Sequential Recruitment of PCAF and BRG1 Contributes to Myogenin Activation in 12-O-Tetradecanoylphorbol-13-acetate-induced Early Differentiation of Rhabdomyosarcoma-derived Cells

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Myogenin and its upstream regulator MyoD are known to be required for myogenic cell differentiation. Although both of them can be expressed in rhabdomyosarcoma-derived RD cells, the cells are unable to undergo full-scale terminal myogenic differentiation. 12-O-Tetradecanoylphorbol-13-acetate (TPA) has been found to be functional in the induction of RD cell differentiation, whereas its mechanism is not fully understood. By using quantitative real-time-based chromatin immunoprecipitation and real-time reverse transcription-PCR-based promoter activity assays, we examined the activation mechanism of the myogenin gene during TPA-induced differentiation of the RD cells. We have shown that a histone acetyltransferase PCAF and ATPase subunit BRG1 of the SWI/SNF chromatin remodeling complex are sequentially recruited to the promoter of the myogenin gene. Both PCAF and BRG1 are also involved in the activation of the myogenin gene. In addition, we have found that the p38 mitogen-activated protein kinase is required for BRG1 recruitment in TPA-mediated myogenin induction. We propose that there are two distinct activation steps for the induction of myogenin in TPA-induced early differentiation of RD cells: 1) an early step that requires PCAF activity to acetylate core histones and MyoD to initiate myogenin gene expression, and 2) a later step that requires p38-dependent activity of the SWI/SNF remodeling complex to provide an open conformation for the induction of myogenin. Our studies reveal an essential role for epigenetic regulation in TPA-induced differentiation of RD cells and provide potential drug targets for future treatment of the rhabdomyosarcoma.

Rhabdomyosarcoma is a malignant tumor of childhood that is thought to derive from muscle precursor cells. Rhabdomyosarcoma-derived RD cells are originated from the embryonal type of rhabdomyosarcoma. The RD cells are defective in myogenic differentiation even though they express both MyoD and myogenin (1). However, once treated with 12-O-tetradecanoylphorbol-13-acetate (TPA), a phorbol ester, RD cells become refractory to growth signals and undergo cell cycle arrest (2). Meanwhile, the TPA-treated RD cells display a morphology resembling that of differentiated myotubes (2). Several protein kinases including protein kinase Ca, extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 mitogen-activated protein kinases (MAPKs) have been implicated in the TPA-induced differentiation of RD cells (3).

As one of the excellent paradigms to study cellular differentiation, myogenic differentiation has been extensively studied in the past decade. Much has been learned about the myogenic regulatory factors (MRFs) of the basic helix-loop-helix protein family, such as MyoD, that govern myogenic differentiation. To mediate muscle-specific gene transcription, MRFs have to dimerize with E2A products (i.e. E12 and E47), which are also members of the basic helix-loop-helix. Together, the heterodimers bind efficiently to the consensus E box (i.e. 5' -CANNTG-3') in the promoter regions of many muscle-specific genes including the myogenin gene. In addition to MRFs, myocytes enhancer binding factor 2 (MEF2s) are also essential for myogenic differentiation (4). Four distinct members of the MEF2 family, namely MEF2A, 2B, 2C, and 2D, bind to a consensus AT-rich sequence (i.e. MEF2 site) in promoters of many muscle-specific genes as either homo- or heterodimers. Previous studies showed that a segment of the proximal mouse myogenin gene (−184/+1) containing an E box, a MEF2, and MEF3 sites is ~88% homologous to that of the humans and is indispensible in myogenin gene expression (5, 6).

Myogenin, and other members of the MyoD family (MyoD, Myf5, and MRF4), possess the ability to convert some of the
non-muscle cells into the myogenic lineage. Expression of myogenin has been considered as one of the earliest molecular markers for cells committed to differentiation in vitro. Depending on the nature of the stimuli, myogenin is either induced or repressed, this in turn determines whether the differentiation program is enhanced or aborted (7). The mammalian SWI/SNF chromatin remodeling complex is required for myogenin gene expression (8–10). The SWI/SNF complex depends upon either BRG1 or BRM, the catalytic subunits with ATPase activity, to hydrolyze ATP to alter chromatin conformation.

p38 MAPK accelerates myogenic differentiation by activating both MyoD- and MEF2-dependent gene transcription (11). Recently, p38 MAPK was also shown to be required for the recruitment of the SWI/SNF complex to the myogenin promoter (12). The role of the p38 MAPK here is to phosphorylate BAF60c, a component in the SWI/SNF complex, which further recruits the ATPase subunit (BRG1 or BRM) of the complex to the myogenin promoter (12). In addition to the SWI/SNF complex, the histone modification enzymes, such as the histone acetyltransferases (HATs), also participate in remodeling of the chromatin structure by modifying histone tails. p300, CBP, and PCAF of the HAT family are of particular interest because these enzymes cannot only modify the histone tails, but also acetylate other transcription regulatory factors including MyoD and MEF2 (13–16). However, it remains unclear which chromatin remodeling factors are involved and how they coordinate with each other in TPA-induced RD cells.

To address the above questions, we focused on the epigenetic regulation of the myogenin gene in TPA-treated RD cells. Our data prompted us to propose a two-step model to explain the TPA-induced Myogenin expression in RD cells: an early step in which PCAF is specifically recruited to the myogenin promoter followed by a p38 MAPK-dependent recruitment of the SWI/SNF complex. Our studies provide potential novel drug targets for treatment of rhabdomyosarcoma in the future.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—RD cells were purchased from ATCC and maintained in Dulbecco's modified Eagles's medium (Invitrogen) supplemented with 10% fetal calf serum, 0.37% NaHCO3, and sodium penicillin and streptomycin sulfate (100 units/ml each) in a 5% CO2 humidified atmosphere at 37°C. For inducing differentiation, stock solution of 100 μM TPA in 5% ethanol kept at 20°C was added with a 1/1,000 dilution to the cultured cells 24 h after plating; equal amount of ethanol without TPA was added to the medium of the control cells.

**Plasmids**—The mammalian expression plasmids were, pcDNA3-HA-p38 (wild type) from Dr. Kun Liang Guan (University of Michigan, Ann Arbor); pcDNA3-antisense p38 from Dr. Gang Pei (SIBS, Chinese Academy of Science); and pBJ5-BRG1(wild type) and pBJ5-BRG1K798R (an ATPase-defective K798R mutant, dominant negative) expression plasmids that were from Dr. Anthony N. Imbalzano (University of Massachusetts Medical School).

**Reagents**—p38 inhibitor SB203580 and TPA were purchased from Sigma. Antibody against p300 was provided by Dr. Q. Li (National Institutes of Health, Bethesda, MD). Antiserum against hBAF60c was raised in rabbits using the full-length BAF60c as an antigen. Anti-myogenin (F5D), anti-β-actin (I-19), anti-MyoD (M-318), anti-BRG1 (H-88), anti-p38 (H-147), anti-phospho-p38 (D-8), and anti-PCAF (E-8) antibodies were purchased from Santa Cruz Biotechnology. Antibodies against pan-acetyl lysine, acetylated histone H3-K14, and acetylated histone H3-K9 were purchased from Upstate Biotechnology (Lake Placid, NY).

**DNA Constructs and DNA Transfection**—An upstream fragment (−1088/+50) of the mouse myogenin gene was fused upstream of the CAT gene in an expression vector modified in the laboratory of Shen and designated as pREP4m-myogenin-CAT. The transfection control plasmid pCMV-β-gal was generated by amplifying the β-galactosidase gene from pRSV-β-gal plasmid (constructed in our laboratory) with primer pairs of the 5′ primer: 5′-CCCAAGCTTTTTCGTCGGGACTGGGTTG-3′ and the 3′ primer: 5′-GCTCTAGAGTCGGGGATAGTCTTCTTG-3′, followed by insertion into the XbaI/HindIII-digested pRC/CMV vector (Invitrogen). Transient transfection of DNA into RD cells was carried out using Lipofectamine 2000 (Invitrogen) in this study. Expression plasmids of p38 and BRG1 genes were individually co-transfected with pREP4m-myogenin-CAT and pCMV-β-gal into RD cells. For promoter activity assay, TPA (100 nM) was added to the medium after transient transfection for 16 h and incubated for another 24 h or the time interval indicated followed by cell harvesting.

**Quantitative Real-time RT-PCR Analysis**—Total RNA was extracted from cells and followed by reverse transcription with a first-strand RT-PCR kit (Promega) per the manufacturer’s instructions. PCR was performed with SYBR® Premix Ex Taq (TaKaRa, Biotech) using the Rotor-Gene RG-3000A (Corbett Research) Real-time PCR System. To detect the induction of the myogenin reporter, the following primers were used: for the myogenin promoter, forward primer, 5′-ACTTTCGCCCGGTTCGA-3′; reverse primer, 5′-CCGC-TCTGCACTCATCAGT-3′; for the control plasmid of pCMV-β-gal, forward primer, 5′-CTTACGGGCTGATTTTGTG3′; reverse primer, 5′-TGTCGTGCTTGGATCTTCCTTTG-3′. The cycle quantity required to reach a threshold in the linear range (Q) was determined and compared with a standard curve for each primer set generated by five 3-fold dilutions of the first-strand cDNA of known concentration. Data represent the mean ± S.D. of normalized promoter activities of myogenin relative to that of pCMV-β-gal in each treatment. To quantitate products generated in the chromatin immunoprecipitation assays or mRNA expression, we used the following primers: primers for myogenin gene, 5′-ATGGAGACCTGATATGAGACATCCCC-3′ (forward, +1/+23) and 5′-GGACACCGACTCTCTTCTTACAC-3′ (reverse, +237/+216). The relative expression at each time interval was normalized against GAPDH (5′-GCTCAGTGGGAGCTGGCCTCC-3′ for-ward, +741/+761) and 5′-TTGGGCGCATAGGGATCCACAC-3′ (reverse, +1050/+1030) using the comparative C T method recommended by the instrument producer. Experiments were repeated at least three times with statistical analyses for each individual experimental set. All values in the experiments were expressed as mean ± S.D.
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**FIGURE 1. The effect of TPA on the differentiation of RD cells.** A, microscopic images (×250 magnification) of RD cells treated with 100 nM TPA were shown at 24 and 96 h as indicated, or without TPA (control). B, Western blot of myogenin expressed in RD cells treated with TPA for the time intervals as indicated on the top of the figure. Whole cell lysates (WCE) were separated on a 12% SDS-PAGE and blotted with antibodies against myogenin and β-actin as indicated on the right. C, real-time RT-PCR detection of myogenin mRNA expressed in RD cells treated with TPA for the time intervals as indicated at the bottom of the figure. GAPDH was taken as internal control using the comparative Ct method as described under “Experimental Procedures.” Each bar represents a normalized mean value from at least three independent experiments at each time point of TPA treatment with standard deviation as error bar shown on the top (mean ± S.D.) in the histogram. D, promoter activity assay of the myogenin gene in RD cells treated with TPA was detected by quantitative real-time RT-PCR analysis with pCMV-β-gal as control for transfection efficiency as described under “Experimental Procedures.” Annotation was as described in C. Each bar represents a normalized mean value from at least three independent experiments at each time point of TPA treatment and was shown as mean ± S.D. in the histogram.

Nuclear Extract, Immunoprecipitation and Immunoblot Analysis—5 × 10⁶ RD cells were collected in 8 ml of culture medium after a different treatment, 32 µl of 0.5 M dithiois(succinimidylpropionate) were immediately added to make a final concentration of 2 mM. After shaking for 5–10 min, glycine was added to a final concentration of 10 mM to stop the cross-linking reaction. Cells were then collected by centrifugation at 1,000 × g for 5 min at room temperature and washed with phosphate-buffered saline. Following repeating this step once more, the pellets were recovered. Nuclear extract preparation was performed as described previously (17). The coimmunoprecipitation assay was carried out by using nuclear extracts (~500 µg of protein) incubated with 2 µl of specific antibody for 2 h at 4 °C on a shaking platform. 20 µl of Protein A-agarose (Santa Cruz) was added to each tube and incubated at 4 °C overnight. Pellets were recovered, and washed three times with RIPA buffer, followed by adding 40 µl of 1× Laemmli buffer (50 mM Tris-HCl, pH 6.8, 80 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol), resuspended and boiled for a 10% SDS-PAGE. Western blot assay was performed as described elsewhere (18).

Chromatin Immunoprecipitation (ChIP) and Quantitative PCR Analyses—ChIP assays were carried out with formaldehyde cross-linking as previously described (17, 19), and detected with PCR and gel electrophoresis. ChIPed DNA was subjected to PCR amplification to yield a positive fragment of 140 bp that was separated on 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Gel images were scanned with an AlphalImager 2000™ as described previously (17). For quantitative assay, standard curve and ChIPed DNA samples were analyzed on a Rotor-Gene RG-3000A Real-time PCR System (Corbett Research, Australia) with PCR Master Mix for SYBR Green assays (TaKaRa, Bio-tech). Primer pairs used for amplification of the myogenin gene were 5’-GATCACATCTAAATCCACTGTA-3’ (forward, –142/-121) and 5’-ACGCCAATCTGCTGGTGCCA-3’ (reverse, –3/-22). The cycle quantity required to reach a threshold in the linear range (Qₜ) was determined and compared with a standard curve for the primer set generated by five 10-fold dilutions of genomic DNA samples of known concentration. In all experiments, the following cycling parameters were used: 95 °C for 10 s, 40 cycles of 95 °C for 8 s, 60 °C for 15 s, and 72 °C for 10 s. The percentage of ChIPed DNA relative to input was calculated and shown as mean ± S.D. from three independent experiments.

**RESULTS**

Enhanced Expression of Myogenin in TPA-treated RD Cells—To study the dynamic effect of TPA on RD cells, we treated the cells with TPA for various intervals of time as indicated. After 24–96 h of TPA treatment, the induced RD cells showed morphological changes including elongation of cells and formation of more multinucleated cells (Fig. 1A), which were in agreement with a previous report (2). Western blotting and quantitative real-time RT-PCR assays showed that myogenin was present at low levels in non-induced parental RD cells (Fig. 1, B and C). As differentiation progressed, the mRNA and protein levels of myogenin gradually increased and reached a peak value after 12–24 h of TPA treatment. We also analyzed the effect of TPA on promoter activity of the myogenin gene. As shown in Fig. 1D, the activity of the gene was enhanced by 2.9-fold 6 h after TPA treatment and by some 6-fold after 12 h of TPA treatment. A similar pattern of TPA-induced myogenin mRNA expression (Fig. 1C) suggested that the myogenin gene is mainly regulated at the promoter level in TPA-treated RD cells.

Enhanced Histone Acetylation at the Promoter Region of Myogenin in TPA-induced RD Cells—To uncover the mechanism by which TPA promotes differentiation of RD cells, we first examined the acetylation status of lysines 9 and 14 in histone H3
H3-K9 and H3-K14) as these residues are related to the activation status of the local chromatin (20). ChIP assays were performed. Sonicated chromosome fragments were pulled down with specific antibodies against acetylated H3-K9 and H3-K14, respectively, followed by real-time PCR analysis using primers in the 5'-flanking region of the myogenin gene. We found that whereas K9 and K14 showed limited acetylation in the first 3 h of TPA induction, the acetylation of K14 reached a higher level at 6 h post-treatment (Fig. 2B). Eventually both sites were significantly acetylated in RD cells after 12 h of TPA treatment (Fig. 2A and B). The enhanced acetylation of histone H3 shown here indicated that HATs are involved in the early stage of TPA treatment.

**PCAF Is the First Histone Acetyltransferase Recruited to the Myogenin Promoter after TPA Treatment**—To determine which HAT is preferentially recruited to the myogenin promotor in response to TPA treatment, we employed chromatin immunoprecipitation assays. Interestingly, we found that both p300 and MyoD consistently associated with the myogenin promotor with or without TPA induction (Fig. 3A). In contrast, PCAF was gradually recruited to the promotor only after 6 h of TPA treatment (Fig. 3A). A quantitative analysis was shown in Fig. 3B. As PCAF is known to interact with and acetylate MyoD (15), we next tested whether this event occurred in TPA-treated RD cells. We found that: 1) PCAF started to accumulate in the nucleus of RD cells after 6 h of TPA treatment (Fig. 3C, top panel); 2) PCAF directly interacts with MyoD, a key regulator of myogenin gene, and vice versa (Fig. 3C, second and third panels); and 3) MyoD was acetylated after 6 h of TPA treatment (Fig. 3C, bottom panel). These results suggested that PCAF is likely responsible for acetylation of both histones (e.g. H3-K9 and H3-K14) and MyoD in the early stage of TPA-induced RD cells.

**SWI/SNF Complex Is Recruited to the Myogenin Promoter at a Later Stage in TPA-treated RD Cells**—To examine whether the TPA-induced expression of myogenin requires the participation of other chromatin remodeling molecules, we focused on the mammalian SWI/SNF complex as it has been implicated in myogenin gene expression (9, 10). Although either BRG1 or Brm could function as the catalytic subunit of the mammalian SWI/SNF complex, only Brg1 was found to be present in RD cells (Fig. 4B, and data not shown).

Expression constructs of the wild type BRG1 (wtBRG1), its dominant negative mutant (dnBRG1), and an empty vector were individually co-transfected with the myogenin reporter plasmid and pCMV-β-gal. Although the wtBRG1 slightly increased the TPA-induced myogenin promoter activity, the dominant-negative BRG1 drastically abolished the TPA induction of the gene to the basal level without affecting the constitutive promoter activity of the gene (Fig. 4A).

By immunoprecipitation and Western blotting, we found that BAF60 physically interacted with Brg1 only after 12 h of TPA induction (Fig. 4B, top row). In addition, we showed by
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**A**

**FIGURE 4.** The involvement of the SWI/SNF chromatin remodeling complex in TPA-treated RD cells. **A**, the effect of BRG1 on the promoter activity of the myogenin gene in RD cells. Expression vector, WT-BRG1, and DN-BRG1 were first transfected into RD cells individually. The cells were then treated with 100 nM TPA for 24 h (TPA) or without TPA (control). The promoter activity of the myogenin gene was shown in the histogram and described legend to Fig. 1D. **B**, Western blot of BRG1 and BAF60 interaction in RD cells treated with TPA for each interval of time as indicated on the top of the figure. Whole cell extracts (WCE) were immunoprecipitated (IP) with antibody against BAF60, and blotted with BRG1 and BAF60 as indicated on the right. C, ChIP assays quantified by real-time PCR in TPA-treated RD cells with specific antibodies against BAF60. Annotation was as described in the legend of Fig. 2.

ChIP assays that BAF60 was efficiently recruited to the promoter region of myogenin in RD cells only after 12 h of TPA induction and that its levels reached a plateau after 24–48 h of TPA treatment (Fig. 4C). Our results suggested that Brg1 is recruited to the myogenin promoter via BAF60 and participates in regulation of the myogenin gene during TPA-induced RD cell differentiation.

**p38 Is Activated by TPA and Plays a Pivotal Role in TPA-induced Myogenin Expression**—The p38 MAPK pathway plays an essential role in muscle cell differentiation (11). Recently, it was found that p38 MAPK promotes myogenic differentiation by controlling the recruitment of the SWI/SNF complex to the myogenin promoter (12). To test whether p38 MAPK is also involved in TPA-induced myogenin gene expression, we employed SB203580 (SB), a specific inhibitor for p38 MAPK. We showed that SB blocked the TPA-induced myogenin expression in RD cells (Fig. 5A). In contrast, basal levels of myogenin expression were insensitive to SB203580. In addition, we found that the protein levels of p38 in both the nucleus and whole cell extracts were enhanced by TPA treatment. Most importantly, the levels of the dually phosphorylated p38 MAPK (i.e. the active form of p38) were also enhanced after 3 h of TPA treatment (Fig. 5B).

Similar results were also obtained from analysis of the myogenin promoter activity in response to TPA treatment. We found that SB effectively reduced the TPA-induced myogenin activity back to the basal levels (Fig. 5C). In addition, the p38 antisense construct also completely repressed the TPA-induced promoter activity of myogenin to the basal levels in RD cells (Fig. 5D). In contrast, the wild-type p38 had no obvious effect. These results confirmed that p38 is indispensable for TPA-induced myogenin induction in RD cells.

Because p38 is known to target the SWI/SNF complex to the myogenin promoter in normal myogenic differentiation, we next examined whether a similar mechanism also worked in TPA-induced RD cells. ChIP assays were performed with or without the p38 inhibitor SB. We found that recruitment of Brg1 to the myogenin promoter was detectable only after 12 h of TPA treatment (Fig. 5E). Importantly, the recruitment of Brg1 was completely dependent on p38 MAPK, in agreement with the findings by Simone et al. (12). In contrast, recruitment of PCAF in response to TPA induction was not affected by SB203580 treatment. The quantitative analysis was shown in Fig. 5E, FA and FB.

**DISCUSSION**

We have shown that p300 constitutively associates with the myogenin promoter in RD cells independent of TPA treatment (Fig. 3A). In contrast, PCAF is recruited to the myogenin promoter in a TPA-inducible manner (Figs. 3A and 5D). As the binding of PCAF to the myogenin promoter correlates with the increase in acetylation of H3–K9, H3–K14, and MyoD (Figs. 2, A and B, and 3B), it suggests that PCAF is likely the HAT that acetylates both histones and MyoD at the early stage of TPA action. Although p300 is also capable of acetylating MyoD (21, 22), it is less likely responsible for MyoD acetylation in RD cells as both p300 and MyoD already associate with the myogenin promoter before TPA induction. However, MyoD is not acetylated until PCAF is recruited into the complex. As both p300 and PCAF can physically associate with each other (23), it raises the interesting possibility that p300 facilitates PCAF recruitment to the myogenin promoter. Whether this is indeed the case remains to be further explored. In addition, it remains unclear how TPA induces PCAF recruitment to the myogenin promoter. Further investigation is needed to address this issue.

Regarding the sequential recruitment of the SWI/SNF complex, our chromatin immunoprecipitation data clearly demonstrated that the mammalian SWI/SNF complex represented by Brg1 is also recruited to the myogenin promoter with another 6-h delay after PCAF recruitment. Presumably, an earlier recruitment of PCAF functions to acetylate both histones and MyoD, which facilitates subsequent recruitment of the SWI/SNF complex. This is consistent with a general mode of action for the SWI/SNF complex, as the SWI/SNF complex in different biological systems is normally recruited to local promoter regions after these regions are pre-acetylated by HATs (9). In the SWI/SNF complex, BAF60c appears to associate with the myogenin promoter earlier than the BRG1, as BAF60 can be
detected to associate with the myogenin promoter as early as 6 h after TPA induction (Fig. 4C). In addition, we found that BAF60 directly interacts with MyoD (data not shown). Based on these data, we suggest that the SWI/SNF complex is recruited to the myogenin promoter via BAF60. Whether the interaction between MyoD and BAF60 is essential in the recruitment of SWI/SNF complex remains to be established.

It has been reported that the p38 pathway in RD cells is defective, which could be responsible for the inability of the cells toward differentiation (11, 24). As the malfunction of defective p38 can be overcome by an ectopic constitutively active mutant of MKKK6 (7), which indicates target elements in the downstream of p38 are not involved in the defect differentiation of RD cells. By using SB to inhibit the activity of p38, we have found that TPA-induced promoter activity of myogenin is abolished, whereas the basal expression remain unaffected. Meanwhile, Brg1 recruitment at the promoter region of the myogenin gene is also repressed by SB after 12–24 h of treatment. These results indicate that p38 activity is essential for an open chromatin conformation on the TPA-induced expression of the myogenin gene. However, the direct target(s) of p38 MAPK in TPA-treated RD cells remains to be explored.

Collectively, the timing for each individual regulator such as PCAF or BRG1 to bind to or modify the myogenin promoter occurred strictly at either 6 or 12 h of TPA treatment. We thus propose that there are two distinct activation steps for the induction of myogenin in the early stage of TPA-induced RD cell differentiation. First, an early step that requires PCAF activity to provide acetylation on core histones and MyoD to initiate myogenin gene expression. Second, a later oriented step, in which p38 dependent activity of the SWI/SNF remodeling complex is required to provide an open conformation for
the induction of myogenin in the TPA-treated RD cells. Our studies reveal an essential role for epigenetic regulation in TPA-induced differentiation of RD cells and provide potential drug targets for future treatment of the rhabdomyosarcoma.

Acknowledgments—We thank Drs. K. L. Guan, A. N. Imbalzano, Gang Pei, and Qiao Li for providing antibodies and plasmids used in the paper.

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