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The transcription factor Sp1 modulates RNA polymerase III gene transcription by controlling *BRF1* and *GTF3C2* expression in human cells

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Specificity protein 1 (Sp1) is an important transcription factor implicated in numerous cellular processes. However, whether Sp1 is involved in the regulation of RNA polymerase III (Pol III)directed gene transcription in human cells remains unknown. Here, we first show that filamin A (FLNA) represses Sp1 expression as well as expression of TFIIB-related factor 1 (BRF1) and general transcription factor III C subunit 2 (GTF3C2) in HeLa, 293T, and SaOS2 cell lines stably expressing FLNA-silencing shRNAs. Both BRF1 promoter 4 (BRF1P4) and GTF3C2 promoter 2 (GTF3C2P2) contain putative Sp1-binding sites, suggesting that Sp1 affects Pol III gene transcription by regulating BRF1 and GTF3C2 expression. We demonstrate that Sp1 knockdown inhibits Pol III gene transcription, BRF1 and GTF3C2 expression, and the proliferation of 293T and HeLa cells, whereas Sp1 overexpression enhances these activities. We obtained a comparable result in a cell line in which both FLNA and Sp1 were depleted. These results indicate that Sp1 is involved in the regulation of Pol III gene transcription independently of FLNA expression. Reporter gene assays showed that alteration of Sp1 expression affects BRF1P4 and GTF3C2P2 activation, suggesting that Sp1 modulates Pol III-mediated gene transcription by controlling BRF1 and GTF3C2 gene expression. Further analysis revealed that Sp1 interacts with and thereby promotes the occupancies of TATA box-binding protein, TFIIA α , and p300 at both *BRF1*P4 and *GTF3C2*P2. These findings indicate that Sp1 controls Pol III-directed transcription and shed light on how Sp1 regulates cancer cell proliferation.

Eukaryotic RNA polymerase III (Pol III)⁵ is comprised of 17 subunits and is responsible for the synthesis of 5S rRNA, tRNA,

U6 snRNA, 7SL RNA, and other noncoding RNA molecules. 5S rRNA and tRNA are highly abundant, accounting for about 15% of total RNA transcripts in cells (1-3). Pol III gene products are involved in the regulation of ribosomal biogenesis, translation, mRNA processing, cellular metabolism, and growth (4-7). The dysregulation of Pol III gene expression closely correlates with a variety of diseases, including cancers and neural defects (8). Abnormally high levels of Pol III products have been observed in a variety of transformed cell types (9). Pol III general transcription factors, TFIIIB and TFIIIC, play essential roles in Pol III transcription initiation (1). Both TFIIIB and TFIIIC are essential for transcription initiation of the genes encoding 5S rRNA and tRNA. TFIIIB is also required for transcriptional initiation of the U6 RNA gene, where the TFIIIB subunit BRF1 is replaced by BRF2 (1, 3). It has been documented that many clinical cancer specimens contain aberrantly high levels of TFIIIB or GTF3C2 (1, 10, 11). Nutrients, signaling pathway factors, and chromatin modifiers are also involved in the regulation of Pol III gene transcription (3, 8, 12–14). However, the regulatory mechanisms of Pol III gene transcription in human cancer cells are not fully understood.

Specificity protein 1 (Sp1), a member of the transcription factor Sp family, is a ubiquitous transcription factor that binds to the GC-box (5'-(G/T)GGGCGG(G/A)(G/A)(G/T)-3') to regulate transcription of many genes, especially TATA-less genes (15). Sp1 plays critical roles in cell metabolism, proliferation, differentiation, senescence, and death. Numerous studies have confirmed that Sp1 is involved in the regulation of human tumorigenesis (15). Many human cancer cell types exhibit aberrantly high levels of Sp1, and the level of Sp1 expression is associated with the state of a tumor and clinical prognosis (16-18). It has been shown that the down-regulation of Sp1 expression from abnormally high levels to normal levels inhibits tumor formation, cancer cell growth, and cell metastasis (19-21). Sp1 regulates the expression of specific genes by interacting with a variety of factors, including general and specific transcription factors, chromatin-remodeling factors, tumor repressors, and

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This article contains Figs. S1–S11.

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⁵ The abbreviations used are: Pol, polymerase; Sp1, specificity protein 1; FLNA, filamin A; BF1, TFIIB-related factor 1; GTF3C2, general transcription factor III C subunit 2; BRF1P1-4, BRF1 promotors 1–4, respectively; GTF3C2P2,

GTF3C2 promoter 2; IP, immunoprecipitation; qPCR, quantitative PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PSBS, putative Sp1-binding site; TBP, TATA box–binding protein; CMV, cytomegalovirus; ANOVA, analysis of variance.

DNA repair factors (15, 22–26). The cellular function of Sp1 can be regulated by protein modifications, including phosphorylation, acetylation, *O*-linked glycosylation, and sumoylation (27–30). Sp1 can enhance or inhibit gene transcription, whereas its stability is affected by the *O*-linked glycosylation and the sumoylation (31–34). A previous study revealed that an activator element of the *Xenopus U6 RNA* gene contains a Sp1-binding site, which is required for transcription of the *U6 RNA* gene at the WT level, indicating that Sp1 has an impact on *Xenopus* Pol III gene transcription (35). However, whether Sp1 regulates the Pol III gene transcription in human cancer cells remains unclear.

Cytoskeletal filamin A (FLNA) is involved in a variety of cellular activities, including cell migration, proliferation, and RNA polymerase I-mediated gene transcription (36). Our previous work has shown that FLNA differentially represses the expression of RNA polymerase III-transcribed genes (37). In the present study, we initially found that knockdown of FLNA enhanced the expression of TFIIB-related factor 1 (BRF1), general transcription factor III C subunit 2 (GTF3C2), and transcription factor Sp1. Both BRF1 promoter 4 (BRF1P4) and GTF3C2 promoter 2 (GTF3C2P2) contain putative Sp1-binding sites. These results suggest that Sp1 could modulate Pol III gene transcription by affecting the expression of BRF1 and GTF3C2 genes. We demonstrate that alteration of Sp1 expression affected expression of BRF1 and GTF3C2, Pol III transcription, and cell proliferation. Luciferase assays confirmed that Sp1 is required for the activities of BRF1P4 and GTF3C2P2. The data from ChIP assays and co-IP assays revealed that Sp1 could promote the occupancies of TBP, TFIIA α , and p300 at the BRF1P4 and GTF3C2P2 promoters by interacting with these factors.

Results

FLNA inhibits BRF1, GTF3C2, and Sp1 expression

Our previous work has shown that FLNA differentially modulates transcription of the Pol III-transcribed genes in transformed cell lines and that FLNA's actin-binding domain can mediate the regulation of Pol III-directed gene transcription (37). When investigating the mechanism by which FLNA regulates Pol III gene transcription, we found that FLNA depletion increased the expression of BRF1 and GTF3C2 genes in SaOS2 cell lines (Fig. 1, A and B). These data suggest that FLNA can regulate Pol III gene transcription using different molecular mechanisms. To verify this finding, we generated HeLa and 293T cell lines stably expressing FLNA shRNA or HA-FLNA and their control cell lines, using the shRNA-expressing lentiviral vectors and packaging vectors. The expression of FLNA, BRF1, and GTF3C2 in these cell lines was analyzed by RT-qPCR and Western blotting. As expected, FLNA depletion enhanced BRF1 and GTF3C2 expression in HeLa and 293T cell lines (Fig. S1, A-D). In contrast, HA-FLNA expression in HeLa and 293T cells reduced BRF1 and GTF3C2 expression (Fig. 1 (C and D) and Fig. S1 (E-G)). These data indicate that FLNA can repress BRF1 and GTF3C2 expression, suggesting that FLNA can regulate Pol III gene transcription by influencing the expression of BRF1 and GTF3C2.

To understand how FLNA regulates BRF1 and GTF3C2 expression, we initially searched the promoters of BRF1 and GTF3C2 from the Eukaryotic Promoter Database (RRID: SCR_002485) and then analyzed transcription factor-binding motifs in both BRF1 and GTF3C2 promoters. Interestingly, there are four predicted promoters for the BRF1 gene, among which promoter 4 is over 14 kb away from the other three promoters (Fig. S2A). There are three predicted promoters for the GTF3C2 gene that overlap each other (Fig. S2B). We found that both the BRF1 promoter 4 (BRF1P4) and the GTF3C2 promoter 2 (GTF3C2P2) contain putative Sp1-binding sites (Fig. 1E and Fig. S3). Next, we tested whether alteration of FLNA expression affected expression of Sp1 expression in the established cell lines by RT-qPCR and Western blotting. Strikingly, knockdown of FLNA enhanced the expression of Sp1 in SaOS2, HeLa, and 293T cells (Fig. 1 (F and G) and Fig. S4 (A-D)). In contrast, HA-FLNA expression reduced the expression of Sp1 in 293T and HeLa cells (Fig. 1 (H and I) and Fig. S4 (E-G)). These results indicate that FLNA can inhibit Sp1 expression in transformed cell lines.

Whether FLNA inhibits expression of Sp1 mRNA at the transcriptional level or post-transcriptional is unclear. We next monitored the half-life of Sp1 mRNA in the 293T cell lines stably expressing Sp1 shRNA or control shRNA after Pol II transcription inhibitor actinomycin D was added into the cell lines. The result revealed that the half-life of Sp1 mRNA showed similar time between the Sp1-depleted cell line and the control cell line (Fig. S4*H*), suggesting that FLNA possibly represses the expression of Sp1 mRNA at the transcriptional level.

We next examined whether the effect of FLNA depletion on Sp1 expression could be extended to a normal cell line. The HUCEC cell lines (HUCEC is a normal cell line for HeLa cancer cells) stably expressing Sp1 shRNA or control shRNA were generated. Sp1 expression and Pol III gene transcription were analyzed by Western blotting and RT-qPCR. Knockdown of FLNA in HUCEC cells enhanced Sp1 expression and transcription of most Pol III-transcribed genes tested in this assay (Fig. S5). These data indicate that FLNA can inhibit both Sp1 expression and Pol III gene transcription in normal cell lines. Collectively, the above results, along with our previous findings (37), confirm that alteration of FLNA expression can simultaneously affect Pol III-directed gene transcription and expression of BRF1, GTF3C2, and Sp1 genes. Because BRF1 and GTFC3C2 belong to a subunit of TFIIIB and TFIIIC, respectively, and their promoters, BRF1P4 and GTF3C2P2, contain putative Sp1-binding sites, we sought to establish whether Sp1 affected the regulation of Pol III gene transcription by influencing BRF1 and TFIIIC2 expression.

Sp1 is required for RNA polymerase III gene transcription

To investigate whether Sp1 affects the regulation of Pol III– directed gene transcription, we generated 293T and HeLa cell lines stably expressing Sp1 shRNA or control shRNA using the Sp1 shRNA–expressing lentiviral and packaging vectors (Fig. 2 (*A* and *B*) and Fig. S6 (*A* and *B*)). Expression of the Pol III–transcribed genes was analyzed by RT-qPCR, and the data are graphically presented in Fig. 2*C* and Fig. S6*C*.





Figure 1. FLNA inhibits expression of BRF1, GTF3C2, and Sp1 genes. *A* and *B*, knockdown of FLNA increased BRF1 and GTF3C2 expression in SaOS2 cells. SaOS2 cell lines stably expressing FLNA shRNA or control shRNA were cultured and used to analyze the expression of FLNA, BRF1, and GTF3C2 by RT-qPCR (*A*) using the primer pairs, BRTF1 *versus* BRTF1 and 3C2RTF1 *versus* 3C2RTR1 (Fig. S2), and by Western blotting with the antibodies indicated in *B*. *C* and *D*, expression of HA-FLNA inhibited BRF1 and GTF3C2 expression in HeLa cells. A HeLa cell line stably expressing HA-FLNA and its control cell line were cultured and used for the analysis of HA-FLNA, BRF1, and GTF3C2 expression by RT-qPCR (*C*) and Western blotting (*D*). *E*, diagram showing putative Sp1-binding sites in *BRF1P4* and *GTF3C2P2*. *F* and *G*, knockdown of FLNA increased Sp1 expression in SaOS2 cells. The SaOS2 cell lines stably expressing FLNA shRNA or control shRNA were cultured and processed as in *A* and *B*. Sp1 expression was determined by RT-qPCR (*F*) and Western blotting (*G*). *H* and *I*, HA-FLNA expression vas analyzed by RT-qPCR (*H*) and Western blotting (*I*). Each column in *A*, *C*, *F*, and *H* represents the mean \pm S.D. (*error bars*) of three independent experiments. *, $p \le 0.05$; **, $p \le 0.01$. *p* values were obtained by one-way ANOVA.

Strikingly, knockdown of Sp1 significantly reduced the expression of the Pol III–transcribed genes examined in this assay, except for the expression of the *7SL RNA* gene, which showed a moderate reduction in 293T and HeLa cells. Together, these data indicate that Sp1 can play a positive role in Pol III– directed gene transcription. Because both *BRF1P4* and *GTF3C2P2* contain putative Sp1-binding sites, we next examined whether Sp1 depletion affected BRF1 and GTF3C2 expression. Knockdown of Sp1 dampened BRF1 and GTF3C2 expression in both 293T and HeLa cells (Fig. 2 (*D*

and *E*) and Fig. S6 (*D* and *E*)). To substantiate these results, we generated a 293T cell line stably expressing mCherry-Sp1 and its control cell line. Pol III gene transcription and expression of BRF1 and GTF3C2 were analyzed using these cell lines. The data from RT-qPCR and Western blotting confirmed that expression of mCherry-Sp1 augmented Pol III gene transcription and expression of *BRF1* and *GTF3C2* genes (Fig. 2, *F*–*H*). A comparable result was also observed using the HeLa cells transiently expressing mCherry-Sp1 (Fig. S7, A–D). In addition, the expression of the RNA-Met



Figure 2. Sp1 is required for Pol III gene transcription. *A* and *B*, Sp1 expression analysis in the 293T cell lines expressing Sp1 shRNA or control shRNA. 293T cells were transfected with the lentiviral vector expressing Sp1 shRNA or control shRNA and the lentiviral packaging vector PH1 and PH2. Sp1 expression in 293T cell lines was analyzed by RT-qPCR (*A*) and Western blotting (*B*). *C*, knockdown of Sp1 inhibited Pol III gene transcription in 293T cells. 293T cell lines stably expressing Sp1 shRNA or control shRNA were cultured and used to analyze Pol III gene transcription by RT-qPCR. *D* and *E*, knockdown of Sp1 inhibited expression of *BRF1* and *GTF3C2* genes. The 293T stable cell lines obtained in *A* and *B* were cultured and used to monitor the expression of *BRF1* and *GTF3C2* genes by RT-qPCR (*D*) and Western blotting with the antibodies indicated in *E*. *shR*, shRNA. *F*, analysis of Western blotting for a 293T cell line stably expressing mCherry-Sp1 and its control cell line. *mCh-Sp1*, mCherry-Sp1. *G*, expression of mCherry-Sp1 enhanced Pol III gene transcription. A 293T stable cell line expressing mCherry-Sp1 and its control cell line obtained in *F* were used for the analysis of Pol III gene transcription by RT-qPCR. *H*, expression of mCherry-Sp1 enhanced BRF1 and GTF3C2 protein expression. A 293T stable cell line expressing mCherry-Sp1 and its control cell line obtained in *F* were used for the analysis of Pol III gene transcription by RT-qPCR. *H*, expression of mCherry-Sp1 enhanced BRF1 and GTF3C2 protein expression. A 293T stable cell line expressing mCherry-Sp1 and its control cell line obtained in *L* were used for the analysis of Pol III gene transcription by RT-qPCR. *H*, expression of mCherry-Sp1 enhanced BRF1 and GTF3C2 protein expression. A 293T stable cell line expressing mCherry-Sp1 and its control cell line obtained in *L* were used for the analysis of Pol III gene transcription by RT-qPCR. *H*, expression of mCherry-Sp1 enhanced BRF1 and GTF3C2 protein expression. A 29



gene showed a more significant response by Sp1 shRNA expression or overexpression than other genes (Fig. 2, *C* and *G*). This suggests that Sp1 modulates Pol III gene transcription in a gene type–specific manner. Altogether, the above data confirm that Sp1 is required for Pol III gene transcription.

Although BRF1 and GTF3C2 act as a subunit of TFIIIB and TFIIIC, respectively, whether alteration of BRF1 or GTF3C2 expression affects Pol III gene transcription is unclear. To address this question, we performed transfection assays using 293T cells and BRF1 or GTF3C2 double-stranded siRNA. Pol III gene transcription was analyzed by RT-qPCR using the cells from transient transfection. Fig. 21 illustrates that knockdown of BRF1 inhibited expression of the Pol III-transcribed genes examined in this assay. In a parallel experiment with GTF3C2 siRNA, knockdown of GTF3C2 by siRNA also repressed Pol III gene transcription (Fig. 2J). Unexpectedly, BRF1 and GTF3C2 depletion can repress the expression of the U6 RNA gene (Fig. 2, *I* and *J*). This result seemed to conflict with the previous conception that BRF and GTF3C2 are not required for transcription initiation of the U6 RNA gene (1-3). However, BRF1 and GTF3C2 might indirectly regulate expression of the U6 RNA gene. Taken together, these data confirm that BRF1 and GTF3C2 can positively regulate Pol III gene transcription, suggesting that Sp1 may regulate Pol III gene transcription by influencing BRF1 and GTF3C2 expression.

As described above, there are four predicted promoters upstream of the BRF1 gene, and BRF1P4 is far from BRF1P1-3. Thus, we next asked whether Sp1 depletion differentially affects the products transcribed by different promoters. To answer this question, we designed and synthesized a new primer pair (BRTF2 and BRTR2) downstream of BRF1P4 (Figs. S2 and S3). Two types of transcription products within the *BRF1* gene were analyzed by RT-qPCR using 293T cell lines with or without Sp1 depletion. RT-qPCR data showed that knockdown of Sp1 reduced both types of products from the BRF1 gene compared with those in the control cell line. However, the level of product detected by the primer pair BRTF2R2 is slightly lower than that detected by the primer pair BRTF1R1 (Fig. S8A). This result is rational because both BRF1P4 and BRF1P1 promoters contain Sp1-binding sites (Fig. S3, A and B), and their transcriptional products can be influenced by Sp1 depletion. In addition, the transcriptional products detected by the BRTF1R1 include those synthesized by the promoters of BRF1P4 and BRF1P1-3. As expected, Sp1 depletion also reduced the products of the GTF3C2 gene detected by the primer pairs 3C2RTF1R1 and 3C2RTF2R2, when compared with those in the control cell line. However, the level of product detected by the 3C2RTF1R1 was significantly lower than that detected by the 3C2RTF2R2 (Fig. S8B). This result is likely due to the difference in the distance of two primer pairs from the promoters. The closer the promoter is, the more abundant the products are because transcription termination or pausing often occurs during transcription elongation.

Pol III gene expression levels closely correlate with the state of cell growth (1). Thus, we next tested the effect of Sp1 expression change on the proliferation of the transformed cell lines. 293T and HeLa stable cell lines were used for cell proliferation assays using cell-counting and MTT methods. The data revealed that knockdown of Sp1 reduced cell proliferation for both 293T and HeLa cells (Fig. 3, A-D). In contrast, the expression of mCHerry-Sp1 in 293T cells promoted cell proliferation (Fig. 3, *E* and *F*). These results indicate that Sp1 can positively regulate cell proliferation in addition to Pol III gene transcription.

Sp1 is involved in the regulation of Pol III–directed gene transcription mediated by FLNA

It was observed that the FLNA-depleted cell lines exhibit increased expression for BRF1, GTF3C2, and Sp1 genes (Fig. 1 and Figs. S2 and S3), and alteration of Sp1 expression affects transcription of the Pol III-transcribed genes in both 293T and HeLa cells. We therefore supposed that Sp1 might also regulate Pol III gene transcription in the FLNA-depleted cells. SaOS2 and HeLa cell lines, stably expressing FLNA shRNA, were further transfected with the lentiviral vector expressing Sp1 shRNA and packaging vectors. The stable cell lines expressing both FLNA shRNA and Sp1 shRNA were screened in 96-well plates using RT-qPCR and Western blotting. The cell lines expressing both FLNA shRNA and Sp1 shRNA showed a reduced level of Sp1 compared with the cell lines expressing FLNA shRNA only (Fig. 4 (A and B) and Fig. S9 (A and B)). Pol III gene transcription was then analyzed by RT-qPCR using the total RNA extracted from the cell lines. As shown in Fig. 4C and Fig. S9C, knockdown of both FLNA and Sp1 caused the repression of Pol III-mediated gene transcription when compared with knockdown of FLNA only, indicating that Sp1 is involved in the regulation of Pol III-mediated transcription in the FLNA-depleted cells. As expected, knockdown of FLNA enhanced Pol III gene transcription compared with the control cell line (Fig. 4C and Fig. S9C). The latter result is consistent with that obtained in our previous work (37). We next examined whether knockdown of both FLNA and Sp1 affected expression of BRF1 and GTF3C2. It was observed that both FLNA and Sp1 depletion inhibited BRF1 and GTF3C2 expression compared with FLNA depletion only (Fig. 4 (D and E) and Fig. S9 (D and E)), whereas FLNA depletion increased their expression compared with the control cell line (Fig. 4 (D and E) and Fig. S9 (D and E)). The result obtained from cell proliferation assays is similar to that obtained from Pol III transcription analysis (Fig. 4 (F and G) and Fig. S9 (F and G)). Taken together, these results confirm that Sp1 is also required for Pol IIIdirected gene transcription, BRF1 and GTF3C2 expression, and cell proliferation regardless of FLNA depletion.

Sp1 positively regulates the activities of the BRF1 and GTF3C2 gene promoters in the cell lines with or without FLNA depletion

Sp1 positively regulates BRF1 and GTF3C2 mRNA expression in 293T and HeLa cells (Fig. 2 (*D* and *H*) and Figs. S6*D* and S7*C*). We therefore asked whether Sp1 modulated *BRF1* and *GTF3C2* gene expression at the transcriptional level. To address this question, we performed transient transfection assays using 293T and HeLa cell lines stably expressing Sp1 shRNA or control shRNA and the vector expressing a reporter gene driven by *BRF1*P4 or the *GTF3C2*P2. Luciferase activity was then monitored using the cell lysate from transient trans-



Figure 3. Sp1 promotes cell proliferation. *A*–*D*, Sp1 knockdown inhibited 293T and HeLa cell proliferation. An equal number of 293T (*A* and *B*) or HeLa (*C* and *D*) cell lines stably expressing Sp1 shRNA or control shRNA were seeded in 12-well or 96-well plates. The cells grown in the 12-well plates were harvested at different time points and counted under a light microscope. The data were processed and presented in *A* and *C*. The cells seeded in the 96-well plates, however, were directly used for MTT assays at different time points. The data were processed and presented in *B* and *D*. *E* and *F*, expression of mCherry-Sp1 enhanced 293T cell proliferation for a 293T cell line stably expressing mCherry-Sp1 and its control cell line was performed by cell counting (*E*) and an MTT assay (*F*). Each datum in the graph (*A*–*F*) represents the mean \pm S.D. (*error bars*) of three independent experiments. *, $p \leq 0.05$. *p* values were obtained by one-way ANOVA.

fection. Luciferase assays showed that knockdown of Sp1 down-regulated the activities of the *BRF1*P4 and *GTF3C2*P2 in both 293T and HeLa cells (Fig. 5, *A* and *B*). In contrast, mCherry-Sp1 expression in 293T cells enhanced the activities of the *BRF1*P4 and *GTF3C2*P2 (Fig. 5*C*). These data indicate that Sp1 is required for the activities of the *BRF1*P4 and *GTF3C2*P2.

We next examined whether the putative Sp1-binding sites (PSBSs) regulated the activities of the *BRF1*P4 and *GTF3C2*P2. Therefore, the effect of the PSBS mutations on the activities of the *BRF1*P4 and *GTF3C2*P2 was analyzed by reporter assays (Fig. 5D). Luciferase assays revealed that mutations of Sp1-binding sites dampened the activities of the *BRF1*P4 and *GTF3C2*P2 in both 293T and HeLa cells (Fig. 5, *E* and *F*), demonstrating that the Sp1-binding sites play a positive role in the activities of the *BRF1*P4 and *GTF3C2*P2. These results confirm that Sp1 regulates transcription of *BRF1* and *GTF3C2*P2.

Because the levels of BRF1, GTF3C2, and Sp1 mRNA were enhanced by FLNA depletion (Fig. 1 and Figs. S1 and S4) we supposed that Sp1 could regulate the activities of BRF1 and TFIIIC2 promoters in the cell line with FLNA depletion. We next performed transfection assays using the SaOS2 or HeLa cell lines indicated in Fig. 5 (*G* and *H*) and the vectors expressing a reporter gene driven by *BRF1*P4 or *GTF3C2*P2. Luciferase assays were then performed using the cell lysate from transient transfection. The data showed that knockdown of both FLNA and Sp1 caused a significant reduction in the activities of *BRF1*P4 and *GTF3C2*P2 compared with knockdown of FLNA only, whereas FLNA depletion elicited a significant increase in promoter activity compared with the control cell line. These results indicate that Sp1 is also required for the transcriptional activities of *BRF1*P4 and *GTF3C2*P2 in the cell lines with FLNA depletion (Fig. 5, *G* and *H*). Together, these data show that Sp1 can positively regulate the activities of the *BRF1*P4 and *GTF3C2*P2 independently of FLNA expression.

Sp1 promotes the recruitment of TBP, TFIIA α , p300, and Pol II at the BRF1P4 and GTF3C2P2 promoters

To understand how Sp1 modulates *BRF1* and *GTF3C2* gene transcription, we initially determined whether Sp1 bound to *BRF1*P4 and *GTF3C2*P2 *in vivo* by ChIP assays. The data from the ChIP assay confirmed that Sp1 could bind to the *BRF1*P4 and *GTF3C2*P2 in 293T cells (Fig. 6A). To verify this result, we performed ChIP assays using a 293T Sp1-depleted cell line and its control cell line. Fig. 6B shows that knockdown of Sp1 sig-





Figure 4. Sp1 is involved in the regulation of the Pol III gene expression in the cell line with FLNA depletion. *A* and *B*, Sp1 expression analysis in the SaOS2 cell line stably expressing both FLNA shRNA and Sp1 shRNA or FLNA shRNA only and the control cell line by RT-qPCR and Western blotting. The SaOS2 cell line with FLNA depletion was transfected with the lentiviral vector expressing Sp1 shRNA and the lentiviral packaging vector PH1 and PH2. SaOS2 stable cell lines expressing both FLNA shRNA and Sp1 shRNA were determined by RT-qPCR (*A*) and Western blotting (*B*) assays. *C*, knockdown of both FLNA and Sp1 repressed Pol III gene transcription. Three SaOS2 cell lines, as indicated in the graph, were cultured and harvested for the analysis of the Pol III gene transcription by RT-qPCR. *D* and *E*, knockdown of both FLNA and Sp1 reduced *BRF1* and *GTF3C2* gene expression. The SaOS2 cell lines indicated in the graph were cultured in the saOS2 cell lines indicated in the graph were cultured in SaOS2 cell lines indicated in the graph were cultured in the saOS2 cell lines indicated in the graph were cultured in the saOS2 cell proliferation. An equal number of cells from three SaOS2 stable cell lines indicated in the graph (*F* and *G*) were cultured in 12-well or 96-well plates. Cell proliferation assays were performed using cell-counting (*F*) or MTT (*G*) methods. Each column in the bar graphs represents the mean \pm S.D. (error bars) of three independent experiments. *, $p \le 0.05$; **, $p \le 0.01$. p values were obtained by one-way ANOVA.

nificantly reduced Sp1 occupancy at the promoters *BRF1*P4 and *GTF3C2*P2 when compared with the control cell line. This was also observed by ChIP assays with the stable cell line expressing both Sp1 shRNA and FLNA shRNA and its control cell lines (Fig. S10, *A* and *B*). These findings indicate that Sp1 can bind to *BRF1*P4 and *GTF3C2*P2 *in vivo* and modulate *BRF1* and *GTF3C2* gene transcription.

It has been shown that Sp1 regulates Pol II-directed gene transcription by interacting with numerous factors, including

TBP, TFIIA, c-MYC, p300, and other factors (15). In this work, we have demonstrated that Sp1 can bind to the *BRF1*P4 and *GTF3C2*P2 and that alteration of Sp1 expression affected BRF1 and GTFC3C2 mRNA expression. However, how alteration of Sp1 expression affected BRF1 and GTF3C2 expression was not fully understood. To answer this question, we performed ChIP assays using a 293T Sp1-depleted cell line and its control cell line with the antibodies against the respective factors shown in Fig. 6 (*C* and *D*). ChIP-qPCR data showed that knockdown of



Figure 5. Sp1 promotes the activities of the *BRF1* **promoter 4 and the** *GTF3C2* **promoter 2.** *A* and *B*, Sp1 knockdown reduced the activities of *BRF1*P4 and *GTF3C2*P2. 293T (*A*) and HeLa (*B*) cell lines stably expressing Sp1 shRNA or control shRNA were transfected with the *BRF1*P4- or *GTF3C2*P2-driving reporter vector, and luciferase assays were performed using the cells from transient transfection. *RLA*, relative luciferase activity. *C*, expression of mCherry-Sp1 enhanced the activities of *BRF1*P4 and *GTF3C2*P2. The *BRF1*P4- or *GTF3C2*P2-driving reporter vectors were transfected into the 293T stable cell line expressing mCherry-Sp1 or the control cell line. Luciferase assays were performed as for *A* and *B*. *D*, diagram showing putative Sp1-binding sites and PSBS mutated bases in the promoters *BRF1*P4 and *GTF3C2*P2. *E* and *F*, effect of Sp1-binding site mutation on the activities of *BRF1*P4 and *GTF3C2*P2. The promoters presented in *C* were cloned into the reporter vectors. *Gand H*, knockdown of both FLNA and Sp1 inhibited the activities of *BRF1*P4 and *GTF3C2*P2. SaOS2 and HeLa stable cell lines as indicated in the graph were transfected with the reporter vectors driven by *BRF1*P4 or the *GTF3C2*P2. and luciferase assays were performed using the cell lines a sindicated in the graph were transfected with the reporter vectors driven by *BRF1*P4 or the *GTF3C2*P2. and HeLa stable cell lines as indicated in the graph were transfected with the reporter vectors driven by *BRF1*P4 or the *GTF3C2*P2. and luciferase assays were performed using the cell lines as indicated in the graph were transfected with the reporter vectors driven by *BRF1*P4 or the *GTF3C2*P2. and luciferase assays were performed using the cell lines as indicated in the graph were transfected with the reporter vectors driven by *BRF1*P4 or the *GTF3C2*P2. and luciferase assays were performed using the cell lines as indicated in the graph were obtained by one-way ANOVA.

Sp1 reduced the occupancies of Sp1, TBP, TFIIA α , p300, and Pol II at the promoters *BRF1*P4 and *GTF3C2*P2 (Fig. 6, *C* and *D*). A consistent result was also obtained from the ChIP assays

with the cell lines expressing both Sp1 shRNA and FLNA shRNA or FLNA shRNA only (Fig. S10, *C* and *D*). These results confirm that Sp1 depletion can inhibit the recruitment of TBP,





Figure 6. Sp1 promotes the recruitments of TBP, TFIIA, p300, and Pol II at the promoters *BRF1P4* **and** *GTF3C2P2. A*, Sp1 binds to the promoters *BRF1P4* and *GTF3C2P2 in vivo.* 293T cells were cultured in 10-cm dishes. At 85% confluence, the cells were fixed and harvested for ChIP assays using the anti-Sp1 antibody or control IgG. Quantitative PCR for each reaction was performed using one-fortieth of the DNA recovered from ChIP assay or 1 ng of genomic DNA using the primers of *BRF1P4* and *GTF3C2P2*. Relative occupancy was obtained by comparing the relative quantity of promoter DNA from a ChIP-qPCR with that from the qPCR with 1 ng of genomic DNA, which is equivalent to 0.01% of input DNA. *B*, Sp1 knockdown reduced Sp1 occupancy at *BRF1P4* and *GTF3C2P2*. The 293T cell lines stably expressing Sp1 shRNA or control shRNA were cultured for ChIP assays. The relative occupancy for Sp1 at the promoters *BRF1P4* and *GTF3C2P2* was obtained as described in *A*. *C* and *D*, Sp1 knockdown reduced the occupancies of TBP, TFIIA α , p300, and Pol II at *BRF1P4* and *GTF3C2P2*. ChIP assay was performed with the cell lines used for *B* and the antibodies as indicated in the graph. *E* and *F*, expression of mCherry-Sp1 enhanced the recruitments of TBP, TFIIA α , p300, and Pol II at *BRF1P4* and *GTF3C2P2*. A 293T stable cell line expressing mCherry-Sp1 and its control cell line were used for ChIP assays as for *C* and *D*. ChIP assays from *C*-*F* were performed as described in *A*. The relative occupancy for individual factors at *BRF1P4* and *GTF3C2P2* was obtained as described in *A*. Each *column* in the graphs represents the mean \pm S.D. (*error bars*) of three independent experiments. *, $p \le 0.05$; **, $p \le 0.01$. *p* values were obtained by one-way ANOVA.

TFIIAα, p300, and Pol II at the *BRF1*P4 and *GTF3C2*P2 regardless of the presence of FLNA. Unexpectedly, the occupancy of c-MYC at these two promoters showed little difference between the Sp1-depleted cells and the control cells (Fig. 6 (C and D) and Fig. S10 (C and D)). To substantiate these results, we performed ChIP assays using a 293T cell line expressing mCherry-Sp1 and its control cell line. As expected, mCherry-Sp1 expression enhanced the occupancies of Sp1, TBP, TFIIA α , p300, and Pol II at BRF1P4 and GTF3C2P2, but did not affect the occupancy of c-MYC at these promoters (Fig. 6, *E* and *F*). The data from ChIP assays confirm that Sp1 promotes the occupancies of TBP, TFIIA α , p300, and Pol II at *BRF1*P4 and GTF3C2P2 but does not affect the occupancy of c-MYC. These findings suggest that Sp1 can positively regulate BRF1 and GTF3C2 gene transcription by promoting the recruitment of specific factors at BRF1P4 and GTF3C2P2.

Sp1 interacts with TBP, TFIIA α , and p300 but not with c-MYC

To uncover the detailed mechanism by which Sp1 regulates Pol III–mediated gene transcription, we initially examined the effect of Sp1 expression on the factors that directly regulate Pol III–mediated gene transcription. HEK293T cell lines expressing Sp1 shRNA or mCherry-Sp1 and their control cell lines were cultured in 6-well plates. Cells were harvested for Western blot analysis. As shown in Fig. 7A, mCherry-Sp1 expression enhanced TBP protein expression, whereas knockdown of Sp1 down-regulated TBP expression in this cell type (Fig. 7B). This result demonstrates that TBP expression is affected by Sp1 expression. Because the TBP promoter 1 contains a putative Sp1-binding site (Fig. S11), alteration of Sp1 expression could influence TBP expression. However, we observed that Sp1 expression change did not affect the expression of POLR3D, a subunit of Pol III (Fig. 7, A and B). To determine whether alteration of Sp1 expression affects the expression of TFIIA α , c-MYC, and p300, we performed Western blot analysis using the 293T cell line with Sp1 depletion or overexpression. As shown in Fig. 7 (A and B), either Sp1 depletion or mCherry-Sp1 expression did not influence expression of TFIIA α , c-MYC, and p300. Thus, the unchanged expression of TFIIA α , c-MYC, and p300 did not contribute to the alteration of the occupancies for these factors observed on BRF1P4 and GTF3C2P2. To understand how alteration of Sp1 expression influences the occupancies of TBP, TFIIA α , and p300 at *BRF1*P4 and *GTF3C2*P2, we performed co-IP assays, using 293T nuclear extract and the



Figure 7. Sp1 interacts with TBP, TFIIA*α*, **and p300**. *A*, effect of the mCherry-Sp1 expression on the expression of transcription factors, POLR3D and p300. A 293T cell line stably expressing mCherry-Sp1 and its control cell line were cultured in 6-well plates. After 48 h, cells were harvested, and protein expression was analyzed by Western blotting with the antibodies as indicated. *mch-Sp1*, mCherry-tagged Sp1. *B*, effect of Sp1 knockdown on the expression of transcription factors, POLR3D and p300. The 293T stable cell lines expressing Sp1 shRNA or control shRNA were used to analyze protein expression by Western blotting as described in *A*. *C* and *D*, Sp1 interacts with TBP, TFIIA*α*, and p300 but not with c-MYC. The nuclear extract from 293T cells was prepared and used for co-IP assays using Sp1 antibody (C) or the antibodies against TBP, TFIIA*α*, *c*-MYC, and p300 (*D*). Western blotting was performed using the protein recovered from IP assays and the antibodies indicated in *C* and *D* in which 5% of the input sample was used as a positive control.

antibodies against the respective factors indicated in Fig. 7 (*C* and *D*). Strikingly, the Sp1 antibody was able to precipitate with Sp1, TBP, TFIIA α , and p300 proteins but not with c-MYC protein (Fig. 7*C*). In reciprocal assays, TBP, TFIIA α , c-MYC, and p300 antibodies were able to precipitate with Sp1 (Fig. 7*D*). c-MYC showed a weaker interaction with Sp1 than TBP, TFIIA α , and p300 (Fig. 7*D*). These data indicate that Sp1 can interact with TBP, TFIIA α , and p300 in 293T cells. Therefore, the data from both ChIP assays and co-IP assays confirm that Sp1 may modulate the recruitments of TBP, TFIIA α , and p300 at *BRF1*P4 and *GTF3C2*P2 by interacting with these factors.

Based on the data obtained in this study, we propose a model whereby Sp1 regulates Pol III–mediated gene transcription. In this model, FLNA inhibits Sp1 expression, but Sp1 can bind to the *BRF1P4* and *GTF3C2P2* through the Sp1-binding site; subsequently recruits TBP, TFIIA α , p300, and Pol II at both promoters by interacting with these factors; and positively regulates *BRF1* and *GTF3C2* gene expression. Next, BRF1 and GTF3C2 proteins are respectively assembled with the other subunits of TFIIIB and TFIIIC, and the resulting TFIIIB and GTF3C2 are recruited at the promoters of the Pol III genes to promote Pol III gene transcription (Fig. 8).

Discussion

In the present study, we have shown that alteration of Sp1 expression in 293T and HeLa cells affected transcription of the Pol III-transcribed genes and expression of BRF1 and GTF3C2 genes. Furthermore, knockdown of BRF1 or GTF3C2 inhibited Pol III-directed gene transcription. These findings suggest that Sp1 regulates Pol III gene transcription by controlling BRF1 and GTF3C2 expression. Unexpectedly, we found that either BRF1 or GTF3C2 depletion could reduce U6 RNA expression (Fig. 2, I and J). This result is in conflict with our current understanding of the transcription of the U6 RNA gene because transcription initiation for the U6 RNA gene does not require BRF1 and GTF3C2 participation (38). Knockdown of BRF1 or GTF3C2 possibly affects the expression of the unknown factors that can directly or indirectly regulate U6 RNA expression. Alternatively, cells might regulate the expression of the U6 RNA gene to adapt to the cellular environmental change caused by the depletion of BRF1 or GTF3C2 through an unknown mechanism. The results obtained in this work confirmed that Sp1 can positively modulate Pol IIIdirected gene transcription in transformed cells with or without FLNA depletion. This finding has extended the roles of Sp1 in the regulation of gene transcription.





Figure 8. Diagram showing the mechanism that Sp1 modulates Pol III gene transcription. Transcription initiation for the tRNA gene is presented in this regulatory model, although Pol III transcriptional initiation contains three typical modes.

Many clinical cancer specimens contain abnormally high levels of Pol III gene products (1, 10, 39). Fast growth for cancer cells requires dynamic protein synthesis, where Pol III products such as 5S rRNA and tRNA play an essential role (40). Thus, inhibition of Pol III gene expression caused by Sp1 depletion could indirectly affect cell proliferation (Fig. 3). It has been documented that abnormally high expression of Sp1 is one of the "hallmarks" for cancer cells (15) and that Sp1 takes a part in the regulation of Pol II gene transcription and cell proliferation by interacting with a number of signaling factors, including EGFR, FGF, and IGF (15, 19, 41, 42). Here we confirm that Sp1 can simultaneously regulate Pol III gene transcription and cell proliferation (Figs. 2 and 3 and Figs. S6 and S7), confirming that there is a link between cell proliferation and Pol III gene transcription. It is well-known that Pol III transcription products have an impact on the efficiency of global protein synthesis that governs the state of cell growth. However, the detailed mechanism underlying this event remains to be investigated. A number of studies have shown that CX-5461 and BMH-21 can inhibit RNA polymerase I gene transcription and show anticancer activities (43, 44). It is also possible that targeting Pol III transcription with inhibitors would be a potential anti-cancer strategy. To this end, understanding the regulatory mechanism of Pol III gene transcription in cancer cells will be important.

Sp1 can interact with general transcription factors, specific transcription factors, and chromatin-remodeling factors to regulate transcription of specific genes (15). In this study, we show that Sp1 promotes the occupancies of TBP, TFIIA α , p300, and Pol II at *BRF1*P4 and *GTF3C2*P2 (Fig. 6 and Fig. S10). Co-IP assays confirmed that Sp1 was able to interact with TBP, TFIIA α , and p300. Thus, the Sp1-binding sites likely attract Sp1 to associate with *BRF1*P4 and *GTF3C2*P2, which subsequently recruit TBP, TFIIA α , and p300 at the promoters. TBP expression change caused by Sp1 depletion or overexpression could

also contribute to the occupancy of TBP at *BRF1*P4 and *GTF3C2*P2. Indeed, Pol II recruitment at the promoters could be indirectly affected by Sp1. Pol II recruitment depends on TBP and TFIIA α required for Pol II–directed transcriptional initiation assembly (45). Furthermore, p300 can act as a transcription coactivator to regulate gene transcription (46). Therefore, Sp1 depletion inhibits *BRF1* and *GTF3C2* gene transcription, possibly by reducing the occupancies of TBP, TFIIA α , p300, and Pol II at the promoters of *BRF1* and *GTF3C2*.

Transcription factor c-MYC can modulate Pol III– directed gene transcription by interacting with TFIIIB (47). In this study, we demonstrated that c-MYC can also be recruited at the BRF1 and GTF3C2 promoters, although knockdown of Sp1 did not affect c-MYC binding to either of promoters. This result suggests that Sp1 and c-MYC regulate BRF1 and GTF3C2 expression independently.

In summary, we found that Sp1 promotes Pol III– directed gene transcription in the cell lines with or without FLNA depletion and that Sp1 modulates this process by affecting *BRF1* and *GTF3C2* gene expression. The data from ChIP assays suggest that Sp1 regulates *BRF1* and *GTF3C2* gene transcription by influencing the occupancies of TBP, TFIIA α , and p300 at *BRF1*P4 and *GTF3C2*P2. Co-IP assays confirmed that Sp1 can interact with TBP, TFIIA α , and p300. These data suggest that Sp1 regulates the occupancies of TBP, TFIIA α , and p300 at *BRF1*P4 and *GTF3C2*P2 by interacting with these factors. This study identifies a novel role of Sp1 in Pol III– directed transcription and sheds light on how Sp1 regulates cancer cell proliferation.

Experimental procedures

Plasmids, gene cloning, cell culture, and reagents

The shRNA-expressing lentiviral plasmids, including pLV-U6-EGFP-Puro and pLV-U6-mCherry-Puro, were purchased



from Inovogen Tech Co. (China). The reporter vector pGL3basic was purchased from Promega Co. Three different Sp1 shRNA cDNA fragments were cloned downstream of the U6 promoter in the pLV-U6-mCherry-Puro plasmid. A HA-FLNA fusion gene was cloned downstream of the CMV promoter in the pLV-U6-CMV-EGFP-Puro plasmid that was modified from the pLV-U6-EGFP-Puro (Inovogen). BRF1P4 and GTF3C2P2, along with their Sp1-binding site mutant derivatives, were cloned into the reporter vector pGL3-basic (Fig. S3 and Fig. 5D). HeLa and transformed HEK293 (293T) cells were cultured in a high-glucose Dulbecco's modified Eagle's medium (HyClone Co.) with the supplement of 10% fetal bovine serum (AusGenex) and 1× penicillin/streptomycin (Thermo Scientific), whereas SaOS2 cells were cultured in a McCoy's 5A complete medium (HyClone Co.). The lentiviral particles expressing five different FLNA shRNA molecules and the lentiviral particles expressing control shRNA were purchased from Santa Cruz Biotechnology, Inc. Luciferase activity detection kits were obtained from Promega Co. Restriction enzymes and PCR and RT-qPCR reagents were purchased from Thermo Scientific. All chemical reagents were obtained from Sigma-Aldrich (Merck).

Generation of stable cell lines

HeLa, 293T, and SaOS2 cell lines that stably express FLNA shRNA or control shRNA were generated by transduction with the lentiviral particles expressing FLNA shRNA or control shRNA (Santa Cruz Biotechnology). These cell lines were obtained by screening from single-cell colonies as described previously (37) and determined using RT-qPCR and Western blotting. To obtain the cell lines stably expressing both FLNA shRNA and Sp1 shRNA, we transfected HeLa and SaOS2 cell lines expressing FLNA shRNA using the lentiviral vectors expressing three different Sp1 shRNA molecules and the packaging vector PH1 and PH2 (Inovogen Tech Co.). The cell lines stably expressing both FLNA shRNA and Sp1 shRNA were screened using 96-well plates and determined by RT-qPCR and Western blotting. Using the same strategy, we generated 293T and HeLa cell lines stably expressing HA-FLNA as well as the cell lines expressing Sp1 shRNA or mCherry-Sp1.

RT-qPCR and Western blotting

Total RNA was prepared with the RNA miniprep kit (Axygen). Reverse transcription was performed in 20 μ l of reaction mixture containing 0.5 μ g of total RNA and 2 units of reverse transcriptase according to the manufacturer's manual (Thermo Scientific). qPCRs for Pol III-transcribed genes were performed with a Bio-Rad real-time detection system, SYBR Green reagent (Roche Applied Science), and the primers as described previously (37). PCR data were analyzed with Bio-Rad CFX manager 3.1. For the detection of BRF1 and GTF3C2 gene expression, qPCR was performed with the primer pairs, BRTF1 versus BRTR1 and 3C2RTF1 versus 3C2RTR1 (Fig. S2). Western blotting was performed using 10 µg of the cell lysate according to the standard procedure. The antibodies for Western blotting were obtained from Santa Cruz Biotechnology, Inc. (BRF1, SC-81405; GTF3C2, SC-81406; Sp1, SC-420), Sigma-Aldrich (HA, H9658; GAPDH, G9545), and Thermo Fisher Scientific (mCherry, PA5-34974).

Cell proliferation assays

Cell proliferation assays were performed with cell counting and MTT methods. For cell-counting assays, an equal number of cells (around 5×10^4) were seeded in the wells of 12-well plates, where each cell type was grown in triplicate. Cell samples were harvested every 24 h and counted with a hemocytometer under a light microscope. For MTT assays, an equal number of cells were seeded in the wells of 96-well plates, and MTT assays were performed every 24 h using MTT assay reagents according to the manufacturer's manual (Beyotime, Shanghai, China). All data for cell proliferation were subjected to statistical analysis.

Transient transfection and luciferase assays

Transient transfection was performed using the TurboFect transfection reagent (Thermo Scientific). 293T, HeLa, and SaOS2 cells were seeded in 12-well plates. 24 h later, cells were transfected with the vectors that express a reporter gene driven by *BRF1*P4 or *GTF3C2*P2 promoters and the vector expressing galactosidase. Cell samples were harvested at 48 h post-transfection and lysed with 50 μ l of the lysis buffer from luciferase detection kits (Promega). 3 μ l of cell lysate were used to determine the activities of luciferase and β -gal. The luciferase activity for each sample was normalized with the activity of β -gal from the same sample. Relative luciferase activity was obtained by comparing the luciferase activity in treatment cells with that in control cells.

ChIP assays

The 293T cell lines with or without Sp1 depletion were cultured in 10-cm dishes, followed by fixing for 10 min with 1% formaldehyde and chromatin fragmentation by sonication. ChIP assays were performed as described previously (37) except that the antibodies were replaced by the antibodies against Sp1, TBP, TFIIA, c-MYC, p300, and Pol II (Sp1, CST #9389S; TBP, SC-204; c-MYC, SC-40; p300, SC-48343; and Pol II, SC-21750). TFIIA α antibody was customized from Boster Co. (Wuhan, China). The DNA from each ChIP sample was eluted with 40 µl of double-distilled H₂O. 1 μ l of the DNA sample was used for a ChIP-qPCR. qPCRs were performed using SYBR Green reagent (Roche Applied Science) and a real-time PCR detection system (Bio-Rad) and the primers for the amplification of BRF1 and GTF3C2 promoter fragments. The relative occupancy was obtained by comparing the relative quantity of promoter DNA from a ChIP-qPCR with that from the qPCR of 1 ng of genomic DNA, which is equivalent to 0.01% of input DNA (the input DNA is the genomic DNA prepared from a ChIP assay sample).

Co-immunoprecipitation assays

293T cells were cultured in five 15-cm dishes. At 90% confluence, cells were harvested, and nuclei were prepared from the cells with buffer A (10 mm Hepes, 1.5 mm MgCl₂, 10 mm KCl, and 0.5 mm DTT) and a Dounce homogenizer. The nuclei were suspended in 2 ml of buffer C (20 mm Hepes, 1.5 mm MgCl₂, 420 mm NaCl, 0.5 mm DTT, 0.2 mm EDTA, and 20% glycerol), followed by dialyzing with buffer D (20 mm Hepes, 100 mm KCl, 0.5 mm DTT, 0.2 mm EDTA, and 20% glycerol). Nuclear extract



was obtained by vortexing for 30 min and centrifuged for 10 min at 12,000 rpm. For Co-IP assays, 10 µg of antibody or normal IgG was added into 200 µl of nuclear extract, and the resulting mixture was incubated at 4 °C overnight on a rotating rocker. 40 µl of protein A/G-agarose (Santa Cruz Biotechnology) were added into the sample containing the antibody. The resulting sample was continually incubated at 4 °C for 4 h and then washed four times with $1 \times$ PBS. When washing was finished, 30 µl of SDS loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, and 0.1% bromphenol blue) was added to the proteinbound agarose beads. The sample was boiled for 10 min at 100 °C in a heat block, and a co-IP sample was obtained by centrifuging for 2 min at 4000 rpm. Ten microliters of co-IP sample was used for Western blot analysis, using the antibodies as described for the ChIP assays, and 5% of input sample (5 μ g of protein) was used as a positive control in the Western blotting assay.

Data analysis

The mean and S.D. for the treatment and control samples in this work were obtained with GraphPad Prism 6.0 software. Each column in the bar graphs represents the mean \pm S.D. of three independent experiments. *p* values were obtained by one-way ANOVA.

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