Cytoskeletal Filamin A Differentially Modulates RNA Polymerase III Gene Transcription in Transformed Cell Lines*

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Cytoskeletal filamin A (FLNA) is an important protein involved in multiple cellular processes. Previous studies have shown that FLNA can promote or inhibit cancer growth and development; however, the mechanisms underlying these events are not fully understood. Here we show that, in both 293T and SaOS2 cells, knockdown of FLNA significantly enhanced transcription of RNA polymerase (pol) III-transcribed genes except for a subset of tRNA genes. In contrast, re-expression of FLNA in an FLNA-deficient melanoma cell line (A7) repressed pol III transcription in a cell type-specific manner. Chromatin immunoprecipitation assays revealed that the repression of pol III gene transcription by FLNA correlates with the decreased occupancy of the RNA pol III transcription machinery at promoters. Immunofluorescence microscopy and coimmunoprecipitation assays revealed that FLNA can associate with the RNA pol III transcription machinery through its actin-binding domain within nuclei. Mechanistic analysis revealed that FLNA suppresses pol III gene transcription by confining the recruitment of the RNA pol III transcription machinery at the promoters of the genes that are sensitive to the alteration of FLNA expression. These findings not only extend the understanding of FLNA function in cells but also provide novel insights into the mechanism by which FLNA represses cell proliferation.

Eukaryotic RNA polymerase (pol) III mediates the synthesis of many non-coding RNAs that include tRNA, 5S rRNA, U6 RNA, 7SL RNA, and other small RNAs. These RNA products account for about 15% of total cellular transcripts and are involved in the regulation of protein biogenesis, RNA splicing, protein transportation, and protein-coding gene transcription (1–3). In cancer, aberrant expression of pol III products strongly correlates with the transformed state (4–6). Abnormal high expression of pol III products in cancer is attributed to increased expression of pol III transcription factors, discharge of repression by tumor suppressors, and activation of oncogene expression (2).

Cytoskeletal filamin A (FLNA) is a 280-kDa protein comprised of two N-terminal calponin homology domains and a long C-terminal rod-like domain. FLNA cross-links with F-actin through the calponin homology domains to maintain the stability of the cellular 3D network. In addition, FLNA acts as a scaffold that binds to over 90 diverse functional proteins to regulate numerous cellular processes (7–9), including cell adhesion, migration, proliferation, angiogenesis, cell signaling, DNA repair, and transcription (10–18). It is well established that FLNA mutations cause several congenital diseases, including defects of the brain, heart, and skeleton (8). FLNA can play dual roles in cancer development; that is, FLNA can promote or inhibit cancer growth and development depending on particular circumstances (10). However, the mechanisms underlying these events are not fully understood.

We showed previously that FLNA can localize to the nucleolus, where it represses RNA gene transcription (18). We also demonstrated that loss of FLNA subsequently leads to an increase in cell proliferation. Indeed, it has been shown recently that increased proliferation of leukemic cells is mediated by pol I transcription escaping the repressive effect of FLNA (19). RNA polymerase I mediates the synthesis of the ribosomal RNAs 5.8S, 18S, and 28S. These rRNAs are required for ribosome assembly, along with the 5S rRNA that is transcribed by RNA pol II. We therefore hypothesized that FLNA might coordinate ribosome production by also regulating pol III-mediated gene transcription. Here we show that FLNA represses RNA pol III gene transcription by decreasing the occupancy of the pol III transcription machinery at promoters and that FLNA binds to components of the RNA pol III transcription machinery in nuclei through β-actin. Mechanistic analysis revealed that FLNA inhibits pol III gene transcription by confining the recruitment of pol III transcription machinery at the promoters through its actin-binding domain.

Results

Knockdown of FLNA Enhances Transcription of RNA Pol III-transcribed Genes, Except for a Subset of tRNA Genes—The roles of FLNA in RNA pol I and II gene transcription have...
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already been characterized by a number of studies (18–21). As described above, we supposed that FLNA could also repress pol III gene transcription because an important component of the ribosome, 5S rRNA, is synthesized by pol III. To determine the role of FLNA in RNA pol III gene transcription, two stable cell lines expressing FLNA shRNA or control shRNA were generated by transduction of FLNA shRNA or control shRNA lentiviral particles into 293T cells, followed by the analysis of gene expression with RT-qPCR. Fig. 1A shows that expression of FLNA was significantly reduced at both mRNA (left panel) and protein (right panel) levels in 293T cells expressing FLNA shRNA compared with cells expressing control shRNA, indicating that FLNA was successfully knocked down by the expression of FLNA shRNA. It is well established that RNA pol III synthesizes a variety of non-coding RNAs (1). Therefore, we initially examined the expression of the 5S rRNA, U6 RNA, and 7SLRNA genes in these cell lines using RT-qPCR. As shown in Fig. 1B, knockdown of FLNA significantly enhanced the expression of these genes and a positive control gene (18S rRNA) (18) but did not affect the expression of the control genes GSK3β and β-tubulin. We next determined whether this effect extends to the other major pol III targets, the tRNAs. Analysis of the expression of 20 tRNA genes revealed that knockdown of FLNA significantly enhanced the expression of 10 tRNA genes (Fig. 1, C–E). However, expression of the remaining tRNA genes only showed a minor increase after knockdown of FLNA (Fig. 1, C–E). These data indicate that knockdown of FLNA in 293T cells enhances the expression of pol III-transcribed genes, except for a subset of tRNA genes, suggesting that FLNA differentially modulates the expression of tRNA genes in 293T cells.

We next determined whether the observation could be reproduced in different cell types. To this end, two SaOS2 stable cell lines expressing FLNA shRNA or control shRNA were generated and analyzed for RNA pol III gene expression using RT-qPCR. As expected, knockdown of FLNA in SaOS2 cells also enhanced expression of the same genes, as shown in 293T cells (Fig. 2, A–D). However, the expression of 10 tRNA genes showed little difference between FLNA shRNA-expressing cells and control shRNA-expressing cells, as observed in 293T cells (Fig. 2, B–D). These data confirm that knockdown of FLNA in SaOS2 cells enhances expression of RNA pol III-transcribed genes, except for a subset of tRNA genes.

To determine whether FLNA indeed regulates pol III gene transcription, three reporter vectors driven by the promoters of 5S rRNA, U6 RNA, and tRNA-Met genes were generated and employed for transient transfection assays. However, we first had to determine whether the reporter gene (luciferase) driven by the pol III gene promoters is efficiently expressed in human cells. To validate the efficiency of reporter gene expression, these vectors and the reporter vector driven by the adenovirus major late (AdML) core promoter were transfected into 293T cells. 36 h post-transfection, the cells were harvested and analyzed for luciferase activity. As shown in Fig. 2E, the promoters of both the 5S rRNA and tRNA-Met genes displayed a similar activity as the promoter of AdML, whereas the promoter of the U6 RNA gene showed much higher activity than the promoter of AdML. The AdML core promoter has been widely applied to the study of gene transcription by RNA polymerase II (22, 23). Thus, these data demonstrate that the reporter vectors driven by pol III gene promoters can be used to analyze gene transcription by RNA pol III.

We next determined the effect of FLNA knockdown on expression of the reporter gene driven by the pol III promoters, as shown in Fig. 2F, 293T stable cell lines expressing FLNA shRNA or control shRNA were used for transient transfection with these vectors, and the transfected cells were analyzed for luciferase activity. Luciferase assays showed that knockdown of FLNA significantly increased expression of the reporter gene driven by the promoters of the 5S rRNA, U6 RNA, and tRNA-Met genes (Fig. 2F), indicating that knockdown of FLNA can enhance transcription activities for the promoters of the 5S rRNA, U6 RNA, and tRNA-Met genes. Taken together, these data suggest that FLNA plays an inhibitory role in transcription of pol III-transcribed genes, except for a subset of tRNA genes.

Expression of FLNA in a FLNA-negative Cell Line Represses Pol III Gene Transcription—To confirm the inhibitory role of FLNA in pol III gene transcription, two melanoma cell lines,
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M2 and A7, were used to analyze pol III gene expression by RT-qPCR. M2 and A7 are an ideal pair of cell lines for this experiment because M2 is naturally FLNA-deficient, and A7 was derived from M2 but stably expresses FLNA (based on information from the ATCC). The expression of the 5S rRNA, 7SL RNA, and U6 RNA genes was initially analyzed. Fig. 3A shows that expression of FLNA in A7 cells significantly down-regulated the expression of these genes as well as 18S rRNA genes (18) but did not decrease the expression of control genes (GSK3B and β-tubulin). We next sought to analyze the expression of 20 tRNA genes in M2 and A7 cells. Strikingly, in FLNA-expressing cells (A7), expression of all 20 tRNA genes was significantly suppressed (Fig. 3, B–D). Taken together, these data indicate that expression of FLNA inversely correlates with pol III gene expression in these melanoma cell lines, confirming that FLNA represses RNA pol III gene transcription. Because knockdown of FLNA did not increase the expression of all tRNA genes in both 293T and SaOS2 cells (Figs. 1, C–E, and 2, B–D), the results imply that FLNA modulates transcription for tRNA genes in a cell type-specific manner.

To confirm that expression of tRNA genes in melanoma cells is indeed distinct from that in 293T or SaOS2 cells, two A7 stable cell lines expressing control shRNA or FLNA shRNA were generated and used for the analysis of pol III gene expression. The data show that knockdown of FLNA enhanced the expression of all detected pol III genes (Fig. 4). These results are consistent with those from the assays with M2 and A7 cells, confirming that FLNA modulates transcription of tRNA genes in a cell type-specific manner.

FLNA Inhibits Recruitment of the Pol III Transcription Machinery at the Promoters of Genes That Are Sensitive to Knockdown of FLNA—To gain insight into the mechanism by which FLNA modulates pol III gene transcription, we asked whether the presence or absence of FLNA alters the occupancy of the pol III transcription machinery at the promoters of pol III-transcribed genes. To address this question, ChIP assays for components of the pol III transcription machinery were performed using 293T stable cell lines expressing FLNA shRNA or control shRNA. The occupancies for BRF1 and TFIIIC2 at the promoters of pol III genes were analyzed by qPCR. As shown in Fig. 5, loss of FLNA by expression of FLNA shRNA in 293T cells significantly increased the occupancies of BRF1 and TFIIIC2 at the promoters of the 5S rRNA, 7SL RNA, and tRNA-Met genes, respectively, but did not change them at the promoter of the tRNA-Cys gene and 5S rRNA coding region (5S CR). Fig. 5 shows that knockdown of FLNA did not change the occupancy of BRF1 at the U6 RNA gene promoter; this is likely because BRF2 replaces BRF1 in transcription factor III B in U6 RNA gene expression (24). However, loss of FLNA still enhanced the recruitment of TFIIIC2 at the U6 RNA gene promoter.
Although the occupancy of TFIIIC2 at the U6 RNA gene promoter in human cells is quite unexpected, it is consistent with that observed previously in yeast (25). In addition, the relative occupancies for BRF1 or TFIIIC2 at the promoter of the tRNA-Cys gene were close to that from the input sample, although tRNA-Cys expression showed little change by knockdown of FLNA (Fig. 1C). Next, the occupancies for polR3D and polR3K at these promoters and the 5S rRNA coding region were analyzed by qPCR. As shown in Fig. 5, loss of FLNA in 293T cells significantly enhanced the occupancies of polR3D and polR3K at the promoters of the 5S rRNA, 7SL RNA, U6 RNA, and tRNA-Met genes as well as 5S CR, respectively, but did not affect them at the promoters of tRNA-Cys gene. The occupancies at pol III gene loci were also examined using positive (anti-TBP antibody) and negative controls (IgG). The data show that the occupancy of TBP at the promoters of the 5S rRNA, 7SL RNA, U6 RNA, and tRNA-Met genes was also enhanced by loss of FLNA; the rest of the data are consistent with those observed in the experiment with TFIIIC2 (Fig. 5). Because expression of the 5S rRNA, 7SL RNA, U6 RNA, and tRNA-Met genes was sensitive to knockdown of FLNA, whereas the expression of the tRNA-Cys gene was not (Figs. 1 and 2), these data show that FLNA inhibits recruitment of the pol III transcription machinery at the promoters of genes whose expression is sensitive to knockdown of FLNA.

FLNA Associates with the RNA Pol III Transcription Machinery—Previous work showed that β-actin associates with the pol III transcription machinery and regulates pol III-driven gene transcription (26). We therefore supposed that actin-
binding FLNA could also associate with the pol III transcription machinery and that the association might contribute to the suppression of pol III transcription. To test this hypothesis, co-localization studies using immunofluorescence microscopy were initially performed using 293T cells and antibodies against FLNA, BRF1, and TFIIIC2. The merged images show that FLNA overlaid with BRF1 and TFIIIC2 (Fig. 6, A and B), suggesting that FLNA is possibly co-localized with BRF1 and TFIIIC2 in the nuclei of 293T cells. Next, immunofluorescence assays were performed using 293T cells and antibodies against FLNA, polR3D, and polR3K. As expected, localizations for FLNA versus pol IIIR3D or FLNA versus polR3K were also observed in nuclei, and yellow patches and spots were observed in the merged images for FLNA and polR3D or FLNA and polR3K (Fig. 6, C and D), implying that the co-localization between FLNA and polR3D or polR3K possibly occurs in the nuclei of 293T cells. Taken together, these data suggest that FLNA is potentially associated with the pol III transcription machinery in 293T nuclei.

To determine whether FLNA directly associates with the RNA pol III transcription machinery, co-IP assays were performed with 293T nuclear extract using antibodies against FLNA and components of the pol III machinery (Fig. 6, E and F). Western blots showed that β-actin and components of the pol III transcription machinery were able to be precipitated by an FLNA-specific antibody (Fig. 6E). In reciprocal experiments, FLNA was also pulled down by antibodies against β-actin and components of the pol III machinery (Fig. 6F). These interactions were specific because lamin B and TAF1 were not precipitated by FLNA antibodies (Fig. 6, E and F). Taken together, these data show that FLNA is associated with the RNA pol III machinery in 293T nuclei.

The Actin-binding Domain of FLNA Is Required for the Repression of Pol III Gene Transcription Mediated by FLNA—We have so far confirmed the association of FLNA with the pol III transcription machinery. We next asked whether the FLNA actin-binding domain (ABD) is required for the repression of pol III gene transcription. To address this question, plasmids encoding GFP-tagged FLNA and its ABD deletion mutant (ΔFLNA-GFP) were transfected into 293T cells. GFP-expressing cells were purified by FACS and tested for expression of several representative pol III-transcribed genes and internal control genes using RT-qPCR. Western blotting showed that GFP and GFP-tagged protein displayed a similar expression level after purification by cell sorting (Fig. 7A). As shown in Fig. 7B, expression of FLNA-GFP in 293T cells significantly reduced the expression of the 5S rRNA, 7SL RNA, U6 RNA, tRNA-Phe, tRNA-Met, and positive control (18S rRNA) genes compared with the expression of control GFP but did not affect the expression of the tRNA-Cys, tRNA-His, and negative control (β-tubulin) genes. Expression of ΔFLNA-GFP in 293T cells showed little difference in the expression of all detected genes compared with the expression of GFP only. These data show that the ABD of FLNA is required for the repression of pol III gene transcription mediated by FLNA.

To understand how the ABD of FLNA mediates repression of pol III gene transcription, immunoprecipitation assays were performed using an anti-GFP antibody and 293T cells that express FLNA-GFP and ΔFLNA-GFP. As shown in Fig. 7C, the ABD deletion (ΔFLNA-GFP) prevented FLNA association with β-actin and, importantly, components of the pol III transcription machinery, indicating that the ABD of FLNA mediates the interaction between FLNA and the pol III transcription machinery. However, we noticed that ΔFLNA-GFP did not completely abolish polR3D binding to ΔFLNA, suggesting that the C terminus of FLNA could contain a weak binding domain for polR3D or a binding domain for an unknown factor that can directly or indirectly associate with polR3D. We next determined whether the ABD deletion of FLNA affects the recruitment of the pol III transcription machinery at the promoters of pol III-transcribed genes. To achieve this, ChIP assays for components of the pol III transcription machinery were performed using 293T cells that express FLNA-GFP and ΔFLNA-GFP. ChIP-qPCR data showed that expression of FLNA-GFP in 293T
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FIGURE 7. The actin-binding domain of FLNA is required for the repression of pol III gene transcription mediated by FLNA. A, immunoblot analysis for HEK293T cells expressing GFP, GFP-tagged FLNA, and FLNA derivatives. Western blotting was performed with antibody against GFP. B, the effect of ABD deletion of FLNA on pol III gene transcription in 293T cells. 293T cells were transfected with vectors expressing FLNA-GFP, ∆FLNA-GFP, and GFP. GFP-expressing cells were purified by FACS and used for detecting gene expression by RT-qPCR. C, analysis of IP for BRF1, TFIIIC2, polR3D, and β-actin using 293T cells expressing FLNA-GFP or ∆FLNA-GFP. IP assays were performed by GFP antibody. The IP samples were detected by Western blotting and the indicated antibodies. D–F, the relative occupancies of BRF1 (D), TFIIIC2 (E), and polR3D (F) and the occupancy of input at detected promoters in 293T cells expressing FLNA-GFP or ∆FLNA-GFP (left panels). The fold changes for the relative occupancy between ∆FLNA-GFP and FLNA-GFP are shown in the right panels. 293T cells were transfected with vectors expressing FLNA-GFP and ∆FLNA-GFP. GFP-expressing cells were purified by FACS and subjected to ChIP analysis. ChIP samples were detected by qPCR. 1 ng of genomic DNA was used to perform qPCR for input, it was equivalent to 0.01% of the sample used for the ChIP assay for each factor. 5S, 5S rRNA gene promoter; 7SL, 7SL rRNA gene promoter; U6, U6 rRNA gene promoter; tRNA-M, tRNA-Met gene promoter; tRNA-C, tRNA-Cys gene promoter; 5S CR, 5S rRNA coding region. Each column represents the mean ± S.E. of three independent experiments. *, p < 0.05; **, p < 0.01. The p values were obtained by t test.

cells significantly reduced the occupancies of BRF1, TFIIIC2, and polR3D at the promoters of the 5S rRNA, 7SL RNA, and tRNA-Met genes but did not affect them at the promoter of the tRNA-Cys gene compared with the expression of ΔFLNA-GFP (Fig. 7, D–F). In addition, occupancy for TFIIIC2 and polR3D at the promoters of the 5S rRNA, 7SL RNA, and tRNA-Met genes was quite sensitive to knockdown of FLNA, and the occupancies of components of the pol III machinery at the promoters of these genes displayed a significant difference between FLNA shRNA-expressing cells and control shRNA-expressing cells; the occupancy change for these factors at the promoters positively correlates with the expression change of these genes (Figs. 1 and 5). However, the occupancies of components of the pol III machinery at the promoter of the tRNA-Cys gene showed little change regardless of the presence of FLNA; the occupancies of these factors were close to that from the input sample (Figs. 5 and 7). In fact, the expression levels of tRNA genes that were insensitive to knockdown of FLNA were normal; therefore, these insensitive tRNA genes cannot be regarded as inactive loci. In addition, tRNAs are essential to the process of translation, and FLNA has been described to regulate RNA polymerase II gene transcription (31–33). Therefore, it is possible that alteration of FLNA expression leads to a variation of expression for many proteins, which, accordingly, requires the differential expression of tRNA genes in cells. Future work will focus on the effect of FLNA on the expression of other genes.
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FLNA knockdown on the genome-wide expression of mRNA, which may provide an explanation for the differential expression of tRNA genes. Nuclear FLNA has now been reported in a number of studies (18, 19, 34–36). In this study, we showed that nuclear FLNA forms a complex with the pol III transcription machinery and that the interaction is mediated by the ABD, suggesting that FLNA-driven repression is mediated by β-actin. Indeed, ABD deletion in FLNA abrogated FLNA binding to β-actin and components of the pol III machinery (Figs. 6E and Fig. 7C). In addition, overexpression of FLNA-GFP in 293 cells inhibited recruitment of the pol III transcription machinery at the promoters of genes sensitive to FLNA expression but overexpression of ΔFLNA-GFP did not (Fig. 7, D–F). These data suggest that FLNA represses pol III gene transcription by interacting with the pol III transcription machinery through its actin-binding domain, subsequently confining their access to the target genes because of abundant FLNA in cells. This inhibitory mechanism, by which FLNA modulates pol III gene transcription, is similar to that described in pol I or pol II gene transcription (18, 21, 31).

It has been described that FLNA can promote or inhibit cancer growth and development (10). A recent study showed that FLNA interacts with HIF-1α to promote tumor growth and angiogenesis (36). However, TIF-90 interacts with the 90-kD isoform of FLNA to inhibit rRNA synthesis and cell proliferation in leukemia cells (19). These observations seem to be in conflict with the function of FLNA in cancer; however, it has been described that the dual functions of FLNA in cancer depend on the isoform of FLNA and its location in cells (10). Our previous work demonstrated that FLNA represses pol I gene transcription and cell proliferation in SaOs2, A7, and 293T cells (18). In this study, we have also shown that FLNA represses transcription of pol III-transcribed genes. Based on these findings, we propose that FLNA inhibits cell proliferation by coordinately repressing both RNA pol I and pol III gene transcription. Thus, our findings provide a novel explanation of how FLNA represses cancer cell proliferation.

Experimental Procedures

Gene cloning, Cell Culture, and Reagents—The 5S rRNA, U6 RNA, and tRNA-Met gene promoters were cloned into the reporter vector pGL3-basic (Promega). 293T and SaOS2 cells stably expressing control shRNA or FLNA shRNA were generated as described previously (18). HEK 293T, SaOS2, M2, and A7 cells were cultured in DMEM, McCoy’s 5A medium, and minimum Eagle’s medium, respectively. Chemical reagents were purchased from Sigma-Aldrich and enzymes from Thermo Scientific.

Quantitative RT-PCR—Total RNA was prepared with the RNA miniprep kit (Axygen), followed by cDNA synthesis with 0.5 μg of total RNA and 2.5 units of reverse transcriptase according to the manual of the manufacturer (Thermo Scientific). 0.5 μl of reaction mixture was used for qPCR. qPCR was performed with Bio-Rad iQ SYBR Green and a real-time detection system. qPCR data were analyzed with CFX Manager 3.1 software (Bio-Rad). Gene expression was calculated by ΔΔCt and normalized by the reference gene GAPDH. The change of gene expression (-fold) for individual genes was obtained by using the gene expression in FLNA shRNA-expressing cells divided by that in control shRNA-expressing cells. The primers used for qPCR are available upon request.

Immunofluorescence Microscopy—Immunofluorescence (IF) assays for FLNA, TFIIC2, BRF1, polR3D, and polR3K in 293T cells were performed with a standard protocol. The specimens were observed under a DeltaVision fluorescence microscope (Manfred) as described previously (18). The images were taken using a ×60 object lens (Olympus) with 512 × 512 pixels. The raw images were subsequently deconvolved with Soft-WORx software (GE Healthcare) and analyzed with MBF ImageJ (National Institutes of Health). Primary antibodies for IF assays were purchased from Abcam (FLNA, ab76289; TFIIC2, ab89113; polR3D, ab86786; polR3K, ab169502) and Santa Cruz Biotechnology (BRF1, SC-81405). Fluorescent secondary antibodies were from Life Tech (catalog nos. A-11034, A-11001, A-11005, and R37117).

Immunoprecipitation and Western Blotting—293T nuclei were prepared according to a standard procedure, followed by sonication with medium power at 4 °C for 5 min (Diagenode), and then centrifuged at 1300 rpm for 10 min. 300 μg of protein from the supernatant was used for IP assays with 10 μg of anti-FLNA antibody or antibodies against BRF1, TFIIC2, polR3D, polR3K, Lamin B (ab16048, Abcam), β-actin (ab6276, Abcam), TAF1(SC-735, Santa Cruz Biotechnology), and GFP (A11120, Life Technologies). IP samples were eluted with 40 μl of 1× SDS loading buffer. 20 μl of eluted sample was used for immunoblotting analysis. 6 μg of protein from nuclear extract was used as input in Western blotting. Western blotting was performed using the same antibodies as in the IP assays.

Transfections, Luciferase Assays, and FACS—Transient transfection was performed with Turbofect (Thermo Scientific). To perform luciferase assays, 293T or 293T stable cell lines were transfected with the vector containing a reporter gene driven by the promoters of the AdML, 5S rRNA, U6 RNA, and tRNA-M genes. The cell lysate from transient transfection was prepared with lysis buffer (Life Technologies), and 3 μg of protein from the lysate was used to analyze luciferase activity with a dual-light detection system (Life Technologies). The luciferase activity was normalized by the activity of β-galactosidase. To determine the effect of expression of FLNA-GFP or its ABD deletion mutant (ΔFLNA-GFP) on pol III transcription, 293T cells were transfected with vectors that express FLNA-GFP, the FLNA ABD deletion mutant (ΔFLNA-GFP), and control GFP. GFP-expressing cells were purified by flow cytometry (BD Biosciences) according to the procedure recommended by the manufacturer.

ChIP Assays—293T stable cell lines expressing control shRNA or FLNA shRNA were used for ChIP analyses for TBP, BRF1, TFIIC2, polR3D, and polR3K. GFP-expressing 293T cells (FLNA-GFP and ΔFLNA-GFP) were used for the ChIP assays for BRF1, TFIIC2, and polR3D. The ChIP assays were performed as described previously (18). The antibodies used for the ChIP assays were as in the IP assays, except for the anti-TBP antibody (SC-204, Santa Cruz Biotechnology). ChIP-qPCR was performed with a Bio-Rad real-time PCR detection system. The data were analyzed with Bio-Rad CFX manager 3.1.
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The relative occupancy for individual factors was obtained using the occupancy of ChIP sample divided by the occupancy of input sample. 1 ng of genomic DNA was used to perform qPCR for input, it was equivalent to 0.01% of sample used for the ChIP assay. The -fold change for the relative occupancy of individual factors was obtained by using the relative occupancy in FLNA shRNA-expressing cells divided by that in control shRNA-expressing cells. The primers used for ChIP-qPCR are available upon request.

Author Contributions—J. W. performed most of the experiments. S. Z. analyzed the RT-qPCR and ChIP-qPCR data. Y. W. performed RT-qPCR for 293T cells. Y. Z. performed RT-qPCR for SaoS2 cells. W. D. conceived the project and designed the experiments, performed the IF and IP assays, and wrote the manuscript.

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