clonal line isolated after transfection with the empty vector (lanes 1, 2, 4, and 5). The blots were additionally probed with a monoclonal antibody that recognizes the closely related BAF155 and BAF170 mammalian SWI/SNF complex subunits. Expression of these subunits is similar in each line. The overall integrity of the complexes in the p270-depleted cells was verified by immunoprecipitation of $^{35}$S-labeled cell lystates with the BAF155-reactive antibody (**Fig. 1B**). Maintenance of expression of the alternative ARID family protein in the conversely depleted cells was confirmed by immunoprecipitation with p270-specific and ARID1B specific monoclonal antibodies (**Fig. 1C**). All selected lines showed a similar degree of depletion. $^{35}$S-methionine pulse labeling indicates that new synthesis of each protein is reduced about ten-fold in the knockdown lines. As a further probe for the overall integrity of the complexes in the knockdown lines, the remaining ARID family product was removed by immune depletion of the cell lysates before immunoprecipitation with the BAF155-reactive antibody (**Fig. 1D**). The results confirm that complex assembly is stable in the absence of either subunit.

**p270-depleted and ARID1B-depleted cells both show impaired induction of the tissue-specific marker alkaline phosphatase.**

MC3T3-E1 cells continue to proliferate for several days after induction with ascorbic acid, then undergo cell cycle arrest at about day 3 post-induction. Expression of the earliest differentiation marker, alkaline phosphatase, can be detected at this time. The knockdown lines were tested for induction of alkaline phosphatase in an *in situ* enzyme assay scored by color development (positive cells stain purple-black). Three independent knockdown lines from each series were tested, and all showed severe impairment of alkaline phosphatase induction; vector-only lines behaved like the parental line.. In multiple independent experiments both series of knockdown lines showed severely reduced induction of alkaline phosphatase at every point tested, both early and late. Representative experiments at day 3 and day 14 are shown in **Fig. 2**. These results indicate that the level of depletion achieved for each target is functionally significant, and that both ARID-containing products are required for normal onset of differentiation. We have used these knockdown lines to explore the role of each protein specifically in cell cycle arrest functions.

**p270-deficient cells fail to undergo normal cell cycle arrest.**

The effect of p270 deficiency *versus* ARID1B deficiency was tested on specific cell cycle arrest functions. We have previously used a gene array approach to identify many of the changes in gene expression that occur in MC3T3-E1 cells as they procede through the differentiation program (35). Expression was assayed on the arrays at days 0, 3, 7, and later times post-induction. Between day 0 and day 7 a number of changes occurred that corresponded with the shut-down of cell cycle activity. Among the most prominent was induction of the cell cycle inhibitor, $p21^{Cip1/Waf1.}$ Several-fold induction of p21 was apparent by day 3 post-induction. When this response was tested here at the protein level, a similar pattern of induction was apparent in the parental line, but p21 expression was not induced in p270-depleted cells. A representative

Western blot depicting results from the MC.p270.KD.CA6 line is shown in **Fig. 3A**. The same pattern was seen with the MC.p270.KD.AA2 and MC.p270.KD.DD2 lines (not shown). In contrast to the p270-depleted cell lines, the ARID1B-depleted lines showed no impairment of p21 induction. Results from a representative line (MC.1B.KD.FD2) are shown in Fig. 3A; the same result was observed with the MC.1B.KD.CA6B and MC.1B.KD.JD6 lines. As a loading control, the blots were also probed with an antibody reactive against the constitutive form of the 70 kDa heat shock protein, hsc70. The hsc70 signal was similar in all lanes.

The gene array also indicated down-regulation of several cyclins as the MC3T3-E1 cells enter growth arrest. These responses were probed here with the MC.p270.KD.CA6 line and the MC.1B.KD.FD2 line (**Fig. 3B**). The array showed down-regulation of B-type cyclins, particularly cyclin B2, by day 3 post-induction. Consistent with the RNA signals, a decreased level of cyclin B2 was apparent in the parental cells by day 4 on the Western blot. However, p270-depleted cells again failed to show the parental response; levels of cyclin B2 remained high. Cyclin C was also sharply down-regulated by day 3 in the gene array probe. Consistent with the RNA signal, down-regulation of cyclin C in the parental cells was clear by day 2 in the Western blot. However, cyclin C levels were unaffected by the induction protocol in the p270-depleted cells. The gene array also indicated down-regulation of cyclin A, but this response was delayed relative to the response patterns of cyclins B and C. On the array, a decreased cyclin A signal was apparent at day 7 post-induction, but not at day 3. The protein probe shows a slight decrease in the cyclin A level in the parental cells by day 6, and no detectable decrease in the p270-depleted cells. The cdc2 kinase gene was not represented on the array, but expression of this gene is of interest as a known E2F target subject to pRb-mediated repression (reviewed in 46). The cdc2 protein product was assayed by Western blotting (**Fig. 3B**), which shows a sharp decrease in protein levels in the parental cells by day 4 post-induction, and no detectable decrease in p270-depleted cells by day 6. In each of these assays, the ARID1B-depleted cells behaved indistinguishably from the parental cells.

A well-characterized marker of proliferation state in differentiating osteoblasts is histone H4 expression (reviewed in 39). Expression of this marker declines dramatically as the cells arrest after induction. This response was also compared in parental MC3T3-E1 and the knockdown cells. Consistent with other markers of cell cycle activity, the histone H4 signal decreased sharply in the parental cells and the ARID1B knockdown line, but remained high in the p270 knockdown line (**Fig. 4**).

The gene expression patterns that accompany induction to the differentiation phenotype in parental MC3T3-E1 cells imply that a sharp decline in cyclin dependent kinase activity would occur by day 4. The activity of cdc2-associated complexes was assayed here directly. The kinase activity shows a sharp decline by day 4 post-induction in the parental cells and the ARID1B-depleted cells, but no decline was detectable in the p270-depleted cells, even at day 6. A representative kinase assay performed with the MC.p270.KD.DD2 line and the MC.1B.KD.FD2 line is shown in **Fig. 5A**. Results from independent experiments with three different p270-depleted lines (MC.p270.KD.AA2, MC.p270.KD.CA6 and MC.p270.KD.DD2) and three different ARID1B-depleted lines (MC.1B.KD.FD2, MC.1B.KD.JD6, and MC.1B.KD.CA6B) were quantified on a phosphoimager and the averages are shown graphically in **Fig. 5B**.

The cell cycle status of the cells was probed directly by assessing the rate of $^{3}$H-thymidine incorporation over several days post-induction. Parental cells, p270-depleted cells, and ARID1B-depleted cells were plated and induced in parallel. At 24 hour intervals, $^{3}$H-thymidine was added to the culture medium for one hour, after which the labeled cells were harvested and assayed for incorporation of the isotope. The results shown for the parental cells are the averages of three independent platings. The results shown for the p270-depleted cells are the averages from three different knockdown lines (MC.p270.KD.AA2, MC.p270.KD.CA6 and MC.p270.KD.DD2), as are the results shown for the ARID1B-depleted lines (MC.1B.KD.FD2,

MC.1B.KD.JD6, and MC.1B.KD.CA6B). The parental cells show a sharp decline in the rate of $^{3}$H-thymidine incorporation by day 4 post-induction, indicating a shut-down of DNA synthesis consistent with cell cycle arrest. The same pattern is seen in the ARID1B-depleted lines. In contrast, the rate of $^{3}$H-thymidine incorporation decreases only slightly in the p270-depleted cells, indicating continued DNA synthesis and failure to undergo normal cell cycle arrest (**Fig. 5C**).

These results identify the p270 subunit as critical for normal cell cycle arrest. In each of the cell cycle assays described here, cloned cell lines containing a functional ARID1B-targeted siRNA sequence behaved exactly like the parental line, indicating that the failure of p270-depleted cells to undergo a normal cell cycle arrest response is a specific effect of p270-deficiency.

**Induction of p21 and repression of E2F-responsive promoters are independent events that each require p270.**

Previous studies have used differential expression of BRG1 and hSNF5 to probe the role of SWI/SNF-related complexes in cell cycle arrest. Several studies, often using cloned rather than endogenous promoters, found BRG1 enhances pRb-mediated repression of E2F-responsive genes, and suggest that SWI/SNF subunits are associated with pRb in repressor complexes (23; 25; 47; 48). Decreased expression of endogenous E2F-responsive gene products such as cyclin A and cdc2 was generally apparent at the protein level when BRG1 expression was restored to naturally deficient tumor cell lines (20; 23), but in another study the effects were modest or cell line specific, at least at the RNA level (19). An upstream effect with the potential to activate pRb was seen consistently; exogenous expression of BRG1 in BRG1-deficient tumor cell lines results in a sharp increase in p21 expression with most other cell cycle inhibitors, including $p16^{ink4a}$, remaining relatively unaffected (19; 20). Expression of hSNF5 in malignant rhabdoid tumor (MRT) lines has likewise been linked with decreased levels of E2F-responsive gene products

such as cyclin A (21; 22; 49). Restoring hSNF5 to MRT cells does not alter p21 expression, but up-regulates the $p16^{ink4a}$ cell cycle inhibitor (17; 22; 46) in contrast to the effect of BRG1. The difference may be a function of cell type rather than subunit effect, however, because siRNA-mediated depletion of hSNF5 in HeLa (cervical carcinoma) or MG63 (osteosarcoma) cells causes a sharp decrease in p21 levels, with no effect on p16 (20).

The failure of p270-depleted cells to induce p21 could theoretically account for all of the cell cycle arrest defects observed in these lines. Repression of E2F-responsive genes and the downstream effects of this repression might be impaired indirectly if cyclin-dependent kinase activity is not appropriately inhibited, leaving targets such as pRb phosphorylated, inactive, and unable to mediate repression of E2F-responsive genes. The effect of the complexes on the p21 promoter is apparently direct, as two studies have demonstrated the presence of BRG1 at the p21 promoter, although a target element in the promoter could not be established (19; 20). A key mechanistic question that remains unclear is whether down-regulation of E2F responsive genes requires the action of the complexes independently of their effects on p21 levels. Chromatin association assays have limited usefulness for these studies because the complexes associate with chromatin widely. This question was therefore addressed here genetically by introducing exogenous expression of p21 in the p270-depleted cells simultaneously with the differentiation signal.

Parental and p270-depleted cells were infected in parallel at the time of ascorbic acid induction with an adenovirus vector expressing p21 or with a negative control virus containing an inactivated E1A gene. The cells were monitored for DNA synthesis as described above. Parental cells that received the p21 expression construct underwent accelerated shutdown of DNA synthesis; $^{3}$H-thymidine incorporation was severely repressed by day 2 post-induction (**Fig. 6 panel A**). Uninfected cells, or cells infected with the negative control virus, showed the same kinetics seen in Fig. 6, i.e., without exogenous p21, DNA synthesis in the parental line remained

high at day 2; a severe decrease was not seen until day 4. In the p270-depleted cells, without exogenous expression of p21, DNA synthesis remains high at least until day 6. However, exogenous expression of p21 caused DNA synthesis to shut down with the same rapid kinetics seen in the parental cells (**Fig. 6 panel B**). (Exogenous expression of p21 was verified by Western blotting, shown in the left hand lanes of **Fig. 6 panel C**; normal induction of p21 in the parental cells and the failure of p21 induction in the p270 depleted cells can be seen in the right hand, 9S-infected, control lanes). The rapid down-regulation of DNA synthesis associated with exogenous p21 expression was expected even in the p270-depleted cells because p21-induced inhibition of cyclin dependent kinase activity is expected to result in the inactivation of essential DNA replication factors. What is of special interest here is the status of E2F-responsive products. This was monitored by Western blotting for the representative E2F-responsive gene products cdc2, cyclin A, and cyclin B2. Expression of cyclin C was also examined. (**Fig. 6 panel C**). The results show that expression levels of cdc2 and the cyclins remain high in the p270-depleted cells despite exogenous expression of p21, indicating that regulation at the p21 promoter and at the E2F-responsive promoters each independently requires the function of the chromatin remodeling complexes, and of p270 specifically, during differentiation-associated cell cycle arrest. The expected repression of the cdc2-associated kinase activity in p21-expressing p270-knockdown cells, despite the maintenance of a high level of cdc2 expression, was verified in a kinase assay (**Fig. 6 panel D**).

## DISCUSSION

The work described here identifies the p270 subunit of mammalian SWI/SNF-related complexes as critical for normal cell cycle arrest in differentiating cells exiting the cell cycle. The evidence of p270 deficiency in certain tumors and tumor cell lines (32), implied indirectly that p270 plays a required role in the tumor suppressor activity of the complex(es). The results presented here establish a specific biological basis for the clinical findings, demonstrating directly that p270 is essential for both the induction of p21 and the repression of E2F responsive genes such as cdc2 during differentiation-associated cell cycle arrest. Involvement in both activation and repression is a feature of SWI/SNF-related complexes generally, presumably determined by the spectrum of transactivators and repressors that recruit the complexes. The demonstration that the complexes are required separately for regulation of at least two distinct steps in proliferation control underscores the carcinogenic potential of cells that have lost function of a required subunit.

These results are particularly significant because previous studies concerning the roles of SWI/SNF complex components in expression of cell cycle markers have largely relied on re-introduction of BRG1 or hSNF5 into tumor cell lines where they were lacking (e.g. 17; 19; 20; 22; 23; 25; 49), rather than monitoring the role of complex components in cells undergoing physiological progression from a proliferative state to cell cycle arrest. The identification of p270 as a subunit required for cell cycle arrest *in vivo* is additionally significant, because unlike BRG1 and hSNF5, p270 is not among those subunits considered to form the "functional core" of the complex(es). The concept of a functional core was based on the observation that BRG1 has a relatively low level of enzyme activity when purified away from other subunits, and that a level of remodeling and ATPase activity similar to that of the intact complex(es) can be reconstituted *in vitro* by assembly of a subset of components consisting of BRG1, BAF170, BAF155, and hSNF5 (50). The *in vivo* requirement for p270 shows that it plays an essential role in the physiological

functions of the complex(es), regardless of whether it contributes directly to the overall level of enzymatic activity.

A cell cycle arrest function has previously been ascribed to the BRG1 and hSNF5 components; however, those subunits do not distinguish between the BAF and PBAF complexes. The p270–depletion phenotype constitutes the first formal evidence of a requirement for the BAF complex series, as opposed to the PBAF complex, in cell cycle regulation. The data presented here shed further light on the question of specificity among the several distinct configurations of the BAF complexes. The alternative ATPase subunits, BRM and BRG1, may be partially redundant in their ability to support cell cycle arrest when exogenously expressed (51), but the most physiological experiments (3; 4) suggest strongly that BRG1-specific complexes and not BRM-specific complexes are essential for this function. The present study indicates that p270-containing complexes, but not ARID1B-containing complexes, are required for cell cycle arrest. Logically, it appears that, of the four combinations made possible by these alternative subunits, it is the BRG1 and p270 combination that plays the major role in cell cycle arrest. hSNF5 is not a determinant of specificity between complexes, but its presence along with BRG1 and p270 is required for the activities that the complex contributes to cell cycle arrest. These findings will help to clarify targets for drug intervention therapies.

The essential biochemical activities contributed by the noncatalytic subunits have yet to be determined. The amino acid sequence of hSNF5 gives little clue to the function of the protein, which remains unknown, except that it can facilitate DNA end-joining *in vitro* (52). In p270, the most obvious structural motif is the approximately 100 amino acid long ARID DNA binding domain, but this large protein (2,285 amino acids) also contains potential protein-protein interaction surfaces (27; 28; 33; 41) that may be more important for its specific function. ARID1B contains an ARID domain that is 80% identical with p270 (alignments can be seen in 30 and 53), and both proteins belong to a subclass of the ARID family that binds DNA without regard to

sequence specificity (34; 53). Thus, a likely scenario is that DNA binding is a function common to both, while sequences outside the ARID determine specificity of function. Structure-function analysis of both these ARID-containing subunits is in progress.

The effects linked individually with BRG1, hSNF5 and p270 are generally similar but not identical. What is not clear is whether differences so far reported are due more to methodology or cell type than to true differences in function between the subunits. Approaches based on re-introduction of specific components into deficient cell tumor cell lines preclude the ability to compare function within a single cell line or in non-transformed lines. The ability to knock down expression is freeing investigators to study the role of the complexes in nontransformed cell lines. BRG1 and BRM can be inhibited by dominant/negative forms with an inactivating mutation in the ATP binding site. Dominant/negative inhibition of BRG1/BRM in NIH3T3 mouse fibroblasts inhibited MyoD-dependent differentiation, but surprisingly did not inhibit concomitant cell cycle arrest; p21 is induced in these conditions and its induction was likewise unaffected by expression of the dominant/negative construct (54). Cell cycle arrest independent of SWI/SNF complex activity may be a phenomenon specific to the function of MyoD however, because a similar dominant/negative approach in BALB/c mouse fibroblasts was sufficient to inhibit C/EBPα-induced cell cycle arrest severely (55). In the latter study siRNA-mediated depletion of hSNF5 or BRM had the same effect on cell growth curves as expression of the dominant/negative construct, but expression of individual genes was not assessed.

The system used here maintains the benefits of probing function in non-transformed cells, and has the additional advantage of relying on an external induction signal to initiate differentiation and cell cycle arrest functions rather than engineered over-expression of a single gene such as MyoD or C/EBPα. The MC3T3-E1 cell system is amenable to knockdown studies targeting each of a succession of subunits, and probing an array of extracellular signals. Further studies are in progress.

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## FIGURE LEGENDS

**Figure 1. Expression of p270 and ARID1B in MC3T3-E1-derived knockdown lines.** A. 100 μg of total cell lysate per lane was separated on 8% SDS-PAGE gels, transferred to PVDF membrane, and probed with either p270-specific or ARID1B-specific monoclonal antibodies, and with a BAF155/BAF170-reactive monoclonal antibody. B. Aliquots of $^{35}$S-labeled cell lysates from parental MC3T3-E1 cells (lanes 1 and 2) or MC.p270.KD.AA2 cells (lane 3) or MC.1B.KDCA6B cells (lane 4) were immunoprecipitated with control antibody (lane 1) or a BAF155-reactive monoclonal antibody (lanes 2 through 4). The identity of the higher molecular weight proteins labeled at the right in the immune complex was verified directly by Western blotting. C. Aliquots of $^{35}$S-labeled cell lysates isolated as described in panel B were immunoprecipitated with p270-specific or ARID1B-specific monoclonal antibodies, as indicated in the figure. D. (I): Aliquots of $^{35}$S-labeled ARID1B-knockdown cells were depleted of p270 by five successive immunoprecipitations with a p270-specific mAb; lanes 1 through 5 show the autoradiogram signal of p270 brought down by each successive immunoprecipitation. (II): Aliquots of $^{35}$S-labeled p270 knockdown cells were depleted of ARID1B by five successive immunoprecipitations with an ARID1B-specific mAb; lanes 6 through 10 show the radiogram signal of ARID1B brought down by each successive immunoprecipitation. The fully depleted lysates from series I and II (sampled in lanes 5 and 10, respectively) were then each immunoprecipitated with a BAF155-reactive antibody (DXD12). Visualization of the immune complexes by autoradiography (lanes I and II) shows the overall integrity of the complexes in the absence of both ARID family subunits.

**Figure 2. Differentiation phenotype of the knockdown cell lines.** Cells were assayed at day 0, day 3, and day 14 post-induction for alkaline phosphatase activity. Conversion of the substrate to a purple color indicates activity of the enzyme. AA2, CA6, and DD2 are identification numbers

for three independently isolated p270-depleted lines. JD6, FD2, and CA6B are identification numbers for three independently isolated ARID1B-depleted lines.

**Figure 3. Expression of p21 and other cell cycle markers in p270-depleted and ARID1B-depleted cells.** Parental, p270-depleted, and ARID1B-depleted MC3T3-E1 cells were harvested at days 0, 2, 4 and 6 post-induction. 100 μg of total cell lysate per lane was separated on SDS-PAGE gels, transferred to PVDF membrane, and probed sequentially with antibodies of each of the specificities shown.

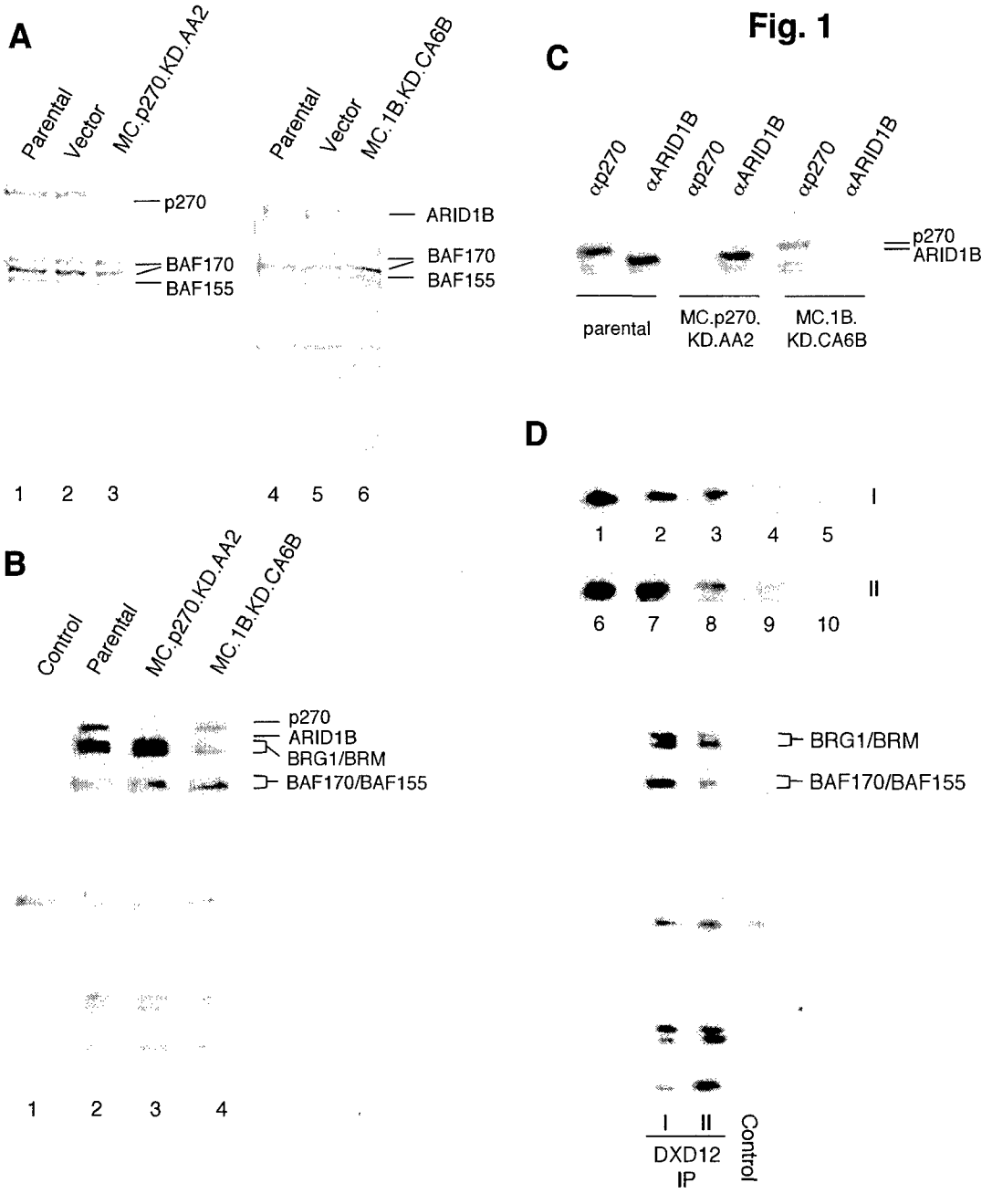
**Figure 4. Expression of histone H4 in p270-depleted cells.** A. Parental, p270-depleted, and ARID1B-depleted MC3T3-E1 cells were harvested at days 0 and 3 post-induction. Total cell RNA was applied to nitrocellulose and probed with a histone H4-specific probe and a GAPDH-specific probe included as a loading control. B. Quadruplicate samples were probed for histone H4 expression as described in panel A, quantified by phosphoimaging, normalized to GAPDH, and plotted relative to day 0. The error bars indicate average deviation.

**Figure 5. cdc2/CDK1-associated kinase activity and DNA synthesis activity in p270-depleted cells.** Parental, p270-depleted, and ARID1B-depleted MC3T3-E1 cells were harvested at days 0, 2, 4 and 6 post-induction. cdc2-specific immune complexes were isolated from 1 mg of total cell lystate, and incubated with $\gamma$-$^{32}$P-ATP and histone H1 as exogenous substrate. A. The reactions were separated on 15% SDS-PAGE gels and visualized by fluorography. B. Reactions performed as described in panel A were quantified by phosphoimaging. Results from three independent experiments with independently isolated cell lines of each knockdown series were averaged, and plotted as kinase activity relative to day 0. The average deviation at each point is indicated by error bars. The solid line indicates kinase activity in the parental cells; the dashed line indicates activity in the p270-depleted cells; the dotted line indicates activity in the ARID1B-depleted cells. C. Parental, p270-depleted, and ARID1B-depleted MC3T3-E1 cells

were labeled with $^{3}$H-thymidine in one hour pulses at days 0, 2, 4 and 6 post-induction, and assayed for trichloracetic acid (TCA)-precipitable counts. Results from three independent platings of the parental cell line, and from three independently isolated cell lines from each knockdown series were averaged within their respective groups and plotted as CPM incorporated. The average deviation at each point is indicated by error bars. The striped bar indicates incorporation in the parental cells; the solid bar indicates incorporation in the p270-depleted cells. The open bar indicates incorporation in the ARID1B-depleted cells.

**Figure 6. Effect of exogenous expression of p21.** Parental and p270-depleted cells were infected in parallel at the time of ascorbic acid induction (day 0) with an adenovirus vector expressing p21 or with a negative control virus containing an inactivated E1A gene (9S). A and B: Cells were assessed for DNA synthesis activity monitored by $^{3}$H-thymidine incorporation; the curves are the averages and average deviation of results obtained with three independent platings of the parental line in parallel with three independent p270 knockdown lines. C: Levels of cdc2, p21, cyclins B2, A, and C, as well as hsc70 were probed by Western blotting in parental and p270 knockdown cells infected with the p21-expressing virus or the control (9S) virus. D: cdc2-associated kinase activity was assayed in parental and p270 knockdown cells infected with the p21-expressing virus or the control (9S) virus. The graphs show the averages and average deviations from triplicate platings of the parental line and three different p270 knockdown lines; the gel shows a representative result.

Fig. 1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MC.1B.KD.CA6B
p270
ARID1B
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BAF155
BAF170
BAF155
1 2 3 4 5 6
B
Control
Parental
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MC.1B.KD.CA6B
p270
ARID1B
BRG1/BRM
BAF170/BAF155
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αp270
αARID1B
αp270
αARID1B
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ARID1B
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BAF170/BAF155
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DXD12
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Control

# Fig. 2

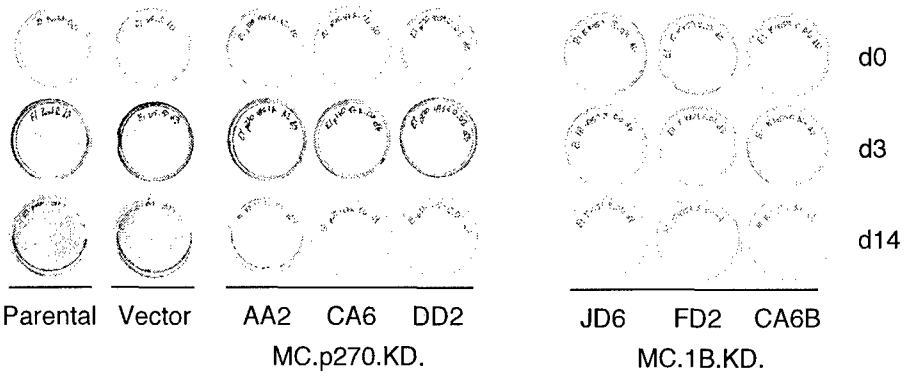
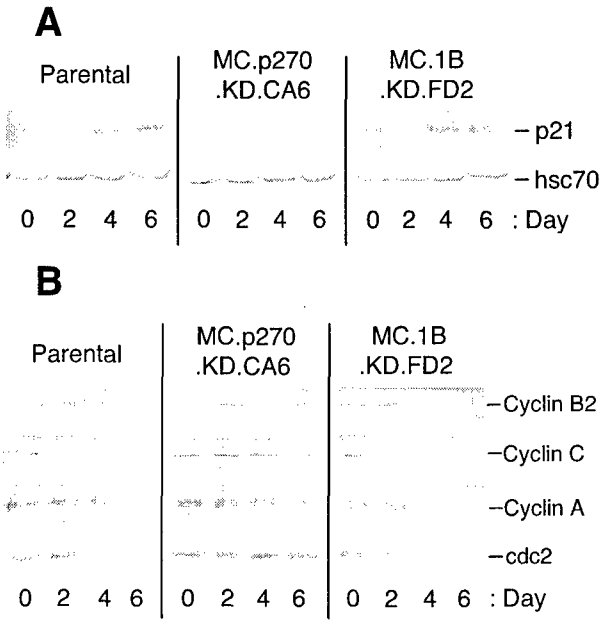

d0
d3
d14
Parental
Vector
AA2
CA6
DD2
MC.p270.KD.
JD6
FD2
CA6B
MC.1B.KD.

**Fig. 3**


A
Parental
MC.p270
.KD.CA6
MC.1B
.KD.FD2
– p21
– hsc70
0 2 4 6 0 2 4 6 0 2 4 6 : Day
B
Parental
MC.p270
.KD.CA6
MC.1B
.KD.FD2
–Cyclin B2
–Cyclin C
–Cyclin A
–cdc2
0 2 4 6 0 2 4 6 0 2 4 6 : Day

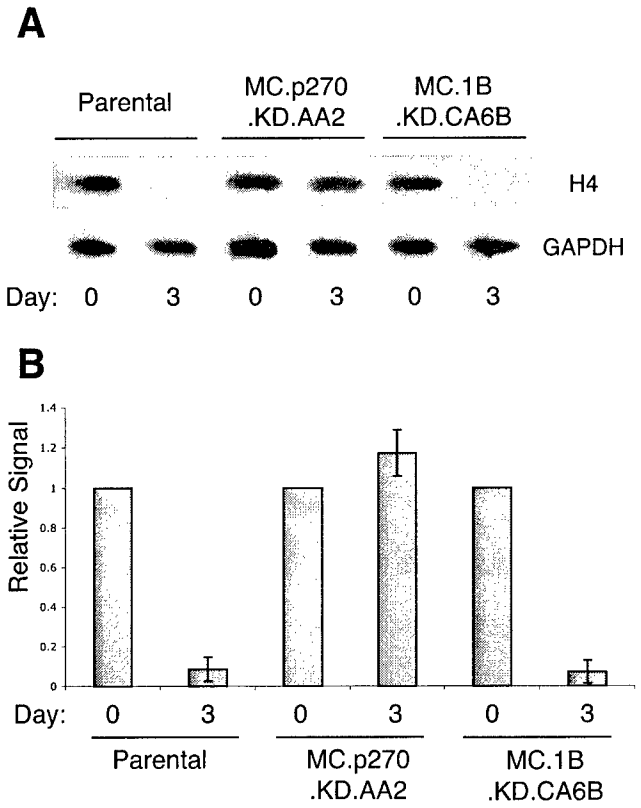
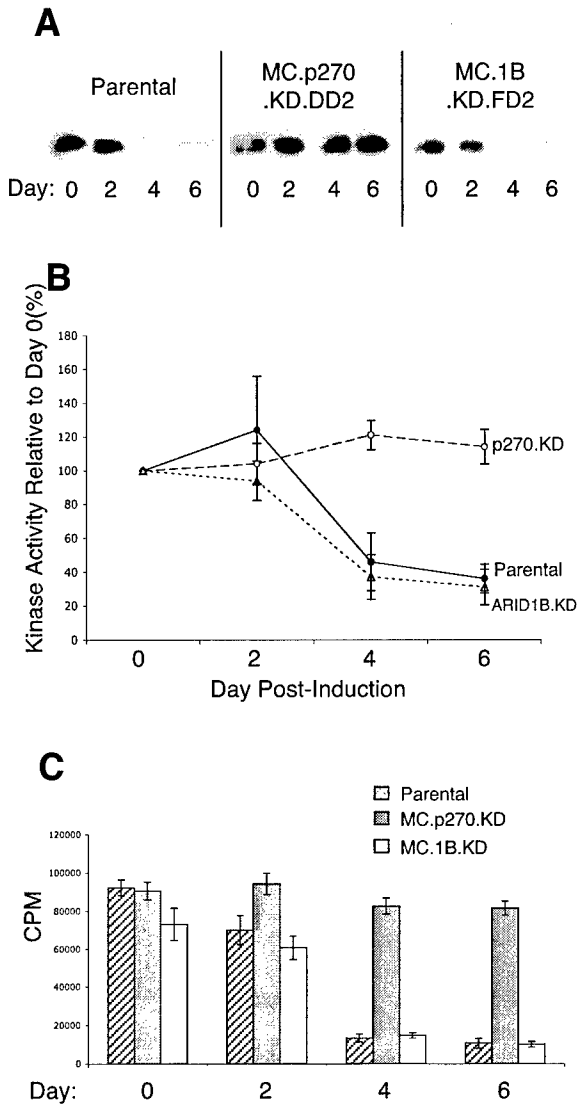

A
Parental
MC.p270
.KD.AA2
MC.1B
.KD.CA6B
H4
GAPDH
Day: 0 3 0 3 0 3
B
Relative Signal
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Day: 0 3 0 3 0 3
Parental
MC.p270
.KD.AA2
MC.1B
.KD.CA6B

**Fig. 4**

Fig. 5


A
Parental
MC.p270
.KD.DD2
MC.1B
.KD.FD2
Day: 0 2 4 6 0 2 4 6 0 2 4 6
B
Kinase Activity Relative to Day 0(%)
180
160
140
120
100
80
60
40
20
0
p270.KD
Parental
ARID1B.KD
0 2 4 6
Day Post-Induction
C
Parental
MC.p270.KD
MC.1B.KD
CPM
120000
100000
80000
60000
40000
20000
0
Day: 0 2 4 6

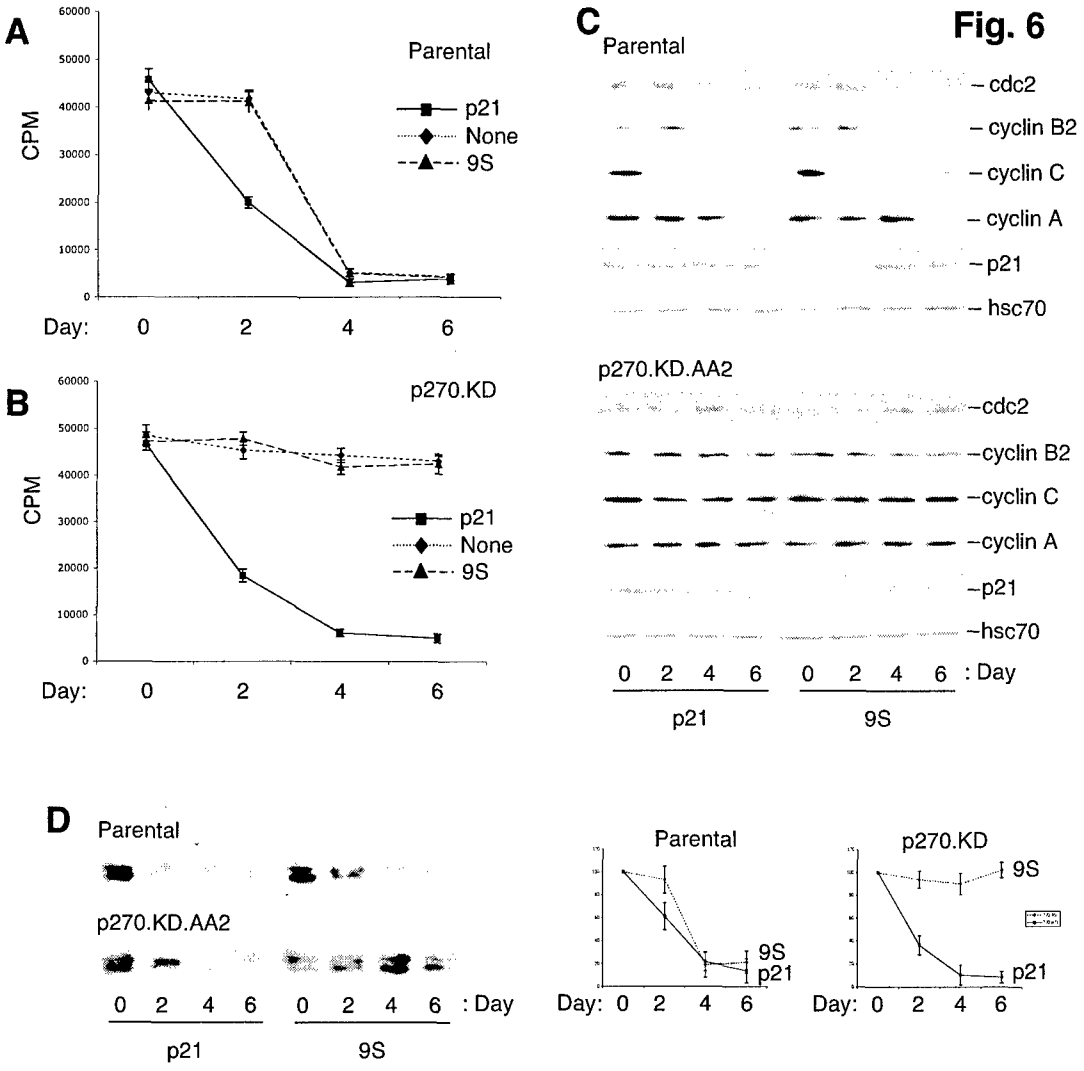

A
Parental
CPM
60000
50000
40000
30000
20000
10000
0
p21
None
9S
Day: 0 2 4 6
B
p270.KD
CPM
60000
50000
40000
30000
20000
10000
0
p21
None
9S
Day: 0 2 4 6
C
Parental
– cdc2
– cyclin B2
– cyclin C
– cyclin A
– p21
– hsc70
p270.KD.AA2
–cdc2
–cyclin B2
–cyclin C
–cyclin A
–p21
–hsc70
0 2 4 6 0 2 4 6 : Day
p21
9S
D
Parental
p270.KD.AA2
0 2 4 6 0 2 4 6 : Day
p21
9S
Parental
9S
p21
Day: 0 2 4 6
p270.KD
9S
p21
Day: 0 2 4 6

Fig. 6

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