

# c-MYC interacts with INI1/hSNF5 and requires the SWI/SNF complex for transactivation function

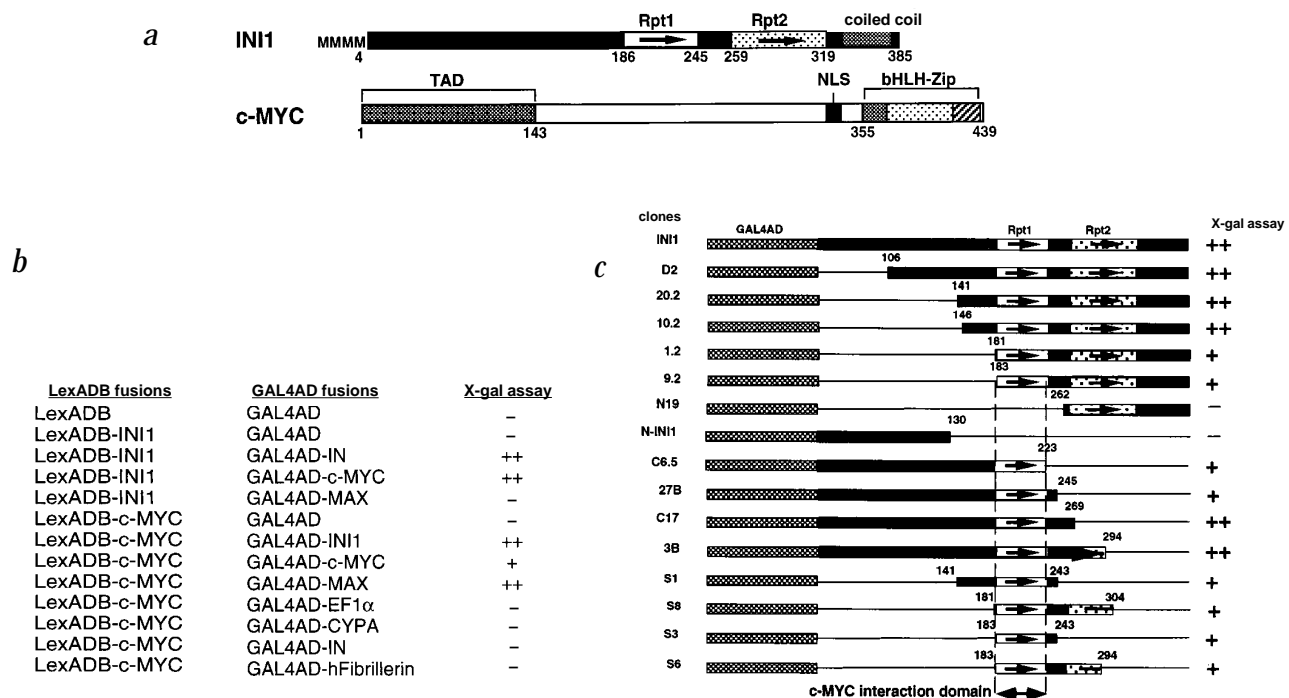
S.-W. Grace Cheng<sup>1</sup>, Kelvin P. Davies<sup>1</sup>, Eric Yung<sup>1</sup>, Ralph J. Beltran<sup>1</sup>, Jin Yu<sup>2</sup> & Ganjam V. Kalpana<sup>1</sup>

Chromatin organization plays a key role in the regulation of gene expression<sup>1,2</sup>. The evolutionarily conserved SWI/SNF complex is one of several multiprotein complexes that activate transcription by remodelling chromatin in an ATP-dependent manner<sup>3-5</sup>. SWI2/SNF2 is an ATPase whose homologues, BRG1 and hBRM, mediate cell-cycle arrest<sup>6,7</sup>; the SNF5 homologue, INI1/hSNF5, appears to be a tumour suppressor<sup>8,9</sup>. A search for INI1-interacting proteins using the two-hybrid system<sup>10,11</sup> led to the isolation of c-MYC, a transactivator<sup>12,13</sup>. The c-MYC-INI1 interaction was observed both *in vitro* and *in vivo*. The c-MYC basic helix-loop-helix (bHLH) and leucine zipper (Zip) domains and the INI1 repeat 1 (Rpt1) region were required for this interaction. c-MYC-mediated transactivation was inhibited by a deletion fragment of INI1 and the ATPase mutant of BRG1/hSNF2 in a dominant-negative manner contingent upon the presence of the c-MYC bHLH-Zip domain.

Our results suggest that the SWI/SNF complex is necessary for c-MYC-mediated transactivation and that the c-MYC-INI1 interaction helps recruit the complex.

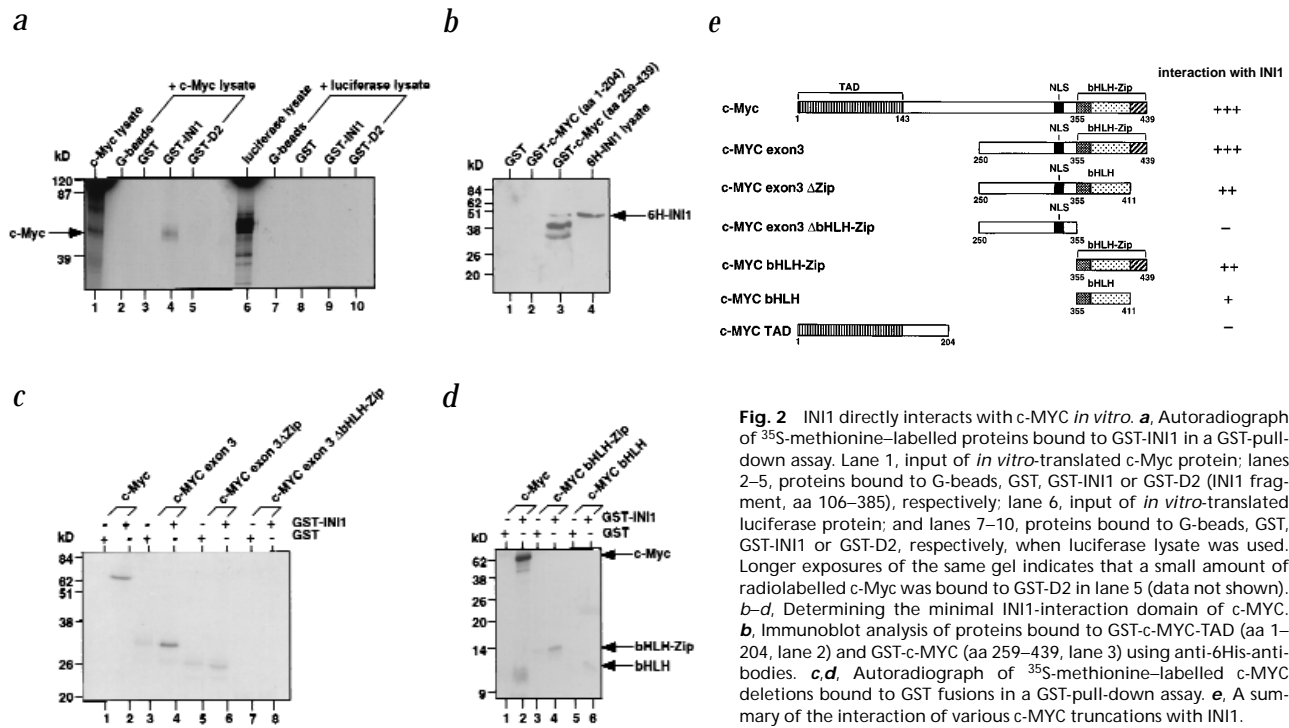
The c-MYC fragment (aa 250–439) obtained in the two-hybrid system included the nuclear localization sequence and the bHLH and Zip domains<sup>12</sup> (Fig. 1a). The INI1-c-MYC interaction was specific, as a LexA-DNA binding domain (LexADB)-INI1 did not interact with either the GAL4-activation domain (GAL4AD) or its fusion with MAX (a bHLH-Zip protein that dimerizes with c-MYC), GAL4AD-MAX. In addition, LexADB-c-MYC interacted strongly with GAL4AD-MAX and GAL4AD-INI1 but not with other GAL4AD fusions tested (Fig. 1b).

INI1 contains two highly conserved imperfect repeats (Rpt1 and Rpt2; Fig. 1a) that are important for interaction with HIV-1 integrase (IN), hBRM and ALL1 (refs 14–16). We mapped the minimal c-MYC-binding region of INI1 in yeast and found that



**Fig. 1** INI1 interacts with c-MYC in the two-hybrid system. **a**, Schematic diagrams of INI1 and c-MYC featuring relevant motifs. Rpt, repeat; TAD, transactivation domain; NLS, nuclear localization sequence; bHLH-Zip, basic helix-loop-helix, leucine zipper domain. **b**, Specificity of interactions of INI1 with c-MYC in the two-hybrid system. Interactions of LexADB- and GAL4AD-fusions of proteins in various combinations was determined by the transactivation of the *lacZ* reporter gene using the X-gal assay. **c**, Minimal c-MYC-interaction domain of INI1. The two-hybrid system was used to determine the minimal c-MYC-interaction domain of INI1. Positive interaction was determined using the X-gal assay. Filled bar, INI1 sequence; Rpt, repeat. Amino acid residues at the junction of the deletions are indicated above each bar. "++", dark blue; "+", light blue; "-", white.

<sup>1</sup>Department of Molecular Genetics, Albert Einstein College of Medicine, 1300 Morris Park Ave, U821, Bronx, New York, New York 10461; and <sup>2</sup>Department of Microbiology, Columbia University, College of Physicians and Surgeons, New York, New York 10032, USA. Correspondence should be addressed to G.V.K. (e-mail: kalpana@aecom.yu.edu).

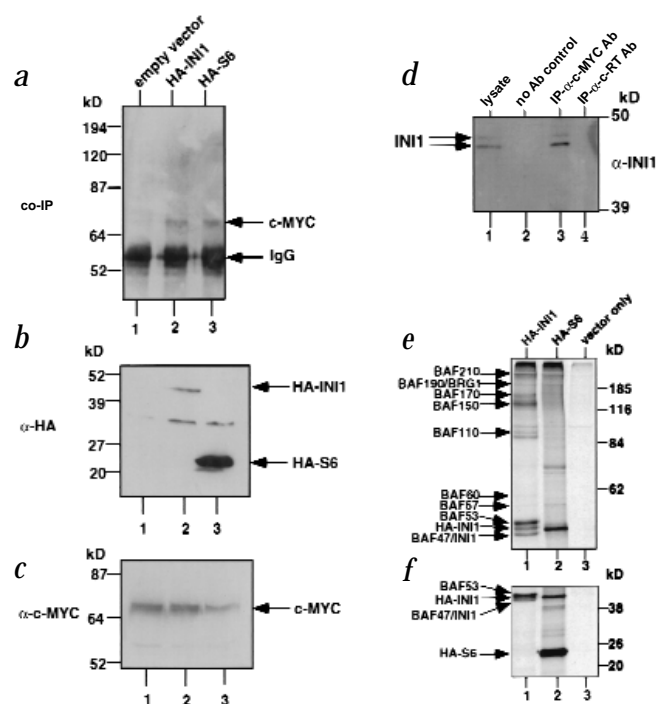
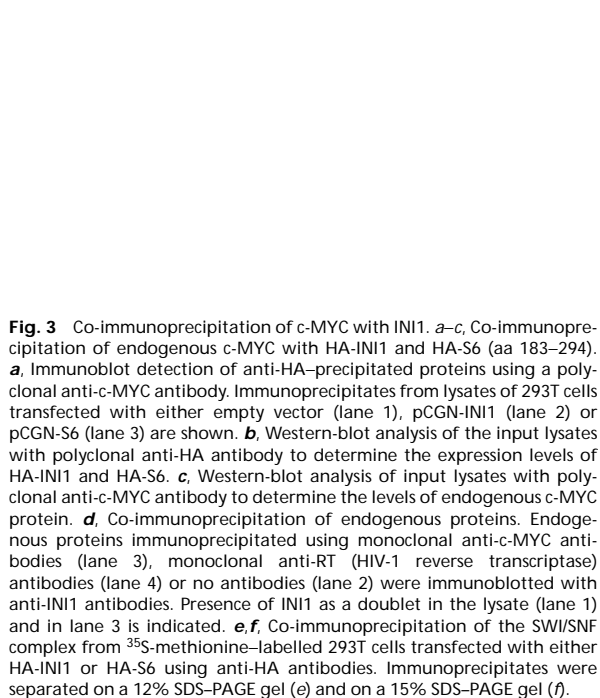


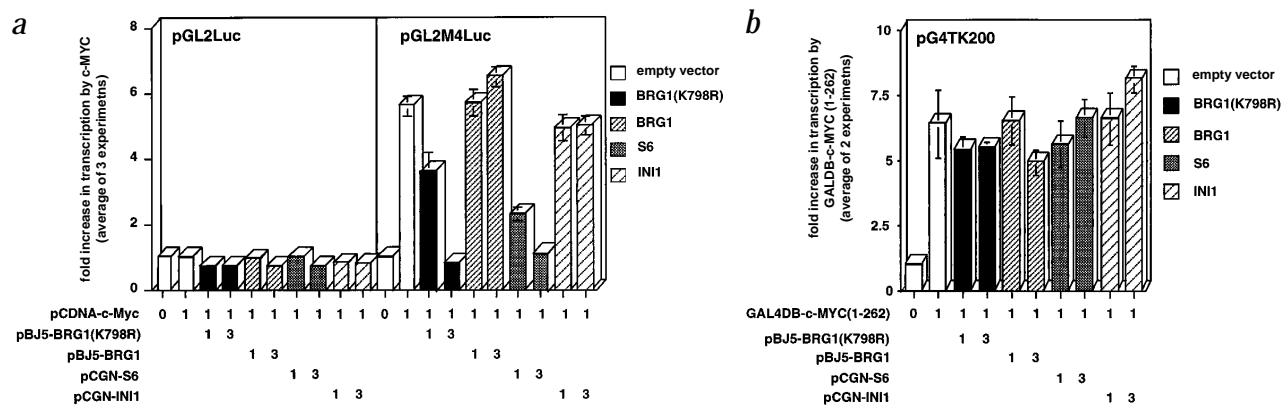
the Rpt1 (aa 181–241) region is necessary and sufficient for the interaction (Fig. 1c).

We performed *in vitro* binding reactions using glutathione-S-transferase (GST)-INI1 (aa 4–385) and <sup>35</sup>S-labelled mouse c-Myc generated by *in vitro* transcription and translation. GST-INI1 was able to specifically bind <sup>35</sup>S-c-Myc (Fig. 2a, lane 4), demonstrating that full-length c-Myc interacts directly with full-length INI1 *in vitro*.

To determine the minimal INI1-interacting domain of c-MYC, we carried out a deletion analysis *in vitro*. GST-c-Myc (aa 259–439) specifically bound a hexa-histidine fusion of INI1 (aa 4–385, 6H-INI1), whereas the GST fusion of the c-MYC transac-

tivation domain (TAD, aa 1–204) did not (Fig. 2b, lanes 3 and 2, respectively). Analysis of additional <sup>35</sup>S-labelled, carboxy-terminal fragments of c-MYC for their ability to bind to GST-INI1 revealed that the bHLH-Zip domain is necessary and sufficient for interaction (Fig. 2c,d). Removal of the Zip region reduced but did not eliminate the interaction with INI1, suggesting that multiple regions within the bHLH-Zip domain are responsible for the interaction (Fig. 2d). The bHLH-Zip domain is critical for c-MYC-mediated transactivation, transformation, S-phase induction and apoptosis<sup>12,13</sup>, indicating that the INI1-c-MYC interaction is functionally significant.





**Fig. 4** Dominant-negative mutants of BRG1 and INI1 inhibit c-Myc-mediated transactivation. **a**, Effect of INI1, BRG1 and their mutants on c-Myc-mediated transactivation. Transcriptional activity was assayed using the luciferase reporter in either pGL2Luc or pGL2M4Luc plasmids by measuring the relative light units. Increase in transactivation by c-Myc in the presence and absence of co-transfected INI1, S6, BRG1 and BRG1(K798R) is indicated as fold increase in relative light units/ $10^6$  GFP-positive cells compared with the relative light units/ $10^6$  GFP-positive cells in the absence of c-Myc, using the same reporter construct. **b**, Effect of INI1, BRG1 and their mutants on the transactivation potential of GAL4DB-c-MYC-TAD(aa 1–262). Transcriptional activity was assayed using the luciferase reporter in the pG4TK200Luc plasmid by measuring the relative light units. Increase in transactivation by GAL4DB-c-MYC(1–262) in the presence or absence of co-transfected INI1, S6, BRG1 and BRG1(K798R) is indicated as fold increase in relative light units/ $\beta$ -galactosidase compared with that in the absence of GAL4DB-c-MYC(1–262).

We performed co-immunoprecipitations of a haemagglutinin-tagged INI1 (HA-INI1) or its truncation, S6, which spans the Rpt1 region (HA-S6, aa 183–294), with endogenous c-MYC using anti-HA antibodies. Both HA-INI1 and HA-S6 were able to co-precipitate endogenous c-MYC from 293T cells (Fig. 3a, lanes 2 and 3, respectively). We also performed co-immunoprecipitation of endogenous proteins from untransfected 293T cells using anti-c-MYC antibodies, and detected INI1 using affinity-purified anti-INI1 antibodies (Fig. 3d). These results indicate that c-MYC interacts with INI1 *in vivo*.

c-MYC is a sequence-specific transactivator that when heterodimerized with MAX binds to a CAC(G/A)TG motif<sup>12,13</sup> (E-box). Repression of transcription at promoters containing E-box by members of the c-MYC network, such as the MAX:MXI1 heterodimers, is thought to be mediated by chromatin condensation via the recruitment of histone deacetylases<sup>17,18</sup>. As SWI/SNF complexes remodel chromatin, we hypothesized that transactivation by c-MYC may involve the recruitment of the SWI/SNF complex to the E-box, mediated by interaction with INI1.

We next studied the dependence of c-Myc-mediated transactivation on the presence of functional components of the SWI/SNF complex, such as BRG1/hSNF2 (refs 19,20). The ATPase activity of BRG1/hSNF2 is essential for the ability of the complex to remodel chromatin; the ATPase-defective mutant, BRG1(K798R), acts as a dominant-negative inhibitor of SWI/SNF-mediated transactivation<sup>19,20</sup>. We tested the effect of BRG1 and BRG1(K798R) on c-Myc-mediated transactivation in HeLa cells. Overexpression of c-Myc stimulated transcription to sixfold over the basal level from a reporter containing four minimal E-boxes (Fig. 4a). This stimulation was inhibited in a dose-dependent manner by co-expression of BRG1(K798R), but not BRG1, reaching near-complete inhibition at higher concentrations (Fig. 4a). In contrast, increasing concentrations of BRG1(K798R) did not significantly affect the transactivation of a reporter deficient in E-boxes (Fig. 4a). Western-blot analysis of total protein revealed equal levels of c-Myc in all transfected cells (data not shown). In addition, neither BRG1 nor BRG1(K798R) had any effect on GAL4-VP16-mediated transactivation from the pG4TK200Luc (ref. 21) reporter plasmid in HeLa cells (data not shown), suggesting that the inhibitory effect of BRG1(K798R) is specific for c-Myc-mediated transcription. Several reports indicate that BRG1(K798R) does not inhibit a number of other promoters and activators<sup>19,20</sup>.

To determine if a deletion fragment of INI1 containing the Rpt1 region might inhibit c-MYC function in a dominant-negative manner, we co-expressed HA-INI1 or HA-S6 with c-Myc in the above transactivation assay. Co-expression of HA-S6, but not HA-INI1, inhibited c-Myc-mediated transactivation in a dose-dependent manner (Fig. 4a) to a level similar to that obtained with BRG1(K798R). Neither HA-INI1 nor HA-S6 significantly affected transcription from the reporter lacking E-boxes (Fig. 4a).

To understand the mechanism of inhibition by the S6 fragment, we compared the ability of HA-INI1 and HA-S6 to co-immunoprecipitate components of the SWI/SNF complex<sup>20</sup> (referred to as BAFs) from <sup>35</sup>S-labelled proteins isolated from transfected 293T cells using anti-HA antibodies. HA-INI1 was able to co-immunoprecipitate components of the SWI/SNF complex. HA-S6 was unable to co-immunoprecipitate most of these proteins, however, including BAF170, BAF150, BAF110, BAF60 and BAF53, indicating its inability to recruit the complete SWI/SNF complex (Fig. 3e,f). On the basis of these results, we hypothesize that the S6 fragment sequesters c-MYC from binding to endogenous INI1 and thereby interferes in a dominant-negative fashion with its ability to recruit the SWI/SNF complex.

To rule out the possibility that the inhibition by S6 and BRG1(K798R) is due to indirect interference by another factor that binds to the c-MYC-TAD, we tested their effect on the transactivation potential of a GAL4DB-c-MYC-TAD (aa 1–262; ref. 23). We found that transcription from the pG4TK200Luc reporter was activated 5–6-fold by GAL4DB-c-MYC-TAD, which was not significantly inhibited by co-expression of either HA-S6 or BRG1(K798R) (Fig. 4b). These results indicate that the inhibition is mediated by the INI1-binding bHLH-Zip and not the TAD domain of c-MYC.

The results of our study—the direct interaction of c-MYC with INI1 and the inhibitory effect of mutants of two different components of the complex, BRG1(K798R) and S6, on c-MYC-mediated transactivation—suggest that high-level transactivation by c-MYC requires the presence of a complete SWI/SNF complex. Further experiments are necessary to determine if the interaction of c-MYC with INI1 and other dimerization partners, such as MAX, is mutually exclusive or complementary. The members of c-MYC network are central to the control of normal cell growth and development and regulate diverse processes such as cellular transformation, differentiation and apoptosis<sup>12,13</sup>. Recruitment

of the SWI/SNF complex, mediated by the interaction of INI1 with c-MYC, may facilitate the transcription of a discrete subset of c-MYC target genes, especially those involved in apoptosis, which might explain the tumour-suppressor activity of INI1.

## Methods

**Yeast two-hybrid analysis.** We generated a bait plasmid, pSH2-1-INI1, by cloning a *Bam*HI-*Sal*I fragment of *INI1* cDNA into pSH2-1, and used it to screen a HL60 cDNA library in the yeast strain CTY10-5 as described<sup>22</sup>. To determine the specificity and minimal c-MYC–interaction domain of INI1, we sub-cloned the *MYC* cDNA fragment of the positive clone (pGADNot-c-MYC) into the pSH2-1A vector (with a +1 frameshift) to generate pSH2-c-MYC, encoding LexADB-c-MYC, and screened it against various GAL4AD fusions.

**In vitro binding studies.** GST and GST-INI1 (ref. 14) were tested for interaction with full-length, <sup>35</sup>S-labelled c-Myc or Luciferase (generated by using the Promega TNT7 rabbit reticulocyte lysate transcription/translation kit) as described<sup>14</sup>. To determine the minimal INI1–interaction domain of c-MYC, we first tested the interaction of GST-c-Myc-exon3 (aa 259–439) and GST-c-MYC-TAD (aa 1–204) with 6H-INI1 (expressed from pQE32-INI1, which was obtained by inserting a *Bam*HI-*Sal*I fragment of *INI1* cDNA into pQE32 vector (Qiagen)). A panel of <sup>35</sup>S-labelled c-MYC fragments, generated *in vitro* by cloning PCR-generated *MYC* deletions into the pCITE-4b(+) vector (Novagen), was tested for interaction with GST-INI1 as described above.

**Co-immunoprecipitation.** pCGN-INI1 and pCGN-S6 (6 µg; E.Y. *et al.*, manuscript in preparation), which encode HA-INI1 and HA-S6, respectively, were transfected into 293T using lipofectin (Life Technologies). Cells were collected after 40 h and sonicated in buffer G (dPBS containing 0.1% IGEPAL, 1 mM DTT, 2 mg/ml BSA, 1 mM PMSF and 1 µg/ml each of pepstatin, aprotinin and leupeptin). Polyclonal anti-HA antibodies (10 µl; Santa Cruz) were added to pre-cleared lysate followed by protein A–sepharose beads (30 µL of a 50% slurry; Sigma) at 4 °C. Bound proteins were washed with buffer G without BSA, separated by SDS–PAGE and immunoblotted with polyclonal anti-c-MYC antibodies (gift of K. Calame).

We performed the co-immunoprecipitation of endogenous proteins as described above from untransfected 293T cells using protein G–agarose (Sigma), anti-c-MYC (C-33) antibodies (Santa Cruz) and affinity-purified

rabbit anti-INI1 antibodies<sup>22</sup>. To co-immunoprecipitate the SWI/SNF complex, we transiently transfected 293T cells with pCGN, pCGN-INI1 or pCGN-S6 (30 µg) using the calcium phosphate reagents (Specialty Media). After 40 h they were metabolically labelled with <sup>35</sup>S-methionine (NEN) for 7 h. Cells were lysed in buffer L (50 mM β-glycerophosphate, 10 mM MgCl<sub>2</sub>, 0.1% IGEPAL, 250 mM NaCl, 1 mM PMSF and 1 µg/ml of each pepstatin, aprotinin and leupeptin in PBS) and diluted (1:1) in buffer L lacking NaCl. We added monoclonal anti-HA-antibody (20 µl; Santa Cruz) to pre-cleared lysates, followed by protein A–sepharose (40 µl, 50% slurry) at 4 °C. Bound proteins were washed with buffer L containing NaCl (125 mM), and immunoprecipitates were analysed by autoradiography.

**Transcription assays.** We used reporter plasmids pGL2M4Luc (ref. 17), pGL2Luc (Stratagene), pTK200 and pTKG4200 (ref. 21). Other plasmids used were: pBJ5-BRG1 and pBJ5-BRG1(K798R) (ref. 19); pCDNA3-Myc (gift of L. Alland and R. Dephino); pGFP (gift of R. Pestell); and pSV-βgal (gift of L. Zhu). HeLa-TetOn cells (Clontech) were transfected by lipofectin with plasmids (14 µg) containing: pGFP or pSV-βgal (1 µg) to measure transfection efficiency; pGL2M4Luc, pGL2Luc or pG4TK200 (1 µg); pCDNA3.1, pCDNA3-c-Myc or pGAL4-c-MYC-TAD (1 µg); and pCGN-INI1, pCGN-S6, pBJ5-BRG1 or pBJ5-BRG1(K798R) (1 or 3 µg). We normalized the difference in the DNA by the addition of empty vectors. After 48 h, we analysed a portion of the cells for transfection efficiency by scoring for GFP-positive cells or for β-galactosidase activity. The remaining cells were lysed in buffer (Promega), protein concentrations were determined (BioRad) and relative light units (RLU) were measured from normalized lysates (10 µl). The transcriptional activity was expressed as RLU/10<sup>6</sup> GFP-positive cells or RLU/unit of β-galactosidase activity.

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