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# The hbrm and BRG-1 proteins, components of the human SNF/SWI complex, are phosphorylated and excluded from the condensed chromosomes during mitosis

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**In yeast, the SNF/SWI complex is believed to regulate transcription by locally altering the chromatin structure. At the present time, three human homologues of yeast SNF/SWI proteins have been characterized: hbrm and BRG-1, homologues of SNF2/SWI2, and hSNF5, a homologue of SNF5. We show here that, during mitosis, hbrm and BRG-1 are phosphorylated and excluded from the condensed chromosomes. In this phase of the cell cycle, the level of hbrm protein is also strongly reduced, whereas the level of BRG-1 remains constant. The mitotic phosphorylation of hbrm and BRG-1 is found not to disrupt the association of these proteins with hSNF5 but correlates with a decreased affinity for the nuclear structure in early M phase. We suggest that chromosomal exclusion of the human SNF/SWI complex at the $G_2$–M transition could be part of the mechanism leading to transcriptional arrest during mitosis.**

*Keywords*: Brahma/chromatin/metaphase/SNF2/transcription

## Introduction

In *Saccharomyces cerevisiae*, the SNF/SWI complex, composed of at least 12 different subunits, is believed to activate transcription by facilitating the access of the transcriptional machinery to promoters occluded in interphase chromatin. The connection between the function of the SNF/SWI complex and chromatin has been established by genetic studies showing that mutations in several chromatin components, including histones H2A, H2B, H3 and H4, suppress the slow growth phenotype of *snf* or *swi* mutants. To date, a direct interaction of the SNF/SWI complex with chromatin has not been clearly established. However, *in vitro* studies have demonstrated that purified SNF/SWI complex is able to stimulate binding of a GAL4 derivative to reconstituted nucleosomal DNA (for review, see Winston and Carlson, 1992; Carlson and Laurent, 1994; Peterson and Tamkun, 1995).

Within the SNF/SWI complex, the SNF2/SWI2 protein is of particular interest. It contains a domain found in several DNA and RNA helicases and has been shown to harbour DNA-dependent ATPase activity (Laurent *et al.*, 1993). This protein is likely to play a key role in the chromatin remodelling activity of the complex. SNF2/SWI2 is highly conserved through evolution and it has allowed the identification of SNF/SWI-like complexes in several other species. In *Drosophila*, the homologue of SNF2/SWI2 is known as Brahma or brm. This protein, a member of the trithorax group, has been shown to regulate the expression of several homeotic genes, possibly by acting on heterochromatin-like structures formed by proteins members of the Polycomb group (Tamkun *et al.*, 1992; Dingwall *et al.*, 1995; Orlando and Paro, 1995; Peterson and Tamkun, 1995). In mammals, several proteins have been proposed as potential homologues of SNF2/SWI2 but, in most cases, sequence similarities are restricted to the helicase-type domain (Okabe *et al.*, 1992; Delmas *et al.*, 1993; Schoor *et al.*, 1993). Only two proteins, known as hbrm (or hSNF2α) and BRG-1 (or hSNF2β) in human and mbrm and mBRG-1 in mouse, show homology to SNF2/SWI2 outside of the potential helicase region (Khavari *et al.*, 1993; Muchardt and Yaniv, 1993; Chiba *et al.*, 1994). These two closely related proteins (75% identical and 86% similar at the amino acid level) are the best candidates for SNF2/SWI2 function in human cells.

The hbrm and BRG-1 proteins both have been shown to enhance transcriptional activation by the glucocorticoid and the retinoic acid receptors through a mechanism that may also involve the retinoblastoma Rb protein (Muchardt and Yaniv, 1993; Chiba *et al.*, 1994; Dunaief *et al.*, 1994; Singh *et al.*, 1995). However, the function of hbrm and BRG-1 may not be restricted to stimulation of nuclear receptor activity. Biochemical studies have shown that HeLa cells contain a 2 MDa complex detected by an anti-BRG-1 antibody. As in yeast, this complex that harbours DNA-dependent ATPase activity is able to alter the structure of *in vitro* reconstituted nucleosomes and can facilitate the binding of TATA binding protein (TBP) as well as several chimeric transcription factors to nucleosomal DNA (Imbalzano *et al.*, 1994; Kwon *et al.*, 1994). One subunit of this complex is the recently identified human homologue of yeast SNF5, known as INI1 or hSNF5 (Kalpana *et al.*, 1994; Muchardt *et al.*, 1995). Both hbrm and BRG-1 can interact with hSNF5, indicating that the two proteins have common molecular partners. However, the lack of direct interaction between hbrm and BRG-1 (C.Muchardt, unpublished observation) further suggests that at least two versions of the SNF/SWI complex may exist in mammalian cells, one containing hbrm and the other BRG-1.

Whereas the human SNF/SWI complex(es) is believed to activate transcription by locally decondensing the interphasic chromatin, it has been known for several decades that the condensation of the chromatin into metaphase chromosomes during mitosis is correlated with a global inhibition of transcriptional activity (Taylor, 1960; Prescott and Bender, 1962; Littau *et al.*, 1964; Johnson

and Holland, 1965). In this phase of the cell cycle, some transcription factors like AP-2 remain attached to the condensing chromosomes (Williams *et al.*, 1988). However, most transcriptional activators are excluded from their normal target sites although they retain their ability to bind DNA (Hershkovitz and Riggs, 1995; Martinez-Balbas *et al.*, 1995). To investigate a possible link between the activity of the human SNF/SWI proteins and the mitotic transcriptional shut-down phenomenon, we have used immunocytochemistry to follow the expression and subcellular localization of hbrm and BRG-1 at different phases of the cell cycle. In interphasic cells, the two proteins were nuclear and expressed at similar levels in all cells. During mitosis, both proteins were excluded from the condensed chromosomes and, in addition, hbrm was partially degraded. The mitotic exclusion of hbrm and BRG-1 could be correlated with phosphorylation of the two proteins. This phosphorylation did not modify their interaction with hSNF5 but appeared to disrupt their association with the nuclear structure before the breakdown of the nuclear envelope in prometaphase. This event is likely to trigger the inactivation of the SNF/SWI complexes during cell division.

## Results

### *Isolation of antibodies recognizing specifically either hbrm or BRG-1*

A prerequisite for our study was the possibility to follow hbrm and BRG-1 independently. The two previously described anti-hbrm and anti-BRG-1 antibodies (Khavari *et al.*, 1993; Muchardt and Yaniv, 1993) were tested on extracts from C33A cells transfected with either hbrm or BRG-1 expression vectors. The C33A cells previously have been shown to contain no endogenous hbrm and low levels of BRG-1 (Muchardt and Yaniv, 1993; Dunaief *et al.*, 1994). Each antibody recognized its anticipated target protein but also had significant cross-reactivity to the other protein (data not shown). We therefore raised new polyclonal antibodies against domains of hbrm and BRG-1 poorly conserved between the two proteins. After affinity purification, the specificity of these antibodies was tested on the above described transfected C33A cell extracts. Western blot analysis showed that the anti-hbrm antibody (α-hbrm) recognized the product of the cytomegalovirus (CMV)-hbrm but not of the simian virus (SV)-BRG-1 expression vector (Figure 1, lanes 2 and 3). In a similar way, the anti-BRG-1 antibody (α-BRG-1) was specific for the product of the SV-BRG-1 expression vector (Figure 1, lanes 5 and 6).

### *The level of hbrm but not BRG-1, is down-regulated during mitosis*

The above described antibodies were use to immunostain an asynchronous culture of MCF-7 cells. This breast cancer-derived cell line has been shown previously to contain easily detectable levels of endogenous hbrm (Muchardt and Yaniv, 1993). In this experiment, in which the cells were fixed with paraformaldehyde, hbrm- and BRG-1-specific immunofluorescence was detected in all cells. In interphasic cells, signals were strictly nuclear, distributed together with the cellular DNA visualized with DAPI. In mitotic cells, representing ~1–2% of the total

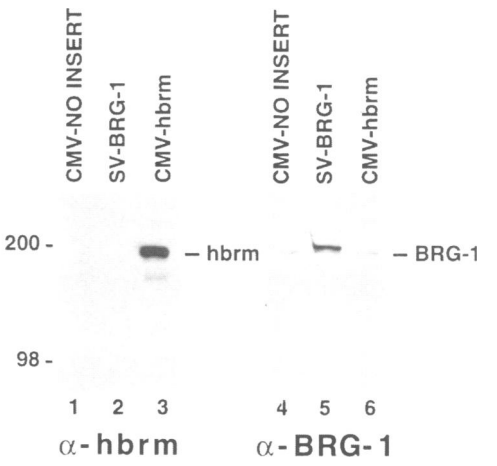


**Fig. 1.** Antibodies specific for hbrm and BRG-1. Extracts from C33A cells transfected with either pCG without insert (lanes 1 and 4), pSVhSNF2β (lanes 2 and 5) or CMV-hbrm/pCG (lanes 3 and 6) were resolved by 5% SDS–PAGE, transferred to nitrocellulose and analysed by immunoblotting with either the purified anti-hbrm (lanes 1–3) or anti-BRG-1 (lanes 4–6) antibodies.

population, the hbrm and BRG-1 signals were found to be excluded from the region containing the condensed chromosomes. When comparing the intensity of fluorescence from one cell to another, it appeared that BRG-1 was expressed at comparable levels in all the cells, whereas the level of hbrm was clearly reduced in mitotic cells (Figure 2A). A decreased hbrm signal was also observed in mitotic HeLa and SAOS-2 cells, indicating that the phenomenon is not cell type specific (data not shown).

To document further the decrease in hbrm levels during mitosis, we analysed extracts from synchronized HeLa cells by Western blotting. These cells are blocked readily in early metaphase with colchicine or nocodazole. Colchicine-arrested mitotic cells were separated from cells in interphase by shake-off, and total extracts from both populations were prepared in 8 M urea. This strong denaturing agent was used because both hbrm and BRG-1 are otherwise difficult to extract from interphasic cells. As shown in Figure 2B, the amount of hbrm protein contained in the mitotic cell extract was greatly reduced as compared with extracts from interphasic or exponentially growing asynchronous cells (lanes 1–3). In contrast, the level of BRG-1 did not vary significantly in any of the extracts (lanes 6–8). A similar result was obtained when cells were arrested with nocodazole (lanes 4, 5, 9 and 10) or just collected from an asynchronous population by shake-off (data not shown). Quantitative RT–PCR on RNA from colchicine-arrested mitotic cells or interphasic cells showed that the level of hbrm or BRG-1 mRNA was not significantly reduced in the mitotic cells (Figure 2C, lanes 1–8). These experiments indicate that the hbrm protein is down-regulated during mitosis, possibly by translational arrest or proteolysis.

### *hbrm and BRG-1 are phosphorylated during mitosis*

In the previous experiment, we also noted that the species detected by either α-hbrm or α-BRG-1 antibodies in the mitotic extracts migrated more slowly than the protein species detected in interphasic extracts, suggesting a

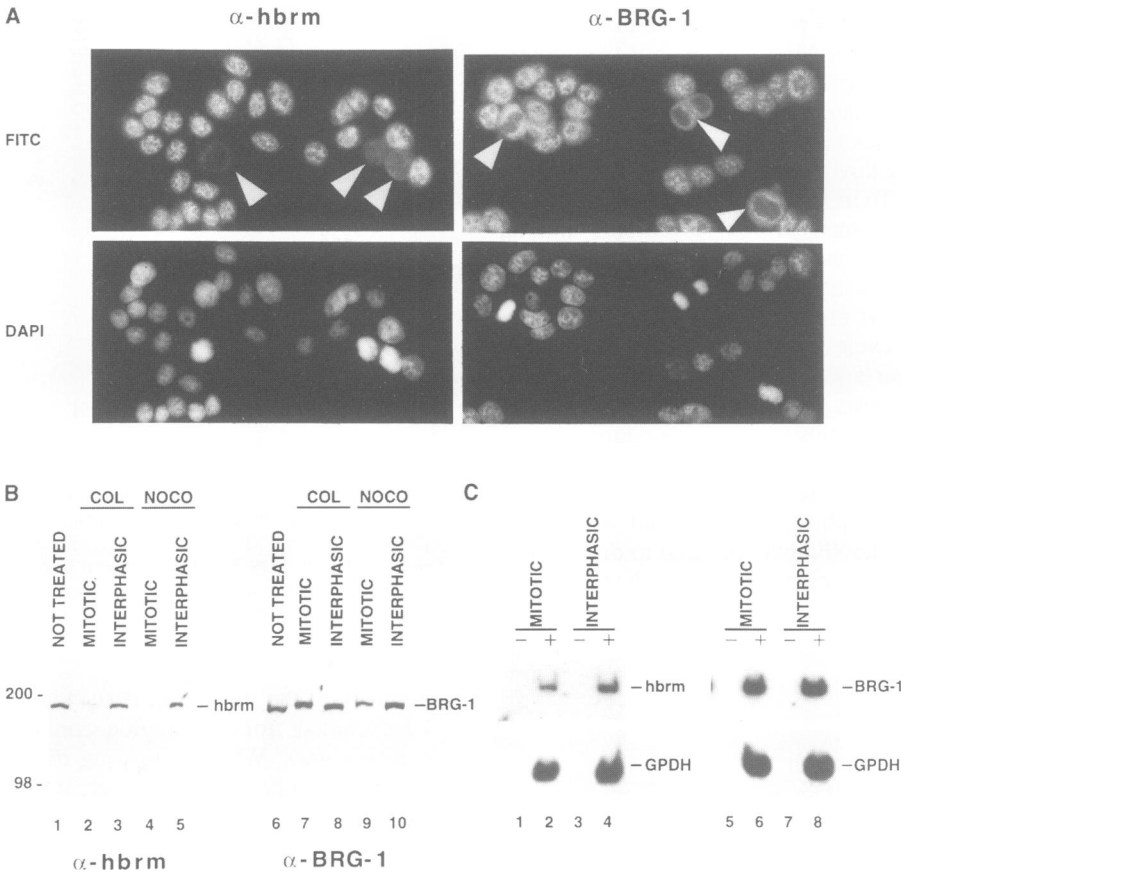


**Fig. 2.** Expression level of hbrm and BRG-1 in mitotic cells. (**A**) MCF-7 cells were fixed with paraformaldehyde and processed as described in Materials and methods, then incubated with either anti-hbrm (top left panel) or anti-BRG-1 (top right panel) antibodies. Cellular DNA was stained with DAPI (bottom panels). Mitotic cells are indicated with an arrow. (**B**) HeLa cells were treated with either colchicine (lanes 2, 3, 7 and 8) or nocodazole (lanes 4, 5, 9 and 10) for 12 h and mitotic cells (lanes 2, 4, 7 and 9) were separated from cells in interphase (lanes 3, 5, 8 and 10) by vigorous shaking. Extracts from both populations as well as extracts from cells grown in the absence of any metaphase-arresting drug (lanes 1 and 6) were analysed by Western blot using either anti-hbrm (lanes 1–5) or anti-BRG-1 (lanes 6–10) antibodies. (**C**) HeLa cells were treated with colchicine and the mitotic floating cells were separated from the adherent interphasic cells. Total RNA was prepared from both populations, and cDNA was synthesized either in the absence (lanes 1, 3, 5 and 7) or the presence (lanes 2, 4, 6 and 8) of reverse transcriptase. The products of these reactions were then amplified by PCR for 13 cycles with either hbrm- (lanes 1–4) or BRG-1- (lanes 5–8) specific primers. Each PCR sample also contained GPDH-specific primers as an internal control of the reaction.

potential post-translational modification of the two proteins during mitosis. Examination of the amino acid sequence of the two proteins showed several putative phosphorylation sites for proline-directed kinases (a serine or a threonine followed by a proline) including a TPXK sequence that is well phosphorylated by the $p34^{cdc2}$ kinase (Pearson and Kemp, 1991; Songyang *et al.*, 1994). To determine if the two proteins were indeed subject to phosphorylation, HeLa cells were treated with colchicine and mitotic cells were collected as described above. Cells were lysed and sonicated in a non-denaturing buffer containing magnesium (IP0.1 buffer, see Materials and methods) and debris was removed by centrifugation. Extracts obtained with this protocol were considerably enriched with respect to both hbrm and BRG-1, facilitating the detection of the mitotic isoform of hbrm. However, with these extracts, quantification was not possible because the slower migrating isoform of hbrm and BRG-1 was extracted preferentially under those mild conditions. Western blot analysis of the extracts after separation on high resolution SDS–PAGE confirmed that the mobility of both hbrm and BRG-1 was slower in mitotic extracts than in interphasic extracts (Figure 3A and B, lanes 1 and

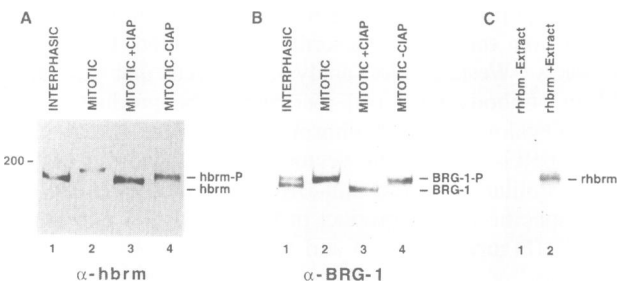


**Fig. 3.** hbrm and BRG-1 are phosphorylated during mitosis. (**A** and **B**) Extracts from colchicine-arrested HeLa cells were incubated at 37°C for 1 h either in the presence (lane 3) or the absence (lane 4) of 20 U of CIAP. These extracts, as well as extracts from mitotic cells (lane 2) or cells in interphase (lane 1) not incubated at 37°C, were analysed by immunoblotting using either anti-hbrm (A) or anti-BRG-1 (B) antibodies. The phosphorylated form of hbrm and BRG-1 are indicated respectively by hbrm-P and BRG-1-P. In (A), the total amount of protein loaded in each lane is adjusted so that hbrm can be detected in all extracts. (**C**) Recombinant HA-hbrm (rhbrm) was incubated with [$\gamma$-$^{32}$P]ATP either in the absence (lane 1) or the presence (lane 2) of HeLa cell extract, immunoprecipitated with anti-HA antibody and resolved by SDS–PAGE. The dried gel was autoradiographed.

2). Alkaline phosphatase treatment shifted the migration of the mitotic hbrm and BRG-1 proteins to that of their interphasic counterparts (Figure 3A and B, lanes 1 and 3), while control incubation without phosphatase had no effect on the mobility of the proteins (Figure 3A and B, lane 4). These results argue strongly that the mobility shift we observed was due to changes in the phosphorylation pattern of the two proteins during mitosis. To confirm that hbrm could be modified by phosphorylation, we incubated recombinant baculovirus-produced haemagglutinin (HA)-tagged hbrm in the presence of total HeLa extract and [$\gamma$-$^{32}$P]ATP. After immunoprecipitation with anti-HA monoclonal antibody, the product of the reaction was analysed by SDS–PAGE. Autoradiography confirmed that the recombinant hbrm protein could be phosphorylated by HeLa extract (Figure 3C, lane 2).

### *Appearance of hypophosphorylated hbrm and BRG-1 proteins correlates with cell entry into $G_1$ phase*

To estimate the time of hbrm and BRG-1 dephosphorylation *in vivo* and to follow the *in situ* movements of the two proteins during cell division, we synchronized HeLa cells as previously using nocodazole. The metaphase block was then released by removing the nocodazole, and samples were collected every hour. As expected from the previous experiments, Western blot analysis of cell extracts in 8 M urea (solubilizing all the hbrm and BRG-1) showed a steady increase in the level of hbrm, whereas the level of BRG-1 remained constant (Figure 4A and D, lanes 2–6). Western blot analysis of cell extracts in IP0.1 (enrichment in hbrm and BRG-1 but no quantitative extraction) showed that hbrm recovered its faster mobility between 1 and 3 h after the release. Appearance of a faster migrating form of BRG-1 occurred at the same time as for hbrm but the slow form of BRG-1 could still be detected up to 5 h after the release (Figure 4B and E, lanes 3–7). Aliquots of the same cultures were also fixed with ethanol and acetic acid, stained with $\alpha$-hbrm or $\alpha$-BRG-1 antibody and propidium iodide and examined by confocal microscopy (Figure 4C and F). For most cells, cytokinesis occurred ~60 min after the release. In dividing cells, the BRG-1 signal was distributed in the entire cytoplasm and excluded from the condensed chromosomes. The low residual hbrm signal showed a similar pattern with additional accumulation in the interzone during mid-anaphase. After 2 h, >90% of the cells had re-entered the $G_1$ phase. At this time, both hbrm and BRG-1 had re-localized to the newly reformed nucleus. Three hours after the release, the signal distribution was unchanged but the level of the hbrm signal was increased. This series of experiments suggests a correlation between the reappearance of dephosphorylated hbrm and BRG-1 species and entry of the cell into $G_1$ phase. In Figure 4, where ethanol/acetic acid was used for fixation, the nuclear hbrm and BRG-1 signals in the interphasic cells were not homogenous. Particulate structures were visible in addition to the evenly distributed signal observed with paraformaldehyde fixation. This granulated aspect was enhanced inadvertently by the confocal microscope image filtering. The pattern of mitotic exclusion was observed with both paraformaldehyde- and ethanol/acetic acid-fixed cells.

### *Phosphorylation of hbrm and BRG-1 does not disrupt their interaction with the human homologue of SNF5*

Using an anti-hSNF5 antibody for immunofluorescent staining of HeLa cells, we have observed that, like hbrm and BRG-1, hSNF5 is excluded from the condensed chromosomes during mitosis (data not shown), suggesting that this protein remains associated with hbrm and BRG-1 during this phase of the cell cycle. To investigate this issue, we used co-immunoprecipitation assays to examine the effect of phosphorylation of hbrm and BRG-1 on their interaction with hSNF5. For this experiment, we constructed a HeLa-derived cell line expressing an HA-tagged version of hSNF5 (H28 cell line). HA-hSNF5 and its potential partners were then immunopurified using an HA-specific monoclonal antibody. Gel filtration showed that the immunopurified HA-hSNF5 eluted as a 2 MDa complex (data not shown), suggesting that this protein remained associated with the other members of the human SNF/SWI complex. Indeed, Western blot analysis of the immunopurified fraction containing HA-hSNF5 allowed us to detect both hbrm and BRG-1 (Figure 5A, lanes 4 and 5). Fractions immunopurified from HeLa cells not expressing HA-hSNF5, using the same monoclonal antibody, did not contain hbrm and BRG-1 (Figure 5B and C, lane 7). The H28 cells were then treated with colchicine and extracts were prepared from either the mitotic cells isolated by shake-off, interphasic cells remaining attached to the plate or untreated H28 cells. Immunopurifications were then performed as above, and the eluted fractions were separated by SDS–PAGE and analysed by Western blot with either $\alpha$-hbrm or $\alpha$-BRG-1 antibodies. As shown in Figure 5B and C, both the dephosphorylated and the phosphorylated forms of hbrm and BRG-1 could be co-immunopurified with HA-hSNF5 (compare lanes 4, 5 and 6 in each panel), indicating that phosphorylation of hbrm and BRG-1 does not disrupt their interaction with hSNF5. The possibility that phosphorylation of the two proteins disrupts contacts with other subunits of the complex cannot be excluded presently.

### *Phosphorylation modifies the extractability of hbrm and BRG-1*

The hbrm and BRG-1 proteins are believed to activate transcription by acting at the level of chromatin. However, direct binding of these proteins to free DNA or to nucleosomal templates has not been demonstrated at this point. We therefore investigated whether hbrm and BRG-1 might be associated with the nuclear structure and whether phosphorylation could disrupt this association. It previously has been described that permeabilization of cells with the non-ionic detergent Nonidet P40 (NP-40) will leave in place transcription factors bound to chromatin while other cellular proteins will be solubilized (Martinez-Balbas *et al.*, 1995). To determine the effect of non-ionic detergent on BRG-1, HeLa cells were pre-treated with a hypertonic buffer in the absence or presence of NP-40 prior to fixation with paraformaldehyde. Immunofluorescent staining of these cells, using the $\alpha$-BRG-1 antibody, showed that BRG-1 resisted extraction in interphasic but not in mitotic cells (Figure 6A). This observation suggests that, in interphasic cells, BRG-1 is bound to the nuclear structure and possibly to the chromatin, and that this

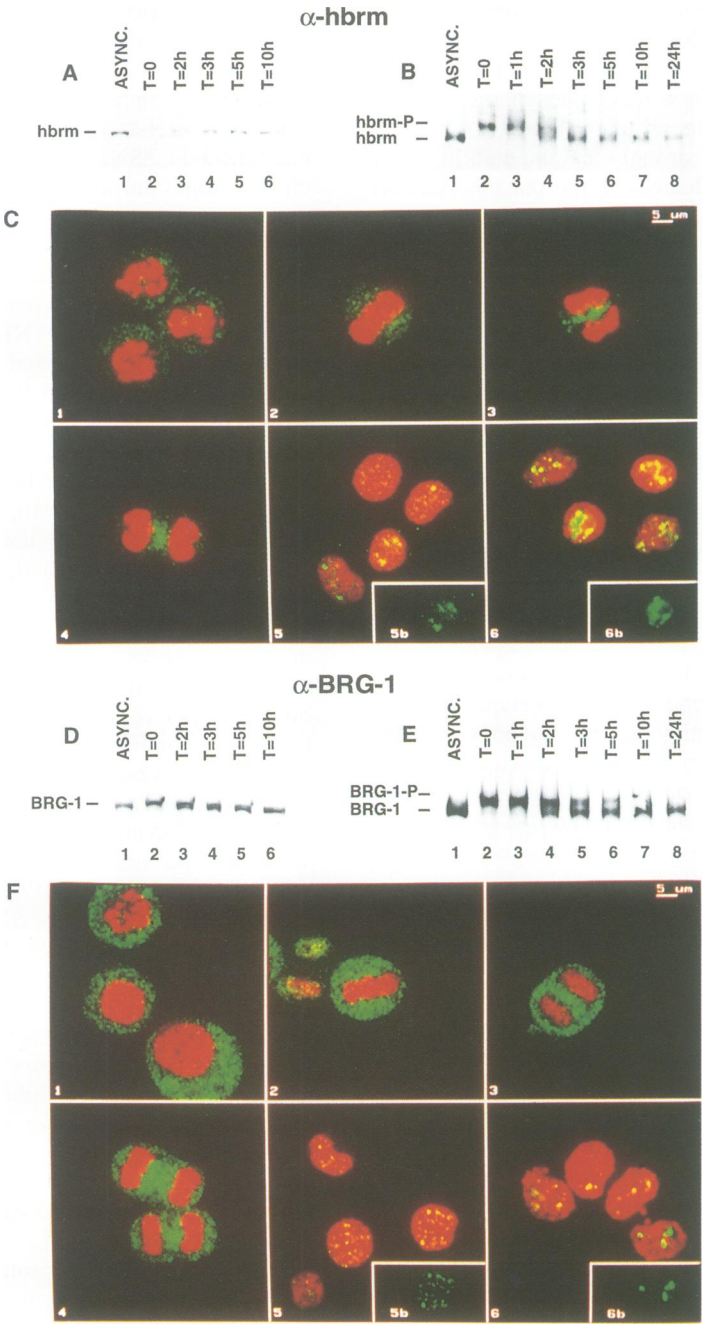


**Fig. 4.** Kinetics of hbrm and BRG-1 dephosphorylation. HeLa cells synchronized in metaphase by incubation for 12 h in the presence of nocodazole were allowed to re-enter the cell cycle by removing the nocodazole and replating on new Petri dishes, some of which contained a glass coverslip. Every hour, the content of two Petri dishes was used to make an extract in either Buffer B or IP0.1. In addition, a coverslip was fixed with ethanol and acetic acid, then stored in PBS. After 24 h, extracts were analysed by Western blotting and coverslips were used for immunofluorescent staining with either the anti-hbrm (**A**, **B** and **C**) or the anti-BRG-1 (**D**, **E** and **F**) antibodies. (A) and (D) show buffer B extracts and (B) and (E) show IP0.1 extracts at relevant time points. (To make the Westerns directly comparable, the same membranes were used in A and D, and in B and E; in B and E, the total amount of protein loaded in each lane is adjusted so that hbrm can be detected in all extracts.) Lane 1 shows extract from cells before the addition of nocodazole (ASYNC). (C) and (F) show confocal microscope images from cells fixed just after the removal of the nocodazole (picture 1) or after 60 min (pictures 2, 3 and 4), 120 min (picture 5) and 180 min (picture 6). The red colour represents the cellular DNA, the green colour either hbrm (C) or BRG-1 (F). In pictures 5B and 6B, one of the cells is represented with just the green coloration. Colours are artificial and intensities have been adjusted for maximum contrast.

binding is relaxed during mitosis. Like BRG-1, hbrm was also found to resist detergent extraction in interphasic cells. However, we could not monitor the extractability of this protein in mitotic cells because of the low intensity of the fluorescence signal.

We showed previously that a phosphorylated form of BRG-1 could still be detected after the nuclear envelope had reformed at the beginning of the $G_1$ phase of the cell cycle (Figure 4). Simultaneous detection of phosphorylated and dephosphorylated forms of BRG-1 was also possible in cells remaining attached to the plate after treatment with nocodazole or colchicine and shake-off (see, for

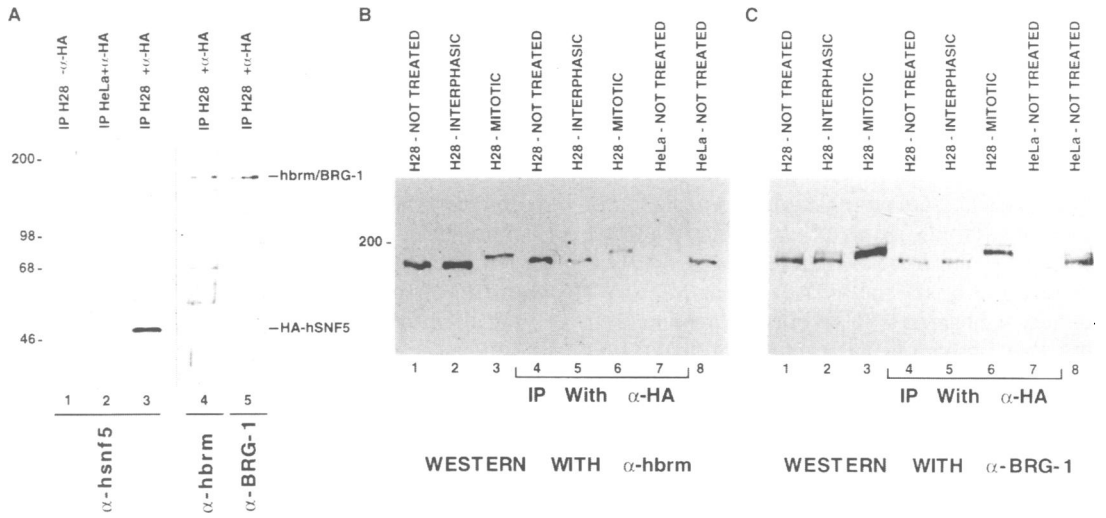


**Fig. 5.** Phosphorylated hbrm and BRG-1 proteins are associated with hSNF5. (**A**) HeLa cells (lane 2) or H28 cells (lanes 1, 3, 4 and 5) were lysed in IP0.1 and immunoprecipitated either in the absence (lane 1) or the presence (lanes 2–5) of anti-HA antibody. After analysis by 9% PAGE, proteins were transferred to nitrocellulose membrane and analysed by Western blot with either α-hSNF5 (lanes 1–3), α-hbrm (lane 4) or α-BRG-1 (lane 5) antibody. (**B** and **C**) H28 cells were treated overnight with colchicine and mitotic cells were separated from interphasic cells by shake-off. Extracts from each population were used for immunoprecipitation as in (A) (lanes 5 and 6). Extracts from either untreated HeLa or H28 cells were also used for immunoprecipitation as a control (lanes 4 and 7). Aliquots of the initial extracts are shown as size markers (lanes 1, 2, 3 and 8). Samples were analysed by 5% PAGE followed by Western blot with either α-hbrm (B) or α-BRG-1 (C) antibody.

example, Figure 3B, lane 1). More than 97% of these cells were found by phase contrast microscopy to contain an intact nuclear envelope. However, flow cytometry analysis of the same population showed that it was composed of a higher proportion of cells with a 4 N chromosomal content as compared with non-treated HeLa cells. We concluded from these observations that the population was enriched in cells in $G_2$ or in prophase. We then used the same cell population to perform NP-40 fractionation. Extracts from cells treated with NP-40 contained exclusively the phosphorylated form of BRG-1 (Figure 6B, lane 3). Only further treatment of the cell debris with IP0.1 buffer and sonication allowed the extraction of dephosphorylated BRG-1 (Figure 6B, lane 4). This experiment shows that, in phases of the cell cycle where the cells have an intact nuclear envelope and contain both phosphorylated and dephosphorylated BRG-1, only dephosphorylated BRG-1 is strongly attached to the nuclear structure.

## Discussion

### *hbrm and BRG-1 are attached to the nuclear structure in interphase but are delocalized during mitosis*

Genetic and biochemical studies have defined the yeast SNF2/SWI2 and *Drosophila* Brahma proteins as components of a complex machinery that regulates transcription by modifying the structure of chromatin in the vicinity of promoters. Two homologues of SNF2/SWI2 and Brahma are present in human cells: hbrm and BRG-1. We have observed that these two proteins have a nuclear localization during interphase but are excluded from the condensed chromosomes during mitosis. The chromosomal exclusion of hbrm and BRG-1 during mitosis is concurrent with phosphorylation of the two proteins. This modification does not disrupt their interaction with their common partner hSNF5, suggesting that phosphorylation

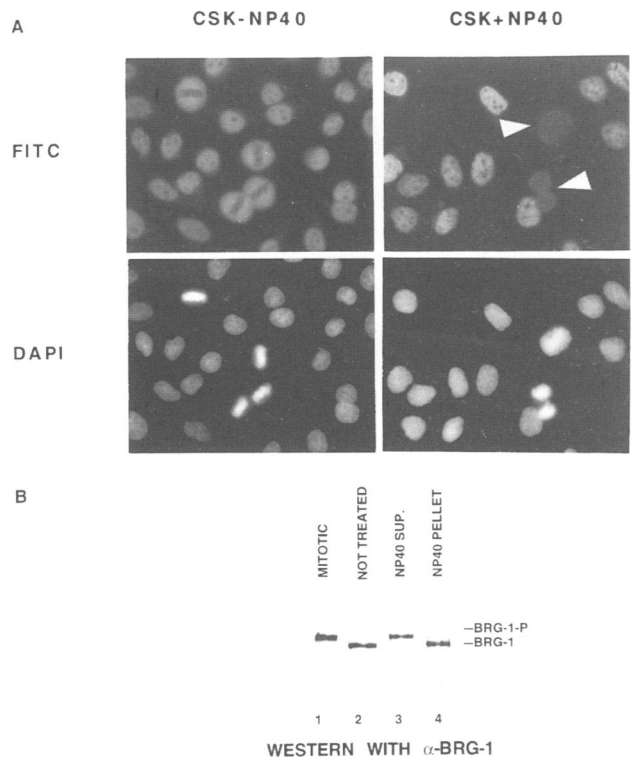


**Fig. 6.** Phosphorylation of hbrm and BRG-1 leads to increased extractability. (**A**) HeLa cells were incubated in CSK buffer either in the absence or presence of NP-40 before fixation with paraformaldehyde and indirect immunofluorescent staining with α-BRG-1 antibody (top panel). Cellular DNA was stained with DAPI (bottom panel). Mitotic cells are indicated with an arrow. (**B**) Cells incubated overnight with nocodazole were washed vigorously in PBS then extracted first with a buffer containing NP-40 (lane 3) then with IP0.1 and sonication (lane 4). Samples were analysed by 5% PAGE and Western blot with α-BRG-1 antibody. Extracts from mitotic cells or asynchronous cells are shown as reference for phosphorylated and dephosphorylated protein (lanes 1 and 2).

does not separate hbrm and BRG-1 from the human SNF/SWI complex. On the other hand, reappearance of hypophosphorylated forms of hbrm and BRG-1 coincides with the re-assembly of the nuclear envelope and nuclear re-localization of the two proteins. In addition, in the phases of the cell cycle where both phosphorylated and dephosphorylated BRG-1 are present simultaneously (early M, early $G_1$ and possibly $G_2$), only phosphorylated BRG-1 can be extracted by non-ionic detergent. These observations suggest that, during the interphase, human SNF/SWI complex, containing hbrm or BRG-1, interacts with nuclear elements and that, upon phosphorylation at the $G_2$–M transition, this interaction is disrupted. In several other cases, phosphorylation modulates the binding of regulatory proteins to nuclear subcompartments. For example, phosphorylation of the tumour suppressor retinoblastoma (Rb) at the $G_1$–S transition will disrupt its association with the nuclear matrix (Mittnacht and Weinberg, 1991; Templeton, 1992; Durfee *et al.*, 1994). In parallel, some transcription factors like Myb and Oct-1 are regulated by mitotic phosphorylation, resulting in decreased DNA affinity (Segil *et al.*, 1991; Luscher and Eisenman, 1992).

### *The hbrm and BRG-1 proteins may have different functions*

The hbrm and the BRG-1 proteins behave very similarly with respect to mitotic phosphorylation and exclusion from the metaphase chromosomes. However, we observed that the level of hbrm was decreased significantly during mitosis and that the protein was resynthesized rapidly in a dephosphorylated form at the $G_1$ phase entry. On the other hand, the level of BRG-1 remained constant throughout the cell cycle, and phosphorylated and dephosphorylated BRG-1 protein co-existed during part of the $G_1$ phase. It is likely that these differences in the regulation of the two proteins reflect variations in their function and possibly in their respective cellular targets. The phosphorylated BRG-1 species present during the $G_1$ phase may form a pool of rapidly available protein gradually distributed to target sites when required. Similarly, the degradation of hbrm may be a way of controlling the return of this protein to its cognate promoters and it is possible that the resynthesis of hbrm only occurs when some required conditions are fulfilled. Alternatively, the degradation of hbrm may create a subpopulation of incomplete SNF/SWI complexes that can be associated either with hbrm or BRG-1 when the cells re-enter the $G_1$ phase.

The origin of the mitotic instability of hbrm is still unclear, since this protein does not contain any obvious peptide sequence previously associated with directed protein degradation. However, we have described earlier an hbrm deletion mutant that lacked the carboxy-terminal end of the protein and that was up to 2-fold more active than the wild-type protein. The increased activity of this mutant appeared to be due to higher stability of the protein (Muchardt and Yaniv, 1993). The region deleted in the stable mutant contained the highly conserved bromodomain but was missing two short sequences exclusively present in BRG-1. These sequences may be required for increased stability of BRG-1. Domain swapping between hbrm and BRG-1 should allow us to address this question.

### *A role for the human SNF/SWI complex in the regulation of active chromatin structure*

Our observations suggest that the human SNF/SWI complex is inactivated prior to chromosome condensation in mitosis, then re-targeted to the chromatin after completion of cytokinesis. It is possible that the SNF/SWI complex creates open chromatin structures in early $G_1$. At this time, the complex may facilitate the expression of specific genes by allowing the access of DNA binding transcriptional activators. At the entry of M phase, the deprogramming of the open structures may be necessary for correct condensation of the chromosomes and some transcription factors may no longer be able to reach their cognate DNA sites. The exclusion of these transcription factors could be part of the mechanism leading to transcriptional arrest during mitosis.

The hbrm and BRG-1 proteins so far have been defined as enzymatic subunits, possibly containing the actual chromatin remodelling activity of the SNF/SWI complex. However, the mitotic modification of the two proteins that is likely to affect the activity of the entire SNF/SWI complex suggests they may also serve as regulatory subunits, only activated or synthesized when an active SNF/SWI complex is required. After this manuscript was submitted for publication, a study by Wilson *et al.* (1996) revealed that, in yeast, the SNF/SWI complex is an integral component of an RNA polymerase II holoenzyme. If this observation can be extrapolated to higher eukaryotes, it raises the interesting possibility that the mitotic modification of hbrm and BRG-1 directly modulates the activity of the human RNA polymerase II.

## Materials and methods

#### *Preparation of anti-hbrm and anti-BRG-1 antibodies*

To produce the hbrm-specific polyclonal antibody (α-hbrm), we inserted a fragment of the mouse mbrm cDNA in pGEX2T in-frame with GST. The fragment of mouse cDNA encoded amino acids 48–214 in the corresponding human sequence. The mouse and the human amino acid sequences are 92% identical in this region. The fusion protein was expressed in *Escherichia coli* and affinity purified on glutathione–Sepharose 4B under conditions recommended by the manufacturer (Pharmacia). The polyclonal BRG-1-specific antibody (α-BRG-1) was produced in a similar way using a fragment from the mouse mBRG-1 cDNA encoding a sequence corresponding to amino acids 39–333 in the human BRG-1. In this region, the homology between mouse and human BRG-1 is 92%. The antibodies were affinity purified using their respective antigens.

#### *Cell culture and synchronization*

HeLa and MCF-7 were grown at 37°C in 7% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 7% fetal calf serum (FCS). For HeLa cell synchronization, one confluent 100 mm dish was passed into a 150 $cm^2$ canted neck flask, then allowed to grow for an additional 24 h. Nocodazole (Sigma) was then added to a final concentration of 0.1 μg/ml. After 12 h, mitotic cells were collected by replacing the medium by Hank's balanced salt solution (HBSS; Gibco) and shaking the flask vigorously. The cells were then washed twice in HBSS, resuspended in pre-warmed fresh medium and replated in a 60 mm dish for extraction with Buffer B (8 M urea, 0.1 M $NaH_2PO_4$, 0.01 M Tris, pH 8) or IP0.1 [20 mM HEPES pH 7.6, 10% glycerol, 25 mM $MgCl_2$, 0.1 mM EDTA, 0.2% NP-40, 0.1 M potassium acetate, 2.25 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM dithiothreitol (DTT)] and in a 35 mm dish containing a poly-L-lysine-coated coverslips for immunofluorescence microscopy. By observation of the DNA after DAPI staining, 95–98% of the collected cells were found to be blocked in metaphase. For collection of interphase cells, cells still attached to the flask were washed twice in phosphate-buffered saline (PBS) with

vigorous shaking and then collected in appropriate buffer. Observation of these cells after DAPI staining showed that 98% of them did not contain condensed chromosomes. When cells were synchronized with colchicine, a similar protocol was used with a final colchicine concentration of 100 μg/ml. Cells treated with colchicine did not re-enter the cell cycle when the drug was removed.

### *Cell extracts and immunoblot analysis*

For quantitative analysis, cells were lysed in 200 μl of Buffer B. For hbrm-enriched extracts, cells were lysed in IP0.1 then sonicated twice for 5 min with vigorous vortexing after each sonication. The extracts were finally cleared by centrifugation. Protein concentrations were determined using the Bio-Rad Bradford reagent. For Western blot analysis, 10 μg of extract was fractionated by SDS–PAGE and transferred to nitrocellulose membranes. The membrane was then blocked with PBS/0.2% Tween 20/10% horse serum and incubated with the various antibodies. Enhanced chemiluminescence (ECL) reagents (Amersham) were used for detection. HA-tagged hbrm was detected using the 12CA5 monoclonal mouse antibody.

### *RNA preparation and RT-PCR*

Total RNA was purified on a CsCl cushion as previously described (Sambrook *et al.*, 1989). Two μg of this RNA was then used for cDNA synthesis using 200 ng of random hexamer primer and 200 U of Moloney murine leukaemia virus reverse transcriptase (RT) in 20 μl under conditions recommended by the RT manufacturer (BRL). A negative control was made under the same conditions but without RT. One-tenth of the reaction or the negative control was amplified for 13 cycles in a two-step PCR (1 min at 95°C and 1 min at 60°C) using *Taq* polymerase from Promega and hbrm-, BRG-1- or GPDH-specific primers (total volume 100 μl). Preliminary experiments have shown that the PCRs were in a linear range between 10 and 15 cycles of amplification. Five μl of the PCR product was resolved on a 10% polyacrylamide gel and analysed by Southern blotting using a *Bam*HI–*Sac*I restriction fragment from the hbrm cDNA and an *Asp*718–*Sac*I fragment from the BRG-1 cDNA (nucleotides 274–548 and 245–599 of the respective published sequences). Primer sequences were: hbrm sense, gccaagtcctggacctccaagtgtc; hbrm reverse, gccaggcaaggtcctttt; BRG-1 sense, cacccaggggcctggagg; and BRG-1 reverse, cctgttgcggacaccgag.

### *Fluorescence microscopy*

MCF-7 cells, grown on coverslips, were fixed in 3% freshly prepared paraformaldehyde in PBS for 10 min. To enhance exposure of hidden epitopes, cells were treated with 6 M guanidine hydrochloride as described previously (Peränen *et al.*, 1993). To determine chromatin affinity, HeLa cells were incubated in ice-cold CSK (PIPES pH 6.8 10 mM, NaCl 100 mM, sucrose 300 mM, $MgCl_2$ 3 mM, EGTA 1 mM) with or without 0.3% NP-40 for 3 min and then fixed in 3.2% paraformaldehyde in CSK for 10 min (Weis *et al.*, 1994; Martinez-Balbas *et al.*, 1995). For confocal microscopy, HeLa cells were fixed with 95% ethanol and 5% acetic acid at –20°C for 10 min, washed twice in PBS then once in PBS/0.05% Tween-20. In all cases, the coverslips were then incubated overnight with either the α-hbrm or the α-BRG-1 antibody in PBS/10% horse serum/0.05% Tween-20. A fluorescein-linked anti-rabbit antibody from Amersham was used for detection, the cellular DNA was labelled with 0.05% DAPI or propidium iodide. The preparations were observed at 63× magnification with a ZEISS axiophot microscope or at 100× magnification with a confocal microscope using a 488 nm laser and a 535 nm narrow band filter for the fluorescein isothiocyanate signal and a 568 nm laser and a 590 nm long band filter for the propidium iodide signal.

### *Permeabilization of cells with NP-40*

HeLa cells incubated with nocodazole overnight were washed vigorously three times with PBS, collected and resuspended in nuclear buffer (60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 15 mM Tris pH 7.4, 0.5 mM DTT, 300 mM sucrose) containing 0.3% NP-40. After 3 min incubation at room temperature, samples were centrifuged and the supernatant constituted the NP-40 extractable fraction. Pellets of treated cells were re-extracted by sonication in IP0.1 buffer.

### *Dephosphorylation and kinase assays*

For dephosphorylation, 10 μg of extract from colchicine-arrested HeLa cells was incubated for 1 h at 37°C in 1× dephosphorylation buffer from BMB (50 mM Tris pH 8.5, 0.1 mM EDTA) in the presence of 20 units of calf intestine alkaline phosphatase (CIAP). For *in vitro* phosphorylation, the protocol was adapted from Lin *et al.* (1991). Recombinant HA-hbrm produced in baculovirus was mixed with 1 μg of 12CA5 monoclonal antibody and 20 μl of a 50% slurry of protein A–Sepharose (Pharmacia) in lysis buffer (50 mM Tris pH 8, 100 mM NaCl, 1% Triton X-100, 2.25 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM PMSF and 0.1 mM DTT). After 2 h, the beads were washed twice in buffer A (same as lysis buffer but with 500 mM NaCl) and twice in HTD buffer (50 mM HEPES pH 7.4, 0.1% Triton X-100 and 1 mM DTT). A plate with confluent HeLa cells was lysed in 200 μl of HTS (50 mM HEPES pH 7.4, 0.1% Triton X-100, 150 mM NaCl, 50 mM β-glycerophosphate, 25 mM NaF, 20 mM EGTA, 15 mM $MgCl_2$). The extract was cleared by centrifugation and 100 μg of the HeLa extract was then incubated with the HA-hbrm beads and 150 μCi of [$\gamma$-$^{32}$P]ATP with a specific activity of 100 Ci/mmol. The reaction was carried out at room temperature for 30 min with shaking, then stopped by adding 1 ml of ice-cold RIPA buffer. The beads were then washed five times with RIPA and resuspended in 15 μl of protein loading solution, analysed by SDS–PAGE and autoradiographed.

### *Immunopurification of the SWI/SNF complex*

Small-scale immunoprecipitations with the 12CA5 monoclonal mouse antibody were performed as previously described (Muchardt *et al.*, 1995). For large-scale immunopurification, 110 mg of HeLa cells expressing HA-hSNF5 (H28 cells) total extract was incubated with 4 ml of cross-linked 12CA5 antibody–protein A–Sepharose beads for 4 h in IP0.1 buffer (the volume ratio of extract to beads was 10:1). Beads were then transferred to a column and extensively washed with IP0.6 buffer (same as IP0.1 but with 600 mM potassium acetate) until no more protein was eluted. The SWI/SNF complex was eluted with 4 ml of HA peptide (2 mg/ml in IP0.1 buffer).

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