Role for Brm in Cell Growth Control

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Introduction

The cell cycle is controlled by a family of protein kinases, whose specific activities depend on the association with protein partners named cyclins. The cyclin-dependent kinases (CDK) and their regulatory cyclin subunits promote passage through each phase of the cell division cycle (1). D-type cyclins participate mainly to the quiescence/G1 phase transition and to the transit to the early G1 phase. E-type cyclins control G1/S transition with a crucial role after cells escape from a quiescent state (2). Cyclin A2 is involved in the G1/S transition and the transit into S phase when associated with cdk2 kinase and the G2/M transition when associated with cdc2. Finally, B-type cyclins contribute to the completion of the G2 phase and mitosis. To safely achieve the cell cycle, cyclin genes are expressed according to a rigorous timing that requires the coordinated activation and repression of specific sets of transcription factors. Among these transcription factors, the chromatin remodeling complex SWI/SNF has revealed interesting features (3). This multisubunit complex that consists of 10 to 12 proteins highly conserved among eukaryotes activates or represses transcription by disrupting histone-DNA interactions in an ATP-dependent manner (4). In mammals, SWI/SNF shows a remarkable diversity brought by the presence of either Brahma (Brm)/SNF2h or Brm-related gene 1 (Brg1)/SNF2h protein that both carry out the ATP-dependent helicase activity of the complex and by an adjustable number of Brg1-associated factors (BAF; ref. 5). Inactivation of Brg1 by homologous recombination in mouse is embryonic lethal at preimplantation stage, but tumors are detected in heterozygous animals (6). In contrast, mice lacking a functional Brm gene are viable but bigger, and derived fibroblasts present a deregulation of cellular growth control especially in response to UV irradiation (7). In human cancer, it is noteworthy that Brm and Brg1 are frequently down-regulated, silenced, or mutated in malignant cells, including cells from bladder, lung, and prostate tumors (8–11). Brm is also systematically down-regulated in rhabdoid tumors lacking SNF5/INI1/SMARCB1/BAF47, one of the subunits of the SWI/SNF complex (12). Consistent with these findings, reexpression of Brm or Brg1 in Brm/Brg1-deficient tumors leads to G1 growth arrest and flat cell formation through interactions with p53 family members (13–15). Brm is also present physically on cyclin D1 and cyclin A promoters upon cell cycle arrest, whereas Brg1 resides on cyclin E promoter in quiescent cells and on cyclin A promoter in proliferating cells (16, 17). Finally, we have shown that Brm controls cyclin A repression directly by modulating the positions of nucleosomes covering the promoter (18).

Despite the accumulated evidence for a role of Brm in cell cycle regulation, the absence of spontaneous tumor formation in Brm-deficient mice has led some authors to hypothesize that only Brg1 functions as a regulator of cell growth. To clarify this issue, we have here examined mouse fibroblasts lacking Brm in conditions of both serum starvation and stimulation. In these cells, we detect alterations in the expression of several genes regulating cell growth with consequences on all phases of the cell cycle, including an imperfect G0, a shorter S phase, and frequent accidents of chromosome segregation in anaphase. Dissection of the mechanism allowing the Brm−/− cells to arrest in a diploid stage upon serum starvation further revealed that these cells can to some extent compensate for the reduced SWI/SNF activity by the use of alternative pathways to control the cell cycle. Altogether, our data suggest that the redundancies in the cell control machinery are sufficient to overcome most consequences of the absence of Brm, but that all steps of the cycle become poorly controlled and less tightly regulated.

Materials and Methods

Cell culture and luciferase. 3T3 fibroblasts were issued from mouse embryonic fibroblast (MEF) cells using the classical 3T3 protocol. Cells were plated at 20,000 per 9-cm dish and grown in DMEM containing 10% heat-inactivated calf serum or left 48 hours in DMEM 0.1% serum to

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become quiescent. Luciferase experiments were done using the Dual Luciferase product (Promega, Madison, WI) according to the supplier’s protocol. Brm-expressing vectors (19) are transfected at 0, 0.5, 1, and 3 hours. Luciferase experiments were done using the Dual Luciferase system (Promega, Madison, WI) following the supplier’s protocol. The following primary antibodies were used: anti-cyclin A (C 4710, Sigma, St. Louis, MO), anti-cyclin D1 (556470, BD Biosciences), anti-cdc2 (C3085, Sigma), anti-cdk2 (sc-163, Santa Cruz Biotechnology), anti-PSTAIRE (P-7962, Sigma), anti-p21 (sc-397, Santa Cruz Biotechnology), anti-p25 (sc-528, Santa Cruz Biotechnology), anti-pRB (554136, BD Biosciences), anti-p107 (sc-318, Santa Cruz Biotechnology), and anti-p27 (sc-317, Santa Cruz Biotechnology).

**In vitro kinase assays.** Cell lysate (100 µg) was incubated for 2 hours at 4°C with 5 µL cyclin E antibody (C4976, Sigma) or 10 µL cyclin A antibody (sc-751, Santa Cruz Biotechnology) and then incubated for 1 hour at 4°C with 30 µL protein A-Sepharose beads. The immunoprecipitates were washed thrice with lysis buffer [100 mMol/L NaCl, 50 mMol/L Tris (pH 7.5), 10 mMol/L EDTA, 1% NP40, 2.5 mMol/L NaPi, 5 mMol/L NaF, 2.5 mMol/L β-glycerophosphate, 1 mMol/L DTT supplemented with protease inhibitors; Boehringer, Mannheim, Germany] and centrifuged at 15,000 × g for 15 minutes. Supernatant protein concentrations were determined using the Bio-Rad Bradford reagent (Richmond, CA). For Western blot analysis, 20 µg of cell extract were separated by SDS-PAGE (7%) or (12%) and transferred to nitrocellulose membranes. After blocking and incubating with antibodies, blots were finally subjected to enhanced chemiluminescence substrate (Lumi-Light, Roche, Indianapolis, IN or SuperSignal West Femto, Pierce, Rockford, IL) following the supplier’s protocol. The following primary antibodies were used: anti-cyclin A (C 4710, Sigma, St. Louis, MO), anti-cyclin B (sc-245, Santa Cruz Biotechnology, Santa Cruz, CA), anti-cyclin C (C4976, Sigma), anti-cyclin D1 (556470, BD Biosciences), anti-cdc2 (C3085, Sigma), anti-cdk2 (sc-163, Santa Cruz Biotechnology), anti-PSTAIRE (P-7962, Sigma), anti-p21 (sc-397, Santa Cruz Biotechnology), anti-p25 (sc-528, Santa Cruz Biotechnology), anti-pRB (554136, BD Biosciences), anti-p107 (sc-318, Santa Cruz Biotechnology), anti-p27 (sc-317, Santa Cruz Biotechnology), anti-c-Myc (9E10), anti-GAPDH (20), and anti β-actin. As secondary antibodies, we used anti-mouse IgG peroxidase conjugated (A9917, Sigma) and anti-rabbit IgG peroxidase conjugated (NA934, Amersham Biosciences, Piscataway, NJ). The signals were measured by ImageJ software and normalized with either GAPDH or β-actin signals.

**Immunostaining and flow cytometry.** Aphidicolin was used at 2.5 µmol/L and nocodazole at 50 µmol/L. The response to drugs was monitored by either the S-phase cells for aphidicolin or the G2 cells unlabeled with BrdUrd for nocodazole. In each case, the number of cells and background was determined using ImageJ software.
remains constant in these compartments. Cells were trypsinized, collected by centrifugation, washed once with PBS, and fixed in cold 70% ethanol overnight at -20°C, with vortexing for the first minute. After washing with PBS, cells were treated with 2 mol/L hydrochloric acid for 20 minutes; washed twice in 1 mol/L borax and once in PBS, 10% FCS, 0.5% Tween 20, and incubated for 1 hour with anti-BrdUrd antibody (5 μL, 347580; Becton Dickinson, Mountain View, CA). After two washes, cells were incubated for 1 hour at room temperature in the dark with FITC-conjugated goat anti-mouse IgG antibody (1:100, Jackson ImmunoResearch, West Grove, PA). All labeled cells were washed twice in PBS, 10% FCS, 0.5% Tween 20 and resuspended in staining solution (PBS, 20 μg/mL, Dna-se-free Rhase, 25 g/mL of propidium iodide). Cells were analyzed by flow cytometry on a Becton Dickinson FACScan with CellQuest software (Becton Dickinson).

**Immunofluorescence.** Cells were grown on glass coverslips, washed twice with PBS, and fixed with PBS containing 3.8% formaldehyde for 10 minutes. After washing with PBS, cells were permeabilized in 0.1% Triton X-100 for 5 minutes. After washing, cells were incubated for 1 hour at 37°C with anti-phospho-Ser10 histone H3 antibody (Cell Signaling, Beverly, MA; 9704, 1:50 in PBS/3% bovine serum albumin), washed in PBS, and incubated for 1 hour at 37°C with FITC-conjugated goat anti-rabbit IgG antibody (1:100; Jackson ImmunoResearch). After washing with PBS, coverslips were mounted with 90% glycerol, 10% PBS, 1 mg/mL p-phenylenediamine, and 100 μg/mL 4,6-diamidino-2-phenylindole (DAPI). Images were acquired on a DMRA microscope using ×40 lens. Digital images were recorded with a 12-bit C4795-NR CCD camera (Hamamatsu, Hamamatsu City, Japan).

**Time-lapse imaging.** Time-lapse epifluorescence microscopy was done on a Leica DM IRBE (Leica, Wetzlar, Germany) inverted microscope equipped with an automatic shutter and green fluorescent protein (GFP) filter sets, a ×40 objective, sample heater (37°C), and a home-made CO₂ incubation chamber. To minimize bleaching and phototoxicity, fluorescence illumination was supplied by a halogen bulb (100 W). Images were captured with a MicroMax 1300 charge-coupled device camera (Princeton Instruments, Roper Industries, Duluth, GA) driven by MetaMorph (version 4.11; Universal Imaging, Marlow, United Kingdom) imaging software, converted to TIF files that were edited with NIH Image software and compiled into QuickTime movies. The exposure time was fixed to 500 milliseconds.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation experiments were done as described in ref. 18. Primers: mP27.1, 5'-CACGTCGAGGAGGGTCTAGGCA-3'; mP27.2, 5'-TACACCTCCGAGTAGTC-3'; mMYC.1, 5'-AGTTGCGCGGAGGGCTC-3'; and m3MYC.1, 5'-TCAGCTCCCTCCTCCGCTCC-3'.

**Results**

**Modified accumulation of cell cycle regulators in brm+/− fibroblasts.** In an earlier study, we have shown that the SWI/SNF subunit Brm was required for repression of the cyclin A1 gene in growth-arrested cells (Fig. 1A and B; ref. 18). This observation prompted us to examine expression of several other cell cycle regulators in brm+/− cells (7) upon serum starvation and subsequent serum stimulation. 3T3 cells either wild type (WT) or lacking a functional Brm gene were cultured for 48 hours in medium containing 0.1% serum and stimulated by readdition of serum to 10% final concentration (Fig. 1A). Under these conditions, we observed decreased levels of the G1-specific cyclin D1 both in the serum-starved and stimulated cells. In addition, cyclin E1, which is activated downstream of cyclin D1 in WT cells, displayed an increased accumulation in the serum-starved brm+/− cells, whereas its expression was not increased upon serum stimulation. A modified pattern of expression of cyclin E1 was also observed using nonimmortalized MEFs (Fig. 1B). Consistent with a regulation of cyclin E and cyclin A by the SWI/SNF complex, we found that reexpression of a WT but not a mutant Brm in serum-starved immortalized brm+/− 3T3 fibroblasts leads to downregulation of reporter constructs carrying the promoters of either cyclin upstream of a luciferase gene (Fig. 1C). Later in the cell cycle, 18 hours after serum stimulation, expression of the G2/M-specific cyclin B1 was stimulated in a similar manner in both WT and mutant cells (Fig. 1A and B). We also examined the expression of members of the Rb family. We noted that the expression profile of pRb was similar to those of cyclin E1 and cyclin A2 and remains constant at all times in the Brm-deficient cells. Nevertheless, in}

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**Figure 2.** S phase is shortened in 3T3 brm+/− fibroblasts. A, DNA content and BrdUrd incorporation analysis by flow cytometry of brm+/− and brm−/− 3T3 cells. Hours indicate time serum addition. Bottom, percentages of cells in each phase of the cell cycle or asynchronous cells in exponential growth (Async.). Mean and SD values of four independent experiments. B, percentage of BrdUrd-positive cells after serum addition. Points, average of four independent experiments; bars, SD. C, diagram of the experimental procedure. D, S phase transit rate determined by the fate of the population in early S phase and BrdUrd positive, following nocodazole block. Points, average of four independent experiments; bars, SD. Lines are the respective linear regressions (R² > 0.98). Using the unpaired Student’s t test, P < 0.03 was obtained for comparison between brm+/+ and brm−/− 3T3 cells 4 hours after nocodazole block.
Expression in 3T3 fibroblasts and MEF cells upon serum starvation. Numbers input material (p27 and c-Myc promoters). PCR templates are chromatin immunoprecipitation bars, FSC G0-G1 MEF values correspond to the forward scatter and side scatter ratios between G0-G1 3T3 fibroblasts. Brm deletion confers increase of G0-G1 cell size and c-Myc relief of repression in quiescence. A, flow cytometry analysis of the size (forward scatter or FSC) and the granularity (side scatter or SSC) of synchronized brm+/+ and brm−/− cells. Columns, average of five independent experiments done in triplicate; bars, SD. Brm deletion confers increase of G0-G1 cell size and c-Myc relief of repression in quiescence. A, flow cytometry analysis of the size (forward scatter or FSC) and the granularity (side scatter or SSC) of synchronized brm+/+ and brm−/− cells. Columns, average of five independent experiments done in triplicate; bars, SD. C, quantitative reverse transcription-PCR analysis of Brm relative to GAPDH mRNA amounts in 3T3 cells treated with scrambled shRNA (3T3 ctrl) or by Brm shRNA (3T3 shRNA). Columns, mean of three independent experiments; bars, SD. Western blot analysis of c-Myc expression in 3T3 fibroblasts and MEF cells upon serum starvation. Numbers correspond to the signal intensity relative to β-actin. Hours indicate time after serum addition. E, chromatin immunoprecipitation analysis of Brm occurrence on p27 and c-Myc promoters. PCR templates are chromatin immunoprecipitation input material (Input), salmon sperm DNA (SS DNA), DNA trapped with anti-HABrm antibody (IPαBrm), or anti-mouse Ig antibody (IPγMAb). F, luciferase expression analysis of c-Myc promoter in 3T3 brm−/− cells upon serum starvation. Columns, mean of six different experiments carried out in triplicate; bars, SD.

Both WT and mutant cells, a gradual phosphorylation of pRb was observed during G1 progression, and the hyperphosphorylated species became detectable mainly 12 hours after serum addition. Rb p130, a marker for quiescence, was present at strongly increased levels in the serum-starved brm−/− cells. Levels of this protein then decreased, whereas the cells reentered the cycle, but they remained high compared with the WT cells. The Rb p107 protein that is present in cycling cells was, like p130, present at high levels in the serum-starved brm−/− cells and remained high after serum stimulation compared with the control cells. Finally, we examined the CDK inhibitor p27. Expression levels of this protein were similar in WT and mutant cells. Taken together, these observations show that inactivation of Brm leads to an extensive reprogramming of the expression of cell cycle regulators that can mainly be visualized in serum-starved cells.

Altered cell cycle control in brm−/− fibroblasts. To examine the consequences of the modified levels of cyclin A, cyclin E, pRb, p130, and p107 on the cell cycle, we next growth arrested WT and brm−/− mutant cells by serum starvation then measured DNA content and bromodeoxyuridine (BrdUrd) incorporation at different times after serum stimulation. After 48 hours of culture in medium containing 0.1% serum, 90% of the WT cells were in G0-G1. Under the same conditions, the brm−/− cells were clearly growth arrested with a decreased efficiency, as only 76% of these cells were diploid (Fig. 2A). The reduced effect of serum starvation on brm−/− 3T3 cells is in agreement with an earlier study showing that brm−/− MEFs show a reduced growth arrest when grown to high confluence (7). These observations suggest that the pathways required to reach growth arrest are maintained in the Brm-deficient cells but are less potent. Careful examination of cell reentry into the cell cycle after serum stimulation further showed that entry into the S phase was delayed by ~1 hour in the mutant cells (P < 0.01; Fig. 2B). In addition, we noted an increased accumulation of cells with a G2-M DNA content 18 hours after serum stimulation in the mutant compared with the WT cells (Fig. 2A, 29.95% versus 22.34%). These observations were compatible with a shorter and possibly less regulated S phase. To further investigate this issue, we followed the population of WT and brm−/− 3T3 cells that had incorporated BrdUrd (i.e., in S phase, >4 hours using flow cytometry). To prevent reentry into the cell cycle after completion of the S phase, we treated the cells with nocodazole, a drug arresting the cycle in mitosis (Fig. 2C). Under these conditions, we observed a faster reduction of S-phase brm−/− cells versus S-phase WT cells during the time of scrutiny (Fig. 2D, P < 0.03). These data confirm, to some extent, that the brm−/− cells transit through S phase more rapidly than the WT control cells.

Altered growth arrest in brm−/− cells. As shown above, serum starvation failed to efficiently growth arrest brm−/− cells. This prompted us to further examine the diploid brm−/− cells obtained after serum starvation. To this aim, we serum-starved 3T3 fibroblasts then compared the size and the internal complexity or granularity of both WT and Brm-mutant cells at different times following serum starvation using, respectively, the forward scatter and the side scatter profiles on the flow cytometer. In this assay, serum-starved brm−/− cells showed a significant increase in size and complexity compared with their wild-type counterparts. This difference could no longer be visualized 6 hours after the serum starvation (Fig. 3A). An increase in granularity was also observed upon serum starvation of WT 3T3 cells after shRNA knockdown of Brm (Fig. 3B and C), as well as in serum-starved brm−/− MEFs (Fig. 3B). In these cells, we also detected a minor but reproducible increase in size (Fig. 3B). These observations suggested that in the absence of Brm, serum-starved mouse fibroblasts do not enter a canonical G0 phase. To gain insight in the mechanism leading to continuous cell growth in conditions of serum starvation, we
examine expression of c-Myc. This proto-oncogene is a major factor affecting growth and cell size in response to serum conditions (21). Predictably, c-Myc was absent from the serum-starved WT 3T3 cells and became detectable only after serum stimulation. In contrast, 3T3 brm−/− cells expressed c-Myc in conditions of serum starvation and did not show induced expression upon serum addition (Fig. 3D). A 2-fold increase in the expression of c-Myc was also observed in serum-starved brm−/− MEFs compared with WT MEFs. Chromatin immunoprecipitation assays showed that Brm was present on the c-Myc but not on the control p27 promoter (Fig. 3E). In addition, reexpression of WT but not mutant Brm resulted in the repression of a reporter construct harboring a luciferase gene (Fig. 3F). These observations show that c-Myc is a direct target for Brm-mediated transcriptional repression in mouse fibroblasts and suggest that imperfect repression of the c-Myc gene in the absence of serum could contribute to increased cell size and complexity of cells lacking Brm.

Compensatory mechanisms of cell cycle control in the absence of Brm. The increased accumulation of diploid cell in response to serum starvation was in contrast to the high levels of cyclin A, cyclin E, and c-Myc detected in the serum-starved cells. We, therefore, investigated whether alternative pathways of cell cycle control were activated in cells lacking Brm. To this end, we first immunoprecipitated cyclin E and cyclin A from either WT or brm−/− 3T3 cells and measured the associated kinase activity in vitro. These experiments revealed that in brm−/− cells, although the levels of cyclin A and cyclin E expression were comparable in serum-starved and serum-stimulated cells, the associated kinase activity was significantly reduced upon serum starvation (Fig. 4A-C). This observation suggested that in the serum-starved cells lacking Brm, increased CDK inhibitor activity could compensate the increased accumulation of CDK complexes. Possibly, CDK inhibition may be provided by the increased accumulation of the Rb proteins p130 and p107 (Fig. 1A) that have been shown to target and poise cyclin A/cdk 2 and cyclin E/cdk2 complexes (22). In addition, we also questioned a possible increased efficiency of the CDK inhibitors p21 and p27 in cells lacking Brm and did immunoprecipitation assays to probe their association with cyclin E and cyclin A. The p27 protein was found to be abundantly associated with cyclin E in serum-starved brm−/− 3T3 cells. This association was not detectable in serum-starved WT 3T3 cells (Fig. 4B). We also observed a modest increase in association of p27 with cyclin A in the serum-starved brm−/− 3T3 cells (Fig. 4D). In contrast, we observed no or little variation in association of p21 with cyclin A and cyclin E (Fig. 4B-D). These observations showed that p27 can associate with cdk2 and cdc2, when cyclin E and cyclin A are abnormally expressed in absence of serum. To some extent, p27 may diminish the associated kinase activities and thus moderate the effect of cyclin E and cyclin A present in brm−/− cells starved from serum.

A longer G2–M phase in cells lacking Brm. To complete the characterization of the effect of Brm inactivation on the cell cycle, we carefully examined the WT and brm−/− 3T3 cells in mitosis. As described above, flow cytometry revealed that serum-stimulated brm−/− cells reached tetraploidy before the WT cells (Fig. 2A). Accelerated entry of the brm−/− 3T3 cells into the M phase could also be visualized using an anti-histone H3 phospho-Ser10 antibody as a mitosis marker (Fig. 5A). This experiment also revealed that phospho-Ser10-positive histone H3 signal was significantly detectable for 2 hours in the brm−/− cells but only for 1 hour in the WT cells. This observation suggested a longer M phase in the cells lacking Brm. To further investigate this issue, we used flow cytometry to follow cells reentering G1 after mitosis. To prevent the cells from initiating a new cycle, growth medium was supplemented with aphidicolin, which inhibits entry and transit through the S phase (Fig. 5B). Under these conditions, the G1-phase WT cell population grew significantly faster than the G1-phase brm−/− population (P < 0.01; Fig. 5C). This data indicates that

Figure 4. CDK activities in Brm-deficient cells. A, cyclin E–dependent kinase activity assessed by in vitro kinase assay in 3T3 fibroblasts. Time indicates hours after serum addition (0, 6, 12, and 18 hours). B, Western blot analysis of cyclin E–associated factors. C, cyclin A–dependent kinase activity settled by in vitro kinase assay in 3T3 fibroblasts. D, Western blot analysis of cyclin A–associated factors.
we use epifluorescence microscopy to examine WT and observed in the absence of Brm was linked to genomic instability, cyclin A (24). To determine whether the reduced kinetic of mitosis genomic instability has also been described in cells overexpressing another core subunit of the mammalian SWI/SNF complex results catastrophe.

*brm*~−/−~ cells exit less rapidly from G2 phase and mitosis compared with the WT cells.

**Inactivation of Brm increases occurrence of mitotic catastrophe.** Inactivation of the hSNFS/INI1 gene coding for another core subunit of the mammalian SWI/SNF complex results in polyploidization and chromosomal instability (23). In addition, genomic instability has also been described in cells overexpressing cyclin A (24). To determine whether the reduced kinetic of mitosis observed in the absence of Brm was linked to genomic instability, we use epifluorescence microscopy to examine WT and *brm*~−/−~ 3T3 cells stably expressing histone H2B fused to GFP. Observation of interphasic *brm*~−/−~ cells revealed the presence of frequent abnormalities in form of micronuclei, nucleus budding, and multinucleation (Fig. 6). Quantification showed that ~26% of *brm*~−/−~ 3T3 fibroblasts but only 1% of the WT cells presented nuclear abnormalities (*n = 200 for each type of cells; Fig. 6A-D). The fraction of cells with aberrant nuclei did not significantly vary with generational age of the cells (data not shown). Similar nuclear abnormalities were also observed in WT 3T3 cells after knockdown of Brm using siRNAs and staining of the DNA with DAPI (Fig. 6E and F). In this case, we observed defects in 19.4% of the *brm*~−/−~ and 5% of the *brm*~+/−~ MEFs (*n = 300; data not shown). We next followed cells through mitosis using time-lapse epifluorescence microscopy. Eleven of the 23 mitotic events analyzed exhibited abnormalities mainly during anaphase. After the separation of chromatids, we detected condensed DNA outside of the two pools of chromosomes (Fig. 7F and movies). Upon reformation of the nuclear envelope, the extra DNA seemed to generate either micronuclei or nuclear lobulation (Fig. 7H and I and movies). 3T3 fibroblasts depleted for Brm by shRNA knockdown showed similar figures. (Fig. 7J-L). Taken together, these data suggest a role for Brm and possibly for the SWI/SNF complex in the maintenance of the genomic integrity. This effect may be linked to the chromatin remodeling activity of the SWI/SNF complex possibly required for correct chromatid segregation. Alternatively, the SWI/SNF complex may act indirectly by controlling expression of cyclin A.

**Discussion**

We have shown here that mouse fibroblasts lacking Brm display defective regulation of several phases of the cell cycle. Upon serum starvation, the growth arrest is incomplete, and the cells have an increased size and maintain high expression levels for cyclin A and cyclin E and c-Myc. In these cells, reduction of the growth rate in the absence of the mitogenic factors present in the serum seems to rely on alternative control pathways that may include activated expression of p130 and p107 and increased association of the CDKI p27 with cyclin E and cyclin A. After serum stimulation, the cells reenter the cell cycle normally but then have a shorter S phase with a delayed onset. Cells are also affected in mitosis with a frequent loss of chromosomal material, suggesting a role for Brm in chromosome stability.

**Brm and cell growth.** In this study, Brm-deficient mouse fibroblasts showed a clear deregulation of multiple regulators of the cell cycle. The perturbations encountered in these cells
included elevated and poorly regulated levels of cyclin A, cyclin E, c-Myc, and p107 family members in absence of serum, and very little or no phosphorylation of p107 during reentry into the cell cycle. Remarkably, despite these perturbations, the cells can maintain a state resembling growth arrest upon serum starvation, and under normal growth conditions, they cycle almost as the wild-type cells with only modification of the duration and the time of onset of the different phases. Several studies have shown that cells can compensate for abnormal expression of cell cycle regulators by modulating the duration of the different phases (for a review, see ref. 25). For example, overexpression of the Drosophila p21/p27 homologues dacapo (dap) in the Wing imaginal disc increases the length of the G1 phase but has little effect on the size of the definitive body structure because the cells react by reducing the length of the S phase (26). This situation is very similar to that observed in the brm−/− cells that during growth arrest show elevated levels of CDK1 p27 activity and, upon reentry into the cycle, a longer G1 and a shorter S phase. It is here tempting to speculate that this compensation may, like in Drosophila, be achieved by affecting the expression of E2F family members. We also speculate that compensation mechanisms may be partially damaged in immortalized and in tumor cells, possibly explaining why the lack of Brm has more obvious consequences on the growth of 3T3s compared with the MEFs. This could also explain why brm−/− mice do no develop tumors, whereas the loss of Brm in cancer cells have been associated with poor prognosis and formation of metastasis (10).

Chromatin immunoprecipitation experiments from several laboratories have shown that cyclin A and cyclin E are direct targets of the SWI/SNF complex (16, 17). These studies further suggest that Brm and the sister protein Brg1 are present on the promoters at different times. Indeed, in proliferating cells, SWI/SNF complexes present on E2F target promoters seems to contain mainly Brg1, whereas Brm is present on these promoters in differentiating cells. In human fibroblasts arrested by serum starvation, E2F-4 and p130 are present at all E2F target promoters under scrutiny, including cyclin A (27, 28). Another study has also shown that, unlike wild-type cells, brm−/− cells can overcome overexpression of p130 (29). Taken together, these observations suggest that in nonproliferating cells, E2F-4/p130 repression may occur through Brm-containing SWI/SNF complexes and raise the possibility that Brm is necessary for p130-mediated cell cycle arrest. The higher levels of p130 in brm−/− cells also suggest a role for Brm in repressing expression of this gene, and a regulatory loop between Brm and E2F-4/p130 may exist. In this regard, we note that the p130 promoter harbors a motif similar to the cyclin A CCRE-CHR repressor sequence (30).

The serum-starved brm−/− cells have an increased size. This is compatible with an earlier study showing that expression of a negative transdominant Brg1 in NIH 3T3 cells resulted in increased size and modified cell shape (31). The increase in cell size may be linked to the higher expression of c-Myc that we observe upon serum starvation of brm−/− cells. The c-Myc protein is a well-characterized regulator of cell size, and its targets include several metabolic enzymes and ribosomal proteins. Possibly, increased expression of c-Myc may also contribute to the larger size of the brm−/− mice (7).

Upon serum starvation of the brm−/− cells, the main form of pRb was hypophosphorylated, but we also detected trace amounts of the hyperphosphorylated species. We cannot exclude that this particular pattern is due to the presence of a small population of cells still cycling despite the lack of serum. Another interesting possibility would be the presence of a cyclin D type–associated kinase activity in these serum-starved cells.

**SWI/SNF and cancer.** The apparently poorly controlled growth of cells lacking Brm may constitute a selective advantage for proliferation of cancer cells. However, Brm-deficient mice do not develop tumors, and it is generally admitted that inactivation or repression of the Brm gene is not a direct cause of cancer formation. We find that brm−/− cells show up-regulated expression of p107 and p130. Possibly, the presence of high levels of these proteins may partly protect the cells from transformation via Brm-independent mechanisms. Preliminary observations also suggest that brm−/− embryonic fibroblasts are able to enter cellular senescence (data not shown), indicating that control of proliferation is maintained in these cells at least to some extent. Further investigations will determine if the state of senescence reached by brm−/− cells resembles canonical senescence.

Cells lacking Brm show intriguing genomic instability. In Drosophila, complete degradation of cyclin A is necessary to correctly insure chromosome separation and the presence of nondegradable cyclin A, or overexpression of cyclin A leads to abnormal mitosis (24). We, therefore, speculate that the sustained expression of cyclin A in the brm−/− cells could be part of the mechanism preventing proper progression through mitosis. Alternatively, Brm may also be directly involved in the expression of members of the chromatin segregation machinery. Why the genomic instability does not lead to tumor formation in mice lacking Brm remains an open question. It has been reported that mice are less prone to develop epithelium tumors, unless p53 and telomerase have been inactivated (32). Possibly, p53 may participate in the protection against damage induced by decreased
SWI/SNF activity. Consistent with this, tumor formation in mice inactivated in the SNF5/INI1 gene encoding another subunit of SWI/SNF is drastically accelerated when the animals also carry a mutation of p53 (33).

Acknowledgments

Received 2/21/2005; revised 3/2/2006; accepted 3/8/2006.

References

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