TFIIB-related factor 1 is a nucleolar protein that promotes RNA polymerase I-directed transcription and tumour cell growth

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Abstract

Eukaryotic RNA polymerase I (Pol I) products play fundamental roles in ribosomal assembly, protein synthesis, metabolism and cell growth. Abnormal expression of both Pol I transcription-related factors and Pol I products causes a range of diseases, including ribosomopathies and cancers. However, the factors and mechanisms governing Pol I-dependent transcription remain to be elucidated. Here, we report that transcription factor IIB-related factor 1 (BRF1), a subunit of transcription factor IIIB required for RNA polymerase III (Pol III)-mediated transcription, is a nucleolar protein and modulates Pol I-mediated transcription. We showed that BRF1 can be localized to the nucleolus in several human cell types. BRF1 expression correlates positively with Pol I product levels and tumour cell growth *in vitro* and *in vivo*. Pol III transcription inhibition assays confirmed that BRF1 modulates Pol I-directed transcription in an independent manner rather than through a Pol III product-to-45S pre-rRNA feedback mode. Mechanistically, BRF1 binds to the Pol I transcription machinery components and can be recruited to the rDNA promoter along with them. Additionally, alteration of BRF1 expression affects the recruitment of Pol I transcription machinery components to the rDNA promoter and the expression of TBP and TAF1A. These findings indicate that BRF1 modulates Pol I-directed transcription by controlling the expression of selective factor 1 subunits. In summary, we identified a novel role of BRF1 in Pol I-directed transcription, suggesting that BRF1 can independently regulate both Pol I- and Pol III-mediated transcription and act as a key coordinator of Pol I and Pol III.

Introduction

Ribosomal RNA (rRNA) synthesis directed by RNA polymerase I (Pol I) accounts for over 60% of the total transcriptional activity in eukaryotic cells. Together with 5S rRNA transcribed by RNA polymerase III (Pol III), cellular rRNA molecules act as essential components of ribosomes to regulate ribosome biogenesis and protein translation (1-3). Dysregulation of Pol I products causes a range of genetic diseases and cancers (4-7). Transcription initiation by RNA Pol I requires the assembly of upstream-binding factor (UBF), selective factor 1 (SL1), transcription initiation factor 1A (TIF-1A), and Pol I at the rDNA promoter, where they form the preinitiation complex (1, 2). In addition to being controlled by general transcription factors, Pol I-dependent transcription is also tightly controlled by oncogenic factors, tumour suppressors, signalling factors, chromatin remodelling factors, and long noncoding RNAs (8-12). It has been reported that the nuclear mitotic apparatus protein NuMA modulates rDNA transcription by mediating the nucleolar stress response in a p53-independent manner (13). The oncoprotein LYAR (ly1 antibody reactive clone) can enhance rDNA transcription by recruiting bromodomain-containing protein 2 and the histone acetyltransferase KAT7 at rDNA loci (14). The lncRNA PAPAS (promoter and pre-rRNA antisense) has been shown to directly interact with rDNA enhancers and repress rRNA synthesis by recruiting CHD4/NuRD to the rDNA promoter (15). The cellular SUMOylation system can inhibit rDNA transcription by downregulating the expression of c-MYC and BRF1 (16). Recently, the Che-1/AATF (Che-1) protein

complex was shown to bind to the RNA polymerase machinery and maintain rDNA gene transcription (17). The Cockayne syndrome group A and B proteins (CSA and CSB) regulate the transcription of ribosomal DNA (rDNA) genes and ribosome biogenesis by inducing nucleolin ubiquitination (18). Despite advances in the understanding of Pol I-directed transcription, the pathways and factors controlling Pol I-directed transcription remain to be identified.

The Pol III general transcription factor IIIB consists of the TBP, BRF1/2, and BDP1 subunits (19). Among these subunits, BRF1 is required for the transcription of *5S rRNA* and *tRNA* genes, while BRF2 is essential for the transcription of the *U6 snRNA* gene (20, 21). Deregulation of TFIIIB expression is closely associated with cancer development (21, 22). During transcription initiation by Pol III, TFIIIC binds to internal box A and B elements within a tRNA-coding region, subsequently recruiting TFIIIB and Pol III to the transcription start site (TSS) (21, 23). It has been shown that tumour suppressors, including p53, RB, and Maf1, can associate with TFIIIB to prevent the assembly of the Pol III transcription machinery and repress Pol III-dependent transcription (24-27). Conversely, the oncoprotein c-MYC targets TFIIIB to activate Pol III-directed transcription (27, 28). Additionally, signalling pathways such as the RAS/ERK, PI3K/AKT, and JNK pathways indirectly regulate Pol III transcription (29-34). Abnormally high expression of TFIIIB subunits has been observed in human cancer cells (35-36). For example, breast cancers and hepatocellular carcinomas exhibit abnormally high expression of BRF1, suggesting that BRF1 is an excellent biomarker for cancer diagnosis (37-39). Previous and recent evidence shows that BRF1 functions as a positive regulator of Pol III transcription (36, 40).

When we previously investigated the role of FLNA in Pol III-directed transcription (40, 41), BRF1 was occasionally observed in the nucleoli of human osteosarcoma cells, although this phenomenon was not our focus in that study. However, this observation prompted us to ask whether BRF1 is a nucleolar protein and what role it plays if it is indeed present in nucleoli. In this study, we show that BRF1 is abundant in the nucleoli of several types of tumour cells and that BRF1 positively regulates Pol I-directed transcription and cell growth *in vitro* and *in vivo*. We further delineated the mechanism by which BRF1 modulates Pol I-mediated transcription using a combined approach.

Results

BRF1 is present in nucleoli in human cell lines

As described above, BRF1 is a subunit of TFIIIB required for Pol III-directed transcription (19, 20). We investigated the role of FLNA in Pol III-mediated transcription and, unexpectedly, observed colocalization of BRF1 and filamin A (FLNA) in the nucleoli of SaOS2 cells (Fig. S1A and unpublished data). Our latest work revealed that colocalization of BRF1 and fibrillarin (FBL; a nucleolar protein marker) was also observed in the nucleoli of SaOS2 cells (Fig. S1B). Based on these data, we hypothesized that BRF1 could be a nucleolar protein, although it was originally found in the nucleoplasm and plays a role in Pol III-mediated transcription (40, 41). To support this hypothesis, we first performed immunofluorescence (IF) assays using HeLa cells and antibodies against BRF1, FLNA, and nucleophosmin (NPM1). Intriguingly,

colocalization of BRF1 with FLNA (or NPM1) was observed in the nucleoli of HeLa cells, indicating that BRF1 is present in the nucleoli of HeLa cells (Fig. 1A and B). We next determined whether this result could be reproduced when nucleolar particles purified from HeLa cells were evaluated by IF staining. As shown in Fig. 1C and D, either NPM1 or FBL was colocalized with BRF1 in the nucleolar particles. We subsequently performed Western blot analysis to examine the presence of BRF1 in the nucleolar fraction purified from HeLa cells. The immunoblot results showed that BRF1 was detected in the nucleolar fraction and that BRF1 displayed a stronger signal in the nucleolar fraction than in the other cellular fractions, indicating that BRF1 is abundant in the nucleoli of HeLa cells (Fig. 1E). Next, we determined whether BRF1 is present in the nucleoli of normal human cells; to this end, we performed IF assays using human primary cervical epithelial cells (HuCEC, ATCC (PCS-480-011)). Fig. 1F shows that BRF1 was localized in the nucleoli of cervical epithelial cells, indicating that BRF1 is present in the nucleoli of normal cervical epithelial cells.

To confirm this observation, we performed IF assays using anti-BRF1 antibodies from different sources (Ab74221, Abcam). Unexpectedly, in most cases, BRF1 was not found to be localized in the nucleoli of HeLa cells (data not shown). It is possible that some antibodies are not suitable for IF assays. In particular, the epitope in a protein that is bound by an antibody is cross-linked, which can severely affect the binding between an antigen and an antibody. For instance, we recently found that one anti-RPA43 antibody could not recognize the RPA43 protein in nucleoli. However, mCherry-RPA43 expression showed that RPA43 is localized in nucleoli (42). Therefore, we first evaluated mCherry-BRF1 expression in HeLa cells by transfecting these cells with vectors expressing mCherry or mCherry-BRF1 (Fig. S2A). Interestingly, mCherry-BRF1 and fibrillarin were colocalized in the nucleoli of HeLa cells (Fig. S2B). In contrast, mCherry alone was not localized to HeLa cell nucleoli (Fig. S2C). A consistent result was obtained in IF assays with an anti-HA antibody in HeLa cells stably expressing HA-BRF1 (Fig. S2D and E). We next investigated whether BRF1 knockdown affects BRF1 localization in nucleoli. BRF1 was silenced by transfection of BRF1 siRNA into HeLa cells, and IF assays were then performed using an anti-BRF1 antibody (SC-81405, Santa Cruz Biotech.). Strikingly, the BRF1 signal disappeared in the nucleoli of most HeLa cells after BRF1 siRNA transfection (Fig. S2F). Taken together, these findings demonstrate that BRF1 is present in nucleoli in both tumour and normal cell lines, suggesting that BRF1 possibly plays an unknown role in nucleoli in human cells.

BRF1 acts as a positive factor in the regulation of Pol I-directed transcription in transformed cell lines

The eukaryotic nucleolus is an important subcellular structure that is responsible for 45S pre-rRNA synthesis, rRNA processing, and the initial assembly of ribosomes (1, 2). BRF1 was found in the nucleoli of human cells, where 45S ribosomal RNA synthesis occurs; therefore, we sought to determine whether BRF1 is required for the synthesis of 45S pre-rRNA. We generated lentiviral particles expressing BRF1 shRNA or control shRNA, and the resulting lentiviral particles were used to infect several transformed cell lines, including HeLa, SaOS2, 293T and M2 cells. RT–qPCR and Western blot

analyses showed that stable expression of BRF1 shRNA was achieved in several cell lines, including HeLa, SaOS2, 293T and M2 (Fig. 2A, B, D, and E; Fig. S3A, B, D, and E). Analysis of Pol I products by RT–qPCR showed that BRF1 knockdown in these cell lines decreased the synthesis of pre-rRNA, 18S rRNA, and 28S rRNA (Fig. 2C and F; Fig. S3C and F), indicating that BRF1 downregulation can inhibit the synthesis of Pol I products. We next determined whether changes in BRF1 expression can affect nascent rRNA synthesis by performing EU (5-ethynyl uridine) assays with HeLa cells. EU incorporation into rRNA was analysed using an EU detection kit (Beyotime, China). Strikingly, BRF1 silencing inhibited EU incorporation into nascent rRNA (Fig. 2G and H), indicating that BRF1 may activate the expression of ribosomal RNA genes.

To verify the positive regulatory role of BRF1 in Pol I–mediated transcription, we generated HeLa, 293T, and M2 cell lines stably expressing HA-BRF1 and the corresponding control cell lines using a lentiviral transduction system. Western blot analysis showed that cell lines stably expressing HA-BRF1 were successfully established (Fig. S4A, C, and E). Clearly, overexpression of BRF1 stimulated Pol I-directed transcription in these cell lines (Fig. S4B, D, and F). Next, the effect of BRF1 overexpression on rRNA synthesis was examined by EU assays. As expected, BRF1 overexpression in HeLa cells enhanced the signal intensity of EU-labelled rRNA (Fig. S5A and B), confirming that BRF1 overexpression enhanced the synthesis of Pol I products. Interestingly, the results of analysis of Pol I products in a normal cell line (HuCEC) with BRF1 depletion or overexpression were consistent with those observed in tumour cells (Fig. S6A and B). Taken together, these findings demonstrate that BRF1 may act as an activator to modulate Pol I-directed transcription in human cell lines.

BRF1 can independently regulate Pol I-directed transcription.

We showed using RT–qPCR analysis and EU assays that BRF1 can positively regulate Pol I-directed transcription. Next, we verified this result using dot blotting, a direct method to detect rRNA expression. Total RNA was extracted from HeLa cell nuclei, and dot blotting was performed using the resulting RNA and probes detecting 45S pre-rRNA. Clearly, BRF1 silencing reduced 45S pre-rRNA synthesis (Fig. 3A and B), whereas BRF1 overexpression enhanced 45S pre-rRNA synthesis (Fig. 3C and D). These data further confirmed that BRF1 can positively modulate Pol I-directed transcription. BRF1 was originally identified as a TFIIIB subunit that positively regulates Pol III-directed transcription (40). We next investigated whether BRF1 regulates Pol I product synthesis independent of changes in Pol III product synthesis. HeLa cells with BRF1 silencing or overexpression and the corresponding control cells were cultured in the presence or absence of 50 μM ML-60218 (a Pol III-directed transcription inhibitor), and Pol I-directed transcription was detected by RT–qPCR. Interestingly, ML-60218 did not significantly affect the activation of Pol I-directed transcription caused by BRF1 overexpression or the inhibition of Pol I-directed transcription caused by BRF1 silencing. However, the synthesis of 5S rRNA, a Pol III product, was severely affected by ML-60218 (Fig. 3E and F). These data suggest that BRF1 can regulate Pol I-directed transcription independently rather than in a manner dependent on feedback from changes in Pol III product levels. To confirm whether BRF1 independently regulates Pol I-directed transcription, we performed *in vitro*

transcription assays using rDNA promoter-driven reporter vectors and HeLa cell nuclear extracts with or without BRF1 depletion with an anti-BRF1 antibody. The results showed that BRF1 depletion reduced the transcriptional activity of the rDNA promoter (Fig. 3G and H). Since *in vitro* transcription was performed using a cell-free system, where Pol III products remained unchanged and did not interfere with rDNA promoter activity, these results from *in vitro* transcription assays confirm that BRF1 can independently regulate Pol I-directed transcription.

Changes in Pol I product levels caused by BRF1 up- and downregulation influenced cell proliferation in vitro and in vivo.

Pol I product levels correlate closely with cell growth and proliferation. Thus, it is necessary to determine whether alterations in Pol I product levels mediated by BRF1 silencing or overexpression contribute to cell proliferation. The effect of BRF1 silencing on cell proliferation was first examined using cell counting and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays. We showed that BRF1 downregulation inhibited the proliferative activity of transformed cell lines, including HeLa, M2, SaOS2, and 293T (Fig. 4A and B; Fig. S7A-F). To confirm this result, we performed EdU (5-ethynyl-2′-deoxyuridine) assays using HeLa cell lines and calculated the rate of EdU-labelled cells. We showed that BRF1 silencing reduced the rate of EdU-labelled cells compared to that in the corresponding control cell line, confirming that BRF1 promotes HeLa cell proliferation (Fig. 4C and D). We next determined whether BRF1 silencing can induce cell death, which might contribute to the decrease in cell growth. The results of flow cytometric analysis revealed that BRF1 shRNA expression did not induce cell death (Fig. S7G-L). This result suggests that BRF1 silencing affects cell growth only by affecting cell proliferation. Using the same strategy, we examined the effect of BRF1 overexpression on cell proliferation. The results of cell counting and MTT assays showed that BRF1 overexpression stimulated proliferative activity in several cell lines, including HeLa, 293T, and M2 (Fig. 4E and F; Fig. S8). Consistent results were obtained in EdU incorporation assays of HeLa cells with BRF1 overexpression (Fig. 4G and H). Additionally, we found that BRF1 can promote the proliferative activity of the normal human cell line HuCEC (Fig. S6C and D). To determine whether the activation of Pol I-directed transcription induced by BRF1 overexpression contributes to the increase in cell proliferation, we performed cell proliferation assays by treating HeLa cells expressing HA-BRF1 with the Pol I-specific inhibitor CX-5461. The results showed that treatment with CX-5461 inhibited cell proliferation and Pol I-mediated transcription in HA-BRF1-expressing cells when compared to control cells or HA-BRF1-expressing cells not treated with CX-5461 (Fig. 4I; Fig. S9). These data indicate that the activation of Pol I-mediated transcription elicited by BRF1 overexpression contributes to the enhancement of proliferative activity.

We showed that both up- and downregulation of BRF1 promote tumour cell proliferation *in vitro*. Whether this result can be reproduced *in vivo* remains unclear. To address this uncertainty, we performed tumour formation assays by injecting HeLa cells expressing BRF1 shRNA or control shRNA into nude mice (n=10 per group). Fig. 5A shows that the tumour size was reduced by BRF1 downregulation. After removal from

nude mice, tumour samples were weighed, and the data were statistically analysed. The results showed that the weights of tumours formed from BRF1 shRNA-expressing HeLa cells were considerably decreased compared to those of tumours from control shRNA-expressing cells (Fig. 5B and C), indicating that BRF1 downregulation inhibited tumour growth *in vivo*. Haematoxylin and eosin staining showed that the cells in the tumour tissues had large and condensed nuclei and were distinct from those in cervical and skin tissues (Fig. 5D). Immunohistochemical staining, immunoblot and RT–qPCR analyses confirmed that the cells in the tumour tissues retained the original features of the corresponding tumour cell line before injection (Fig. 5E-G). Collectively, these data indicate that BRF1 silencing-mediated inhibition of Pol I-dependent transcription contributes to suppression of tumour growth, although the contribution of changes in Pol III product levels to this event cannot be excluded.

BRF1 binds to the Pol I transcription machinery

To understand how BRF1 modulates Pol I-directed transcription, we examined the connection between BRF1 and the Pol I transcription machinery using a combined approach. Immunofluorescence (IF) assays were first performed using HeLa cells and antibodies against BRF1 or components of the Pol I transcription machinery. IF staining showed that BRF1 was colocalized with TIF-1A, RPA40, and UBF in the nucleoli of HeLa cells (Fig. 6A and B; Fig. S10A). Next, IF staining was performed on nucleolar particles purified from HeLa cells. The results showed colocalization of BRF1 and components of the Pol I transcription machinery, such as TIF-1A, RPA43, and TBP, in the nucleolar particles (Fig. 6C and D, Fig. S10B), suggesting potential interactions between BRF1 and the Pol I transcription machinery components. To determine whether BRF1 interacts with these components, we performed coimmunoprecipitation (co-IP) assays using an anti-BRF1 antibody. Fig. 6E shows that the anti-BRF1 antibody was able to precipitate TIF-1A, UBF, TAF1A, TBP, and RPA43 proteins from HeLa cell nuclear extracts. In reciprocal co-IP assays, the BRF1 protein was precipitated by the antibodies against the indicated components (Fig. 6F and G). To verify the reliability of the BRF1 co-IP results, we tested whether BRF1 can bind to other subunits of SL1 and Pol III transcription factors by performing co-IP assays using antibodies against BRF1, TAF1B, and GTF3C2. Western blot analysis showed that BRF1 also interacts with the SL1 subunit TAF1B and the TFIIIC subunit GTF3C2 (Fig. S11). Taken together, these findings show that BRF1 can bind to components of the Pol I transcription machinery, suggesting that BRF1 can modulate Pol I-dependent transcription by interacting with the Pol I transcription machinery.

BRF1 binds to the rDNA promoter and modulates Pol I transcription by affecting the assembly of the Pol I transcription machinery at the promoter

To obtain more details about how BRF1 regulates Pol I-directed transcription, we determined whether BRF1 binds to the rDNA promoter (or the *45S rRNA* gene promoter) by performing ChIP assays. Fig. 7A shows that BRF1 can bind to the rDNA promoter, rRNA-coding region and intergenic spacer region in HeLa cells, although its occupancy at the promoter is, on average, higher than that at the rRNA-coding and intergenic spacer regions. Interestingly, BRF1 exhibited considerably high occupancy at the promoters of *45S rRNA* and *5S rRNA* genes compared with control IgG, indicating

that BRF1 can concurrently bind to these two promoters although BRF1 occupancy at the rDNA promoter showed slightly higher than that at the *5S rRNA* promoter (Fig. 7B). Since BRF1 has been shown to interact with the Pol I transcription machinery, we next determined by sequential ChIP assays whether BRF1 can bind to the rDNA promoter along with components of the Pol I transcription machinery. Fig. 7C shows that BRF1 occupied the rDNA promoter together with UBF, TIF-1A, TBP, and RPA43. Given that BRF1 binds to the rDNA promoter, we next determined whether alteration of BRF1 expression affects rDNA promoter activity. The rDNA promoter sequence was inserted into the pGL3-Basic reporter vector (Fig. 7D). The promoter–driving reporter vectors were transfected into HeLa cell lines expressing BRF1 shRNA or HA-BRF1 and the corresponding control cell lines. RT–qPCR showed that BRF1 knockdown repressed rDNA promoter activity. In contrast, BRF1 overexpression induced rDNA promoter activity, indicating that BRF1 can directly regulate rDNA promoter activity (Fig. 7E and F). As expected, in the transfection experiments using the vector containing a 45S rRNA cDNA fragment driven by the rDNA promoter (Fig. 7G*)*, a consistent result was obtained (Fig. 7H). To understand how alteration of BRF1 expression modulates Pol I-directed transcription or rDNA promoter activity, we performed ChIP assays using HeLa cell lines with BRF1 depletion or overexpression and the corresponding control cell lines. Recruitment of the Pol I transcription machinery components to the rDNA promoter was analysed by qPCR. Fig. 7I shows that BRF1 downregulation reduced the recruitment of TBP, TIF-1A, UBF, TAF1A, and RPA43 to the rDNA promoter. In contrast, BRF1 overexpression enhanced the recruitment of these components to the promoter (Fig. 7J). These data suggest that BRF1 modulates rDNA transcription by controlling Pol I transcription machinery assembly at the rDNA promoter.

BRF1 regulates the expression of TBP and TAF1A

To understand why changes in BRF1 expression influence Pol I transcription machinery assembly at the rDNA promoter, we analysed the expression of Pol I transcription machinery components by Western blotting using HeLa cell lines with BRF1 knockdown or overexpression and the corresponding control cell lines. Clearly, BRF1 knockdown reduced the expression of the selective factor 1 subunits TBP and TAF1A but did not affect the expression of TIF-1A, UBF, or RPA43 (Fig. 8A and B). Conversely, BRF1 overexpression enhanced TBP and TAF1A expression but did not affect the expression of the other factors analysed (Fig. 8C and D). These findings indicate that BRF1 can regulate Pol I transcription machinery assembly at the rDNA promoter by controlling TBP and TAF1A expression. Based on the findings of this study, we proposed a model of the mechanism by which BRF1 regulates Pol I-directed transcription in human cells. On the one hand, BRF1 can interact with Pol I transcription machinery components, and the resulting complexes are recruited to the rDNA promoter to regulate Pol I-directed transcription (Fig. 8E-①). On the other hand, BRF1 can regulate the expression of the SL1 subunits TBP and TAF1A, which accordingly controls Pol I transcription machinery assembly at the rDNA promoter and Pol I-directed transcription (Fig. 8E-②). In summary, BRF1 can regulate Pol I-dependent transcription through the two pathways shown in Fig. 8E.

Discussion

BRF1 has been identified as a subunit of TFIIIB and can activate Pol III transcription in nuclei (19, 20, 39, 40). In this study, however, we show that BRF1 is abundant in nucleoli in several types of human cells (Fig. 1; Figs. S1 and 2). It has been reported that some of Pol III products are also localized near nucleoli (43), prompting a question about the specific localization of BRF1: is BRF1 localized 'near' or 'in' nucleoli? Interestingly, we observed that BRF1 and the components of the Pol I transcription machinery were colocalized in the nucleoli of HeLa cells, indicating that BRF1 is present in nucleoli (Fig. 6A-D). We demonstrated that BRF1 acts as a positive factor in the modulation of Pol I transcription (Fig. 2; Figs. S3 and S5). Thus, in this study, we identified a novel role of BRF1 in basal transcriptional regulation. This finding and the findings from previous studies suggest that BRF1 can independently modulate Pol I- and Pol III-mediated transcription by interacting with the Pol I and Pol III transcription machinery, respectively. Other factors, such as oncogenic factors, tumour suppressors, and signalling factors, have previously been confirmed to regulate both Pol I- and Pol III-mediated transcription (6, 22, 27). However, these factors modulate Pol I and Pol III transcription by directly or indirectly regulating the activity of general transcription factors (27). The findings of this study suggest that BRF1 acts as a direct link between Pol I and Pol III transcription, allowing Pol I- and Pol III-dependent transcription in human cells to be coordinated via control of the BRF1 expression level.

We showed that knockdown of BRF1 inhibited proliferative activity in several human cell types. Conversely, BRF1 overexpression enhanced proliferative activity (Fig. 4; Figs. S6-S8). Additionally, BRF1 downregulation reduced the sizes and weights of tumours formed in nude mice (Fig. 5). These results indicate that BRF1 can promote tumour cell growth *in vitro* and *in vivo*. Previous studies have shown that BRF1 is abnormally highly expressed in alcohol-associated cancers, including hepatocellular carcinoma and breast cancer (37-39, 44), suggesting that BRF1 is a potential diagnostic biomarker for cancer in the clinic and a therapeutic target in alcohol-associated cancers (37-39). Indeed, tamoxifen has been found to suppress alcohol-induced BRF1 expression and Pol III transcriptional activity in ERα^+ breast cancer (45). A recent study showed that the compound betaine impedes the activation of both BRF1 expression and Pol III transcription induced by alcohol and subsequently inhibits tumour cell growth (46). In this study, we found that BRF1 promoted cell proliferation by stimulating Pol I-mediated transcriptional activity, whereas the pol I inhibitor CX-5461 severely suppressed the increase in cell proliferation and the activation of Pol I-directed transcription induced by BRF1 overexpression. This finding suggests that anticancer drugs could be developed based on the inhibition of BRF1 expression and Pol I- and Pol III-mediated transcriptional activity.

We demonstrated that BRF1 interacted with the Pol I transcription machinery components and bound to the rDNA promoter along with these components (Fig. 6, Figure 7A-C). Alteration of BRF1 expression influenced rDNA promoter activity and the occupancy of the Pol I transcription machinery components at the rDNA promoter (Fig. 7D –J). Additionally, we found that alteration of BRF1 expression affected the protein expression of TBP and TAF1A (Fig. 8A-D). These findings suggest that BRF1

modulates Pol I-directed transcription by affecting the expression of SL1 subunits. Since BRF1 also interacts with components of the Pol I transcription machinery (Fig. 6E-G, Fig. S11C), it is possible that BRF1 silencing reduces the stability of the Pol I transcription machinery components, which consequently impacts the assembly of the Pol I transcription machinery at the rDNA promoter and hence Pol I-dependent transcription (Fig. 8E). However, whether BRF1 directly binds to these factors is unclear. If a direct interaction does occur, which component of the Pol I transcription machinery does BRF1 directly interact with? It has been shown that human TAF1B (yeast RRN7) is a TFIIB-like factor in the core Pol I machinery (19, 47), whereas BRF1 is a TFIIB-like factor in the core Pol III machinery (20, 21); however, whether BRF1 can substitute for the TAF1B subunit to regulate Pol I-mediated transcription remains to be elucidated. Whether there is a difference between the mechanisms by which BRF1 and TAF1B regulate Pol I-directed transcription remains to be addressed in future work.

In summary, we identified a novel function for BRF1 in Pol I-directed transcription, suggesting that BRF1 can independently modulate Pol I- and Pol III-mediated transcription. The findings from this study provide novel insights into the mechanism of Pol I-directed transcription and the mechanism of the coordination between Pol I- and Pol III-directed transcription.

Materials and Methods

Plasmids, cells, and reagents

The pLV-U6-EGFP-Puro lentiviral plasmid was purchased from Inovogen Tech. Co. (Beijing, China). Three different cDNA fragments encoding BRF1 shRNAs were inserted into the plasmid downstream of the U6 promoter. The pGL3-Basic plasmid containing a reporter gene was purchased from Promega. The SaOS2, HeLa, 293T, and M2 cell lines were obtained from ATCC and cultured in the corresponding media supplemented with 10% foetal bovine serum (Biowest) and 1× penicillin/streptomycin (HyClone). DNA and RNA extraction kits were purchased from Axygen. Biological reagents, including restriction enzymes, reverse transcription enzymes, PCR reagents, and transfection reagents, were obtained from Thermo Fisher Scientific. All other chemicals were purchased from Sigma–Aldrich (Merck).

Immunofluorescence assays

SaOS2 and HeLa cells were cultured on small round coverslips (14 mm in diameter) in their corresponding complete media. At 60% confluence, the culture medium was removed, and the cells were washed twice using 1× PBS solution prior to fixation for 10 min using 4% formaldehyde freshly prepared with 1× PBS. Thereafter, immunofluorescence (IF) staining was performed to assess colocalization of BRF1 and nucleolar proteins or Pol I-related components of the Pol transcription machinery using a method described previously (41, 48). Cell samples were observed under a DeltaVision fluorescence microscope and imaged with a Z-stack tool and a 60× objective (Olympus). Deconvolution of the original images was then performed with SoftWoRx software (GE Healthcare), and the resulting images were analysed with ImageJ software (NIH).

For IF assays with nucleolar particles, nuclei were isolated from 2×10^7 HeLa cells by

using buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM DTT) and a Dounce tissue homogenizer with a tight pestle. Nuclei were pelleted by centrifuging the cell lysate at 1000 rpm (Beckman GS-6 centrifuge, GH-3.8 rotor) and were then resuspended in 1 mL of S1 solution (0.25 m sucrose and 10 mM $MgCl_2$). The resulting nuclear suspension was slowly added to 2 mL of S2 solution (0.35 m sucrose, 10 mM $MgCl_2$) and subjected to centrifugation at 2500 rpm. The nuclear pellets were resuspended in S2 solution and subjected to disruption for 6 cycles of 10 sec each using an ultrasonicator (ScientZ-IID, Ningbo, China) with the smallest tip. Nucleoli were purified via sucrose gradient centrifugation using S3 solution (0.8 M sucrose and 10 mM $MgCl_2$). After centrifugation for 10 min at 3500 rpm, the supernatant was discarded, and the nucleolar pellet was resuspended in 500 μL of S2 solution and centrifuged for 5 min at 2500 rpm. The nucleolar pellet was retained and resuspended in 200 μL of S2 solution, and highly purified nucleoli were eventually obtained. Nucleoli (20 μL) were fixed with 100 μL of 4% formaldehyde-PBS solution. After fixation, nucleolar samples were subjected to IF staining using an anti-BRF1 antibody and antibodies against nucleolar proteins or the components of the Pol I transcription machinery. Samples were observed under a confocal fluorescence microscope and imaged with a 100× objective (Olympus). The primary antibodies used in the IF assays were purchased from Abcam (anti-FLNA, ab76289; anti-fibrillarin, ab166630; anti-nucleophosmin, ab183340; anti-UBF, ab244287; anti-RPA40, ab196657; and anti-RPA43, ab99305) or Santa Cruz Biotech (anti-BRF1, SC-81405). Fluorescent secondary antibodies were obtained from Thermo Scientific (Cat. Nos. A-11001, A-11005, A-11034, and R37117).

Transfection, generation of cell lines, and analysis of rRNA expression

For transient transfection assays, three BRF1 small interfering RNAs (siRNAs) were synthesized by GENEWIZ (Suzhou, China). HeLa and 293T cells were cultured in 12-well plates for 24 hours prior to transfection assays. Two microlitres of TurboFect (Thermo Scientific) was mixed with a 60 pmol siRNA mixture (20 pmol of each siRNA), and the resulting suspension was added to the cells in each well. Forty-eight hours post-transfection, cells were harvested and used for analysis of BRF1 expression and Pol I-mediated transcription. To generate cell lines stably expressing BRF1 shRNA or HA-BRF1, we transfected 293T cells using lentiviral vectors expressing BRF1 shRNA or HA-BRF1 and the packaging plasmids pH1 and pH2 (Inovogen, Beijing). After 48 hours, the medium containing lentiviral particles was collected and filtered through sterile 0.45-μm filters. The filtered medium was used to infect HeLa, M2, and SaoOS2 cells that were cultured in 12-well plates. The cell lines stably expressing BRF1 shRNA or HA-BRF1 were screened by puromycin selection in 96-well plates. Cell lines with stable expression were identified by immunoblotting with the anti-BRF1 antibody. For rRNA expression analysis using cell lines stably expressing BRF1 shRNA or HA-BRF1 or the corresponding control cell lines, total RNA was extracted from 5×10^6 cells using an Axygen RNA extraction kit, and cDNA synthesis was performed with a 20 μL reaction mixture containing 0.25 μg of total RNA and 0.5 μL of RT M-MuLV reverse transcriptase (New England Biolab.). When the reverse transcription reaction was complete, the cDNA mixture was diluted 5-fold with ddH_2O. Quantitative PCR was performed using 1 μL of diluted cDNA and 10 μL of 2× SYBR Green

MasterMix in a real-time PCR detection system (Bio-Rad) with GAPDH as the reference gene. PCR data were processed with CFX Manager 3.1 software (Bio-Rad). Relative expression was quantified by comparing the abundance of the target gene in the treated samples to that in the control samples after normalization to the GAPDH abundance in the corresponding samples, with the transcript abundance in the control samples arbitrarily set as 1. The primers used for RT–qPCR are listed in Table S1. For analysis of newly synthesized rRNA, HeLa cells with BRF1 depletion or overexpression were cultured in 12-well plates and labelled with 5-ethynyl uridine (EU) for 2 hours prior to fixation with 3.7% formaldehyde solution freshly prepared with 1× PBS. EU-labelled RNA was detected in accordance with the manual provided in the Cell-Light EU Apollo 555 *in Vitro* Imaging Kit (RiboBio, Guangzhou). Cell samples were observed under a fluorescence microscope (Olympus, Japan) and imaged with a 10× objective. The fluorescence intensity of nucleoli and the nucleoplasm in an equal area was quantified with ImageJ software. The relative fluorescence intensity of a single nucleolus was calculated using the following formula: (fluorescence intensity of a single nucleolus – fluorescence intensity of the nucleoplasm in an area equal to the nucleolar area) × proportion of Pol I products within the total rRNA (0.983). A scatter plot was generated by determining the relative fluorescence intensity of individual nucleoli using GraphPad Prism 6 software.

Dot blotting

HeLa cell lines expressing BRF1 shRNA or HA-BRF1 and the corresponding control cell lines were cultured in 10 cm dishes. At 90% confluence, cells were harvested, and nuclei were purified from HeLa cells using the method described above. Next, total RNA was extracted from nuclei using an RNA extraction kit (Axygen). One microgram of total RNA was spotted in small circles on a piece of nylon membrane (5 cm × 7 cm), and the membrane was dried at 65 °C for 1 hour. Probes were prepared in a 40 μL reaction mixture containing 10 U of Klenow enzyme, 25 pmol of biotin-labelled random hexamer primers and 500 ng of template DNA amplified from the introns of 45S pre-rRNA. Dot blot hybridization was performed using standard procedures described previously (49). After hybridization, the membrane was incubated for 1 hour in a 5% skim milk-PBS solution containing 1 μL of an anti-biotin HRP-linked antibody and was washed twice with 1×PBS and detected with ECL reagent.

In vitro transcription

The rDNA promoter sequence amplified from human genomic DNA was inserted into the pGL3-Basic reporter vector. HeLa cells were cultured in 10-cm dishes. At 90% confluence, cells were harvested, and nuclei were purified using the method described above. The resulting nuclei were resuspended in 400 μL of buffer D (20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF) and subjected to vortexing for 20 min. The nuclear extract was obtained by centrifugation for 10 min at 12000 rpm. *In vitro* transcription assays were performed using the rDNA promoter-driven plasmids and 25 μL of nuclear extract according to the nonradioactive method described previously (50). Relative transcriptional activity was obtained by comparing the promoter activity in the nuclear extracts with and without BRF depletion.

Cell proliferation assays

Cell proliferation assays were performed in the cell lines expressing BRF1 shRNA or HA-BRF1 using different methods, including cell counting, MTT colorimetry, and EdU incorporation. In the MTT assay, proliferative activity was detected as described previously (40). In the EdU incorporation assay, we followed the manual provided in the BeyoClick EdU Cell Proliferation Kit (Beyotime, China). Briefly, HeLa cells expressing control shRNA or BRF1 shRNA were seeded in 24-well plates. After 24 hours, the culture medium was replaced with complete medium containing 10 mM EdU, and the cells were incubated for 2 hours. After incubation, the cells were fixed for 15 minutes using 4% paraformaldehyde, permeabilized for 10 minutes with a PBS solution containing 0.3% Triton X-100 and stained with Click Additive Solution for 30 minutes. Then, the cell samples were washed 3 times with 1× PBS solution and stained with Hoechst 33342 for 10 minutes. After drying, the stained samples were imaged under a fluorescence microscope (Olympus IX71-F22FL/DIC, Japan). EdU-labelled cells and total cells were counted in a minimum of five images per sample. The rate of EdU-positive cells was determined by comparing the number of EdU-labelled cells to the number of total cells.

Mouse models of tumorigenesis

Twenty of the five-week-old female BALB/c nude mice were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China). The mice were housed under specific pathogen-free (SPF) conditions in a temperature-controlled room (25 °C) on a 12 h light/dark cycle at 60% humidity. At six weeks old, nude mice were randomly grouped into two groups (10 mice/group) and were injected subcutaneously in the right flank near the hind leg. Each mouse was injected with 1×10^7 HeLa cells expressing control shRNA or BRF1 shRNA. Beginning 7 days post-injection, the lengths and widths of the tumours were measured with Vernier callipers every 3 days; tumour volumes were calculated using the following formula: $V=\frac{\pi}{6}\times(\text{length}\times\text{width}^2)$. At the end of the fifth week, mice were euthanized, and the tumours formed were excised, weighed, and photographed. Immunohistochemical and haematoxylin and eosin (H&E) staining were performed on the tumour tissues. Animal experiments were approved by the Animal and Medical Ethics Committee of Tianyou Hospital affiliated with Wuhan University of Science and Technology. Animal experiments were conducted according to the Guide for The Care and Use of Laboratory Animals (NIH).

Haematoxylin and eosin staining and immunohistochemistry

Three tumour samples randomly selected from each group were fixed with formalin, embedded in paraffin, and sectioned manually according to a standard procedure. Tissue sections were deparaffinized before haematoxylin and eosin (H&E) staining was performed as described previously (51). Tissue sections were observed and imaged under a bright-field microscope. For immunohistochemical (IHC) analysis, tissue sections obtained as described above were deparaffinized by two incubations with xylene and then rehydrated using ethanol at different concentrations and double-distilled water (ddH_2O). IHC analysis was performed using an anti-BRF1 antibody

based on a method described previously (52). IHC samples were observed and imaged under a bright-field microscope at 400x magnification.

Immunoprecipitation and immunoblotting

HeLa cells were cultured in six 10-cm dishes. At 90% confluence, cells were harvested, and nuclei were isolated as described above. The nuclear pellet was suspended in 1 mL of lysis buffer (50 mM Tris-HCl (pH 7.9), 250 mM NaCl, 1 mM DTT, 1% NP-40, and 2 mM PMSF) and then vortexed for 20 minutes. After centrifugation for 10 minutes at 13000 rpm, the supernatant (nuclear extract) was retained for immunoprecipitation (IP) assays. IP assays were performed using normal IgG, an anti-BRF1 antibody, and antibodies against UBF (ab244287, Abcam), TIF-1A (ab251933, Abcam), TBP (ab28175, Abcam), TAF1A (SC-393600, Santa Cruz Biotech.), GTF3C2 (SC-81406, Santa Cruz Biotech.), TAF1B (CSB-PA684476ESR1HU, Cusabio), and RPA43 (Ab99305, Abcam). Briefly, 200 μL of the nuclear extract was incubated with 5 μg of the indicated antibody at 4 °C overnight prior to incubation with 40 μL of protein A/G agarose (Santa Cruz Biotech.) at room temperature for 2 hours. After incubation, the samples were washed 4 times with a modified RIPA buffer (0.05% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA, 0.5 mM EGTA, 300 mM NaCl, and 10 mM Tris-HCl (pH 8.0)). Antibody-bound proteins were eluted with 40 μL of 1* SDS loading buffer. After boiling for 10 min, 10 μL of each eluted sample was used for Western blot analysis, and 5% of the input was used as the positive control.

Reporter assays and ChIP assays

A cDNA fragment encoding the 5' region (approximately 400 nt) of 45S rRNA was amplified by RT–PCR and inserted downstream of the rDNA promoter in the plasmid pGL3-rDNAP (see Fig. 7F). HeLa cells stably expressing BRF1 shRNA or control shRNA were seeded in 12-well plates and cultured overnight prior to transfection. The rDNA promoter-driven reporter vectors (1.5 μg/well) were transfected into HeLa cells. Forty-eight hours post-transfection, cells were harvested, and reporter gene expression was analysed by RT–qPCR using a pair of primers targeting the luciferase gene (Fig. 7D-F) or a pair of primers targeting a 45S pre-rRNA fragment (forward) and the luciferase gene (reverse) (Fig. 7G, and H).

ChIP assays were performed using HeLa cell lines stably expressing BRF1 shRNA or HA-BRF1 and the corresponding control cell lines as described previously (40) with antibodies specific for BRF1, TBP, TIF-1A, TAF1A, UBF, and RPA43. After decrosslinking, DNA was recovered from ChIP samples using a QIAGEN PCR Purification Kit and eluted with 40 μL of ddH_2O. One microlitre of DNA recovered from the ChIP assay (1/40) was used for qPCR, while 1 ng of genomic DNA (equal to 0.02% of the input) was used as the positive control. Relative enrichment (or the percentage of the 0.02% input value) was determined by comparing the quantity of promoter DNA in the 1 μL ChIP sample to that in the 0.02% input sample. Sequential ChIP assays were performed according to a method described previously (53). Briefly, HeLa cells were cultured in 10-cm dishes; at 90% confluence, the cells were fixed and harvested for ChIP assays using an anti-BRF1 antibody. After eluting the products from the first immunoprecipitation reaction, chromatin was diluted and incubated with

antibodies against the Pol I transcription machinery components. When the second round of ChIP was complete, DNA was purified from the ChIP products as described above. Quantitative PCR was performed using 1 μL samples of DNA from the second round of ChIP and 0.5 ng of genomic DNA (0.01% of the input DNA) was used as the positive control. All antibodies used for the ChIP assays were the same as those used for the co-IP assays. The primers used for ChIP–qPCR are listed in Table S1.

Experimental design and statistical analysis

All experiments, including IF assays, RT–qPCR, Western blot analysis, cell proliferation assays, IP assays, ChIP assays, and reporter assays, were designed and performed with three biological replicates. For RT–qPCR, cell proliferation assays, tumorigenesis assays, reporter assays, and ChIP assays, the mean and standard deviation (SD) values were calculated with GraphPad Prism 6.0 software. Representative images selected from IF assays, IP assays and protein expression analyses by Western blotting are presented in this manuscript. Each column or point in the bar and point–line graphs represents the mean±SD of three biological replicates. *P* values were calculated by Student's *t* test performed with data for the control and treatment groups or, alternatively, by two-way ANOVA.

Acknowledgements

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Conflicts of interest

The authors declare that they have no conflicts of interest in relation to the work.

Ethics statement

Animal experiments were approved by the Animal and Medical Ethics Committee of Tianyou Hospital affiliated with Wuhan University of Science and Technology. The animal protocols abided by the Animal Welfare Guidelines (NIH).

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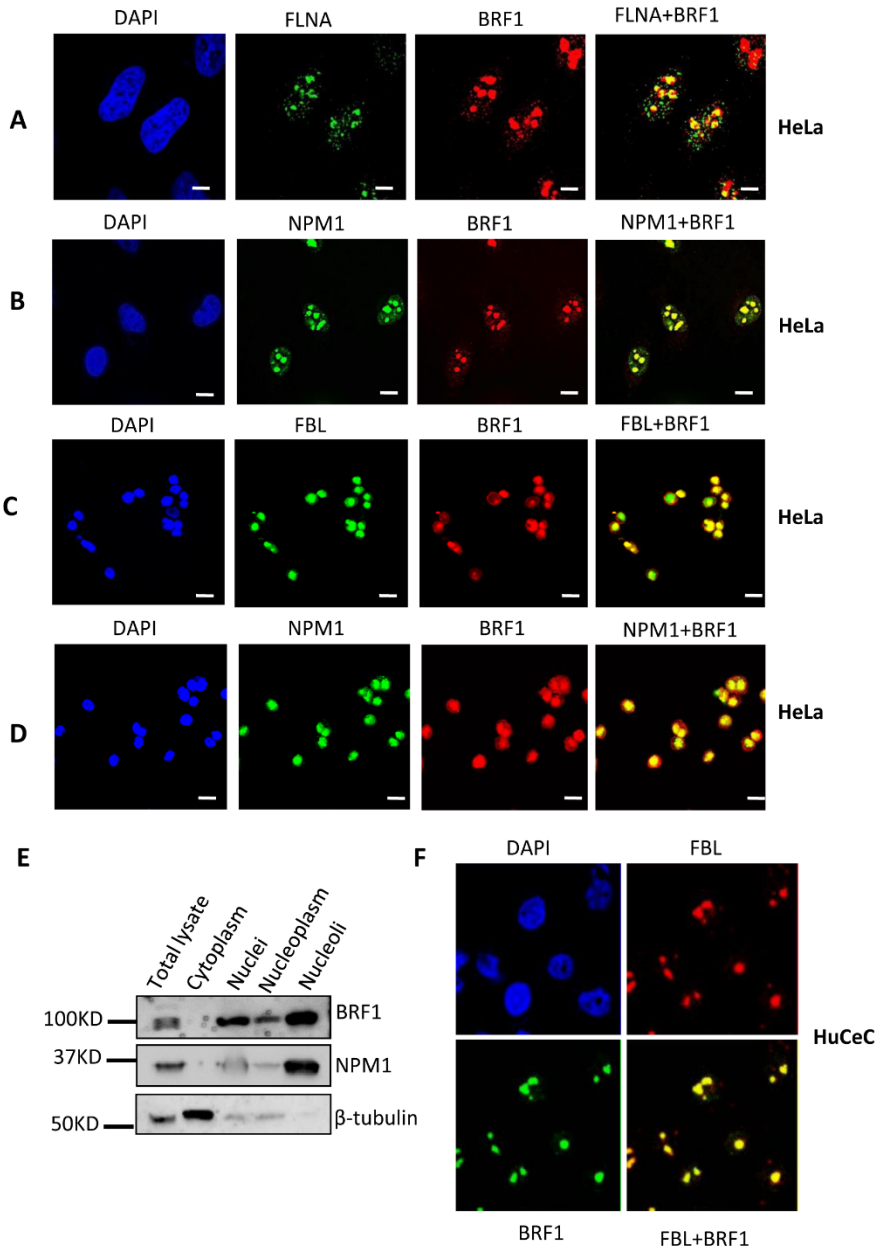
Figure legends

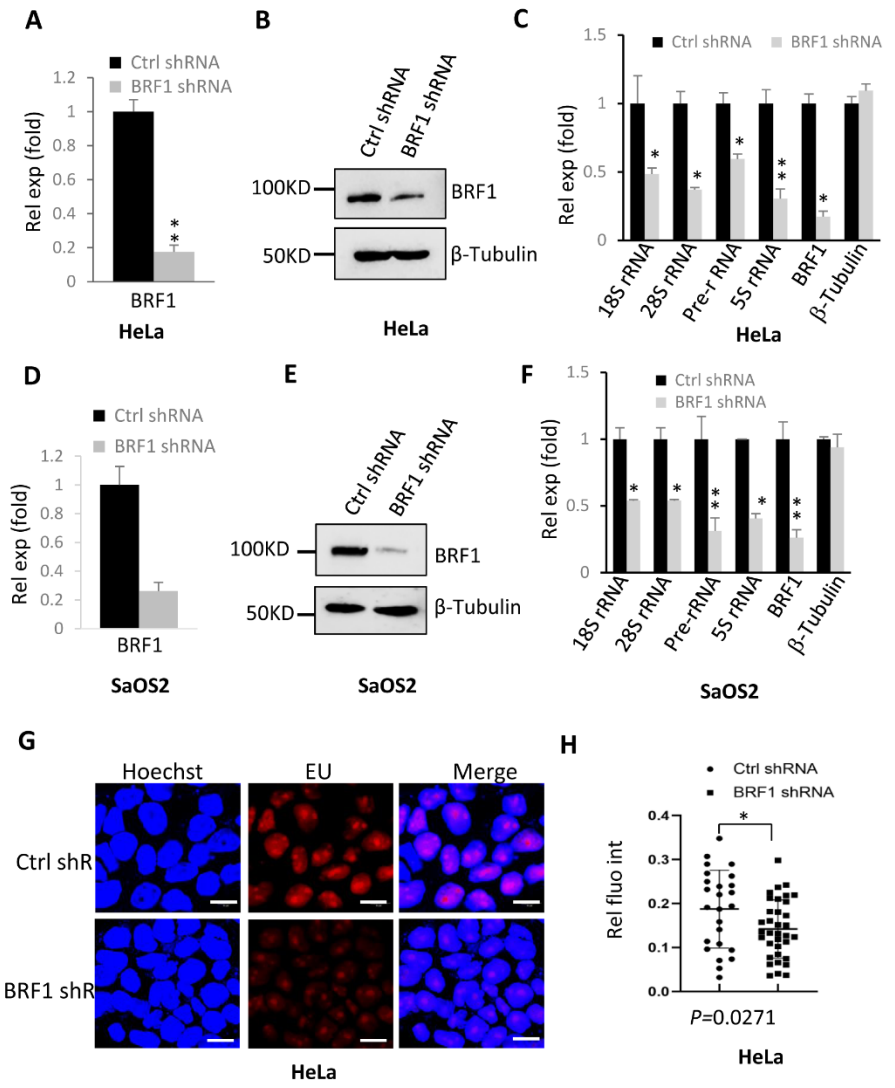
Figure 1. BRF1 is localized to nucleoli in HeLa cells.



(**A**) BRF1 and FLNA were colocalized in the nucleoli of HeLa cells. Immunofluorescence (IF) staining was performed using HeLa cells and antibodies against BRF1 and FLNA. The IF samples were observed under a DeltaVision fluorescence microscope and imaged with a 60x objective (Olympus). (**B**) BRF1 and NPM1 were colocalized in the nucleoli of HeLa cells. IF staining was performed as described in *A* with the anti-NPM1 antibody replacing the anti-FLNA antibody. (**C**) Analysis of the colocalization of BRF1 and FBL in nucleolar particles purified from HeLa cells. Nucleoli were purified from HeLa cells, and IF assays were performed using the resulting nucleolar samples and the indicated antibodies. The IF samples were observed under a confocal fluorescence microscope and imaged with a 100x objective (Olympus). (**D**) Analysis of the colocalization of BRF1 and NPM1 in nucleolar particles purified from HeLa cells. IF staining was performed as described in (C) with the anti-FBL antibody replaced by the anti-NPM1 antibody. (**E**) Immunoblot analysis of the

nucleolar fraction and other cellular fractions obtained from HeLa cells. (**F**) Colocalization assay of BRF1 and FBL in human cervical epithelial cells (HuCEC). IF assays were performed with human cervical epithelial cells and the indicated antibodies. The IF samples were observed and imaged under a confocal fluorescence microscope. The scale bars in A, C and D represent 1 μm; the scale bars in B and D represent 2 μm; and the scale bars in all amplified (AMP) images represent 0.3 μm.

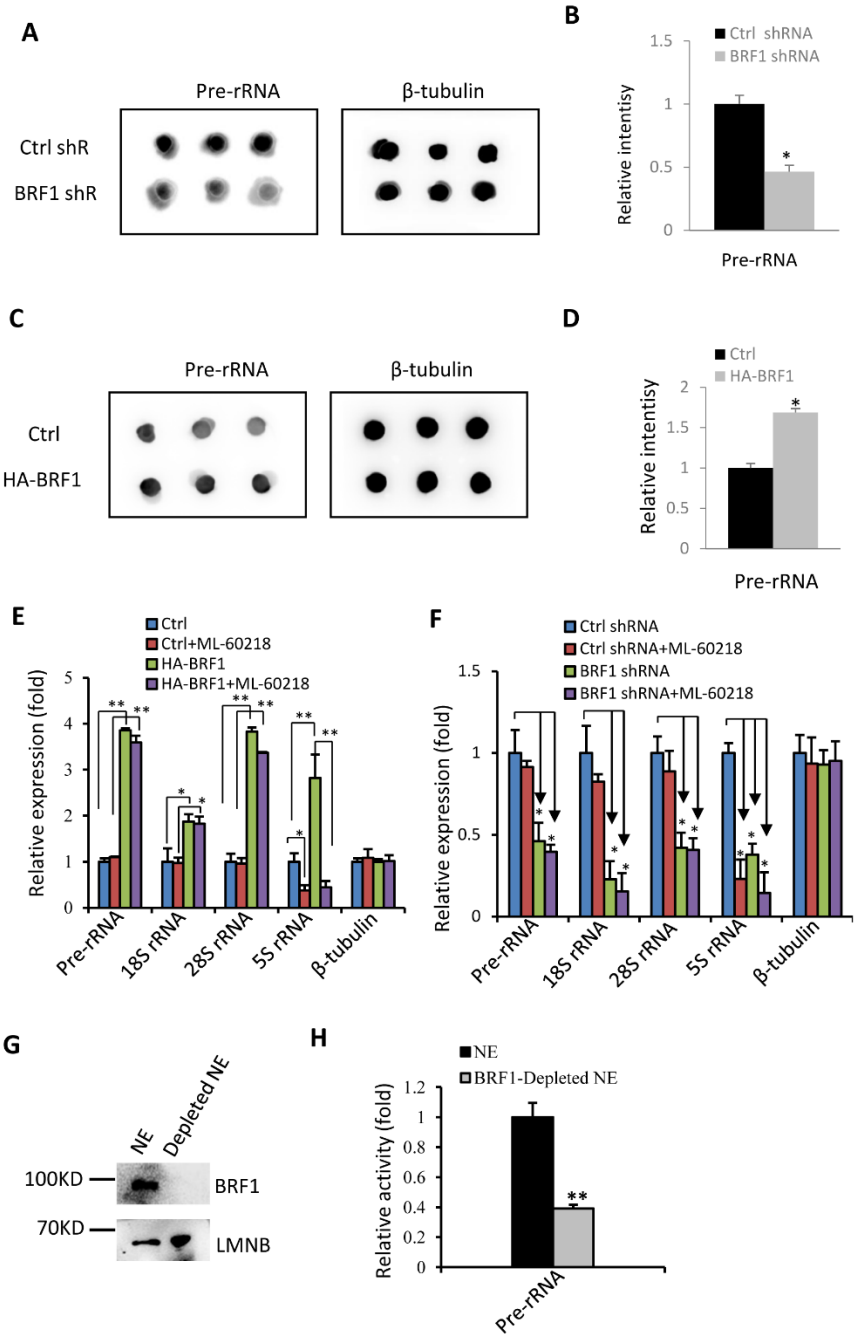
Figure 2. BRF1 knockdown inhibited Pol I-directed transcription.



(**A**, **B**) Generation of HeLa cell lines stably expressing BRF1 shRNA or control shRNA. HeLa cells were infected with lentiviral particles expressing BRF1 shRNA or control shRNA. Cell lines stably expressing BRF1 shRNA or control shRNA were screened in 96-well plates and confirmed by RT–qPCR (A) and Western blotting (B). (**C**) Stable expression of BRF1 shRNA inhibited Pol I-mediated transcription in HeLa cells. Total RNA was extracted from the cell lines described in (A) and (B), and rRNA gene expression was analysed by RT–qPCR. (**D-F**) BRF1 depletion by shRNA

repressed Pol I transcription in SaOS2 cells. SaOS2 cell lines stably expressing BRF1 shRNA or control shRNA were generated using a lentiviral transduction system and confirmed by RT–qPCR (D) and Western blotting (E), and Pol I products were monitored by RT–qPCR (F). (**G** and **H**) Result of an EU assay showing the effect of BRF1 depletion on rRNA synthesis. HeLa cell lines stably expressing BRF1 shRNA or control shRNA were labelled with 5-ethynyl uridine (EU). The samples from the EU assays were observed and imaged with a fluorescence microscope (G), and the intensity of red fluorescence representing nucleoli was determined with ImageJ software (H). The scale bar in each image represents 50 μm. *, $P \leqq 0.05$; **, $P \leqq 0.01$; *P* values were obtained by Student's *t* test performed with data for the control and treated samples.

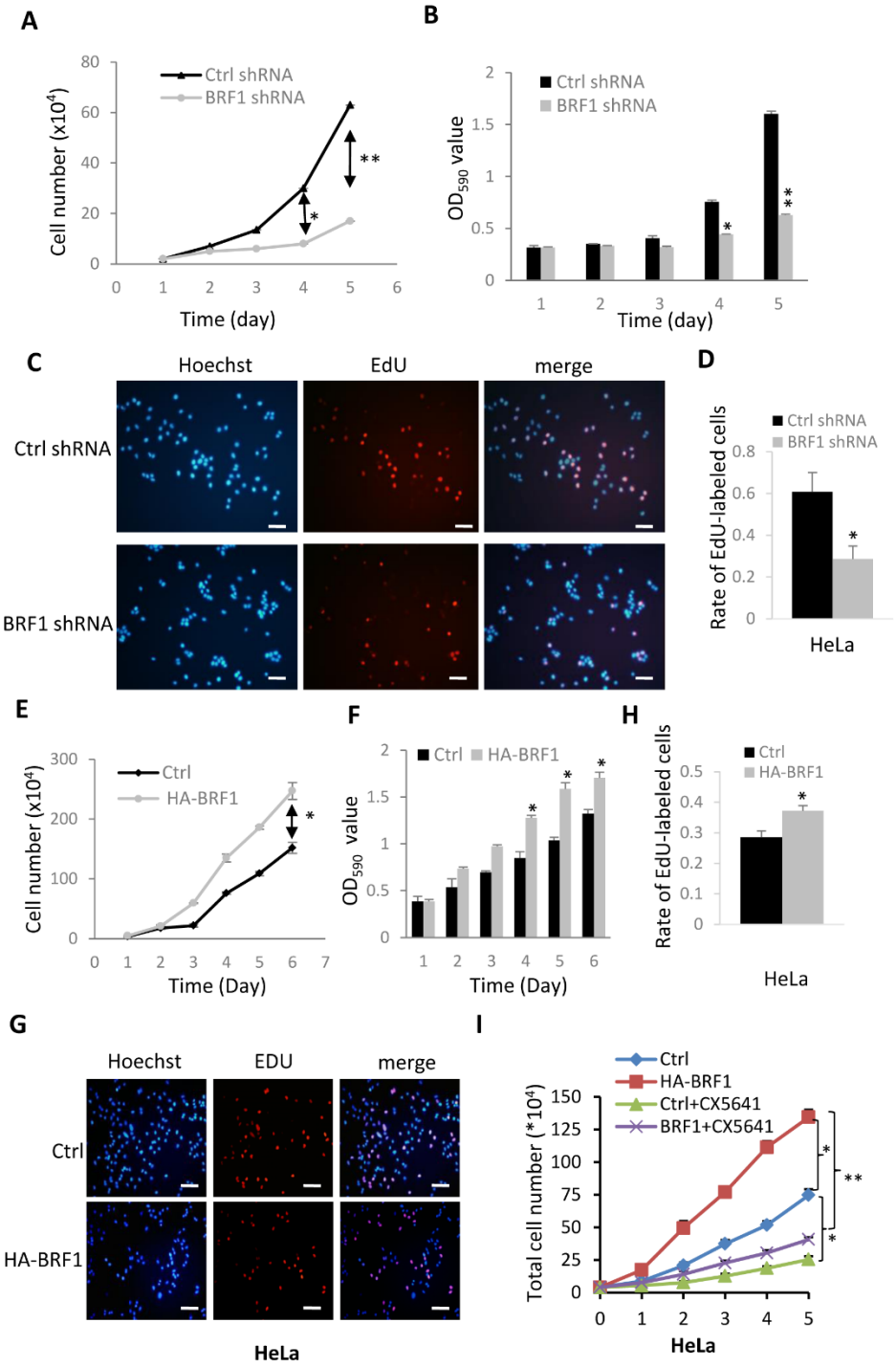
Figure 3. BRF1 can directly regulate Pol I-directed transcription.



(**A**) Results of dot blotting for 45S pre-rRNA (left panel) and β-tubulin mRNA extracted from HeLa cells expressing BRF1 shRNA or control shRNA. (**B**) Quantitative analysis of the dot blots obtained in A. (**C**) Results of dot blotting for 45S pre-rRNA (left panel) and β-tubulin mRNA in HeLa cells expressing HA-BRF1 shRNA and the corresponding control cells. (**D**) Quantitative analysis of the dot blots obtained in (C). (**E**) Treatment with a Pol III-specific inhibitor did not significantly affect the activation of Pol I transcription induced by BRF1 overexpression. RT–qPCR was performed using RNA extracted from the indicated HeLa cell lines treated with or without 50 μM ML-60218. (**F**) Treatment with the Pol III-specific inhibitor did not significantly enhance the inhibition of Pol I transcription caused by BRF1 silencing. RT–qPCR was performed using the RNA extracted from the indicated HeLa cell lines treated with or

without 50 μM ML-60218. (**G**) Western blotting was used to detect BRF1 enrichment in the nuclear extract (NE) from HeLa cells with BRF1 depletion. (**H**) *In vitro* transcription assays of the rDNA promoter-driven reporter gene, where transcription of the *luciferase* gene *in vitro* was detected by RT–qPCR. *, $P \leqq 0.05$; **, $P \leqq 0.01$; *P* values were obtained by Student's *t* test performed with data from the two groups as indicated.

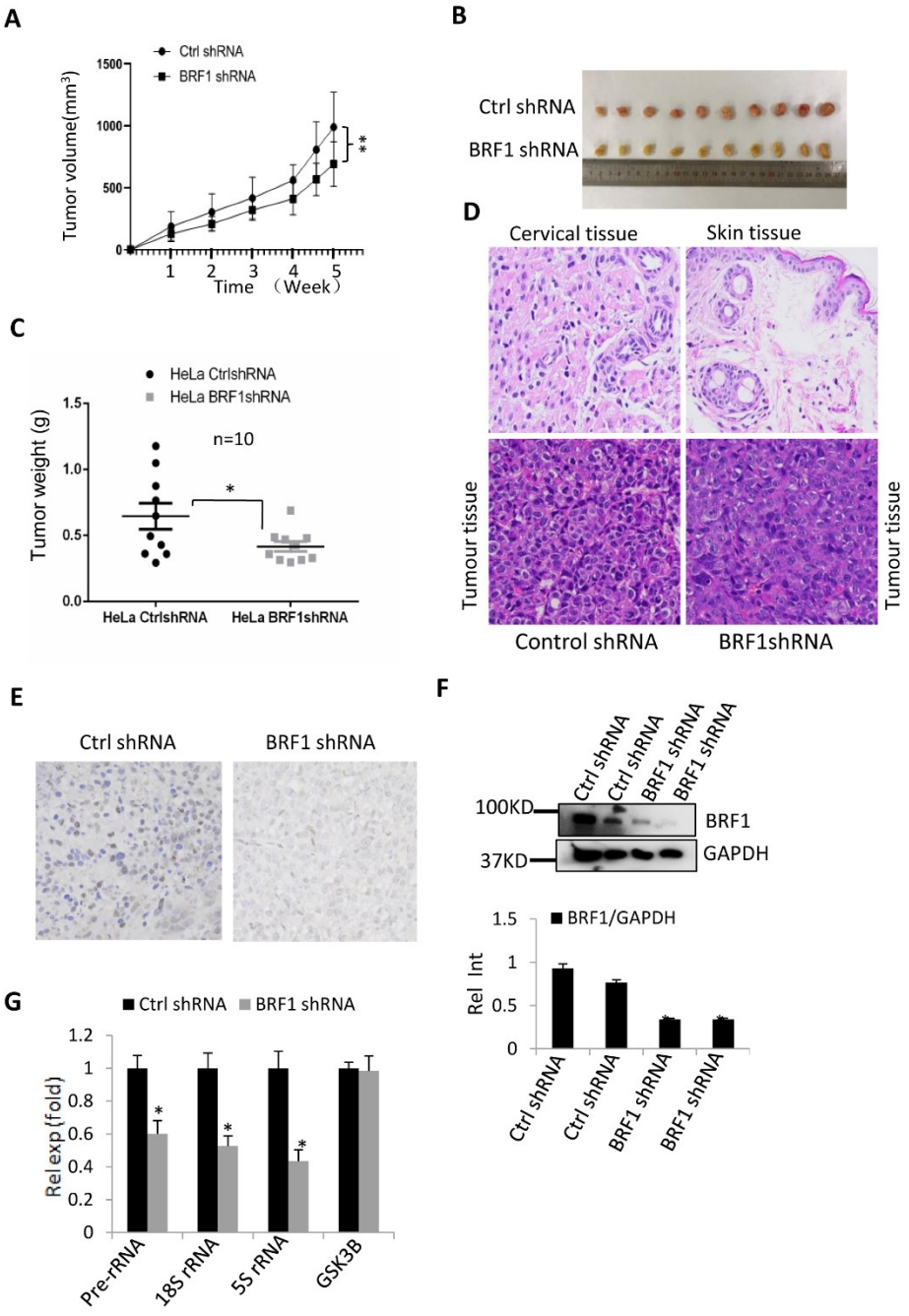
Figure 4. **BRF1 promotes cell proliferation by increasing Pol I transcription**.



(**A**, **B**) Knockdown of BRF1 suppressed HeLa cell proliferation. HeLa cell proliferation

was analysed using cell counting (A) and an MTT assay (B). (**C**, **D**) Evaluation of HeLa cell proliferation by an EdU incorporation assay. HeLa cell lines stably expressing BRF1 shRNA or control shRNA were labelled with EdU, and the effect of EdU incorporation was monitored by using an EdU detection kit and imaged under a fluorescence microscope (C). EdU–labelled cells in the images were counted under a microscope, and the data were analysed with GraphPad 6.0 (D). The scale bars in the images represent 200 μm. (**E**, **F**) BRF1 overexpression enhanced HeLa cell proliferation. HeLa cells stably expressing HA-BRF1 and the corresponding control cells were seeded in 12-well and 96-well plates. Cell proliferation was analysed using cell counting (E) and an MTT assay (F). (**G**, **H**) Result of an EdU incorporation assay showing the effect of BRF1 overexpression on HeLa cell proliferation. HeLa cells stably expressing HA-BRF1 and the corresponding control cells were cultured in 12-well plates. EdU incorporation assays were performed as described in (C) and (D). EdU-labelled cells were imaged (G), and the data were statistically analysed (H). The scale bars in the images represent 200 μm. (**I**) CX-5461 suppressed the increase in proliferative activity induced by BRF1 overexpression. HeLa cells expressing HA-BRF1 and the corresponding control cells were cultured in medium containing 4 μg of CX-5461. Cells were harvested every 24 hours and used for cell counting. *, $P \leqq 0.05$; **, $P \leqq 0.01$; the *P* values in D and H were obtained by Student's *t* test performed with data for the control and treated samples. The *P* values in the other subfigures were obtained by two-way ANOVA.

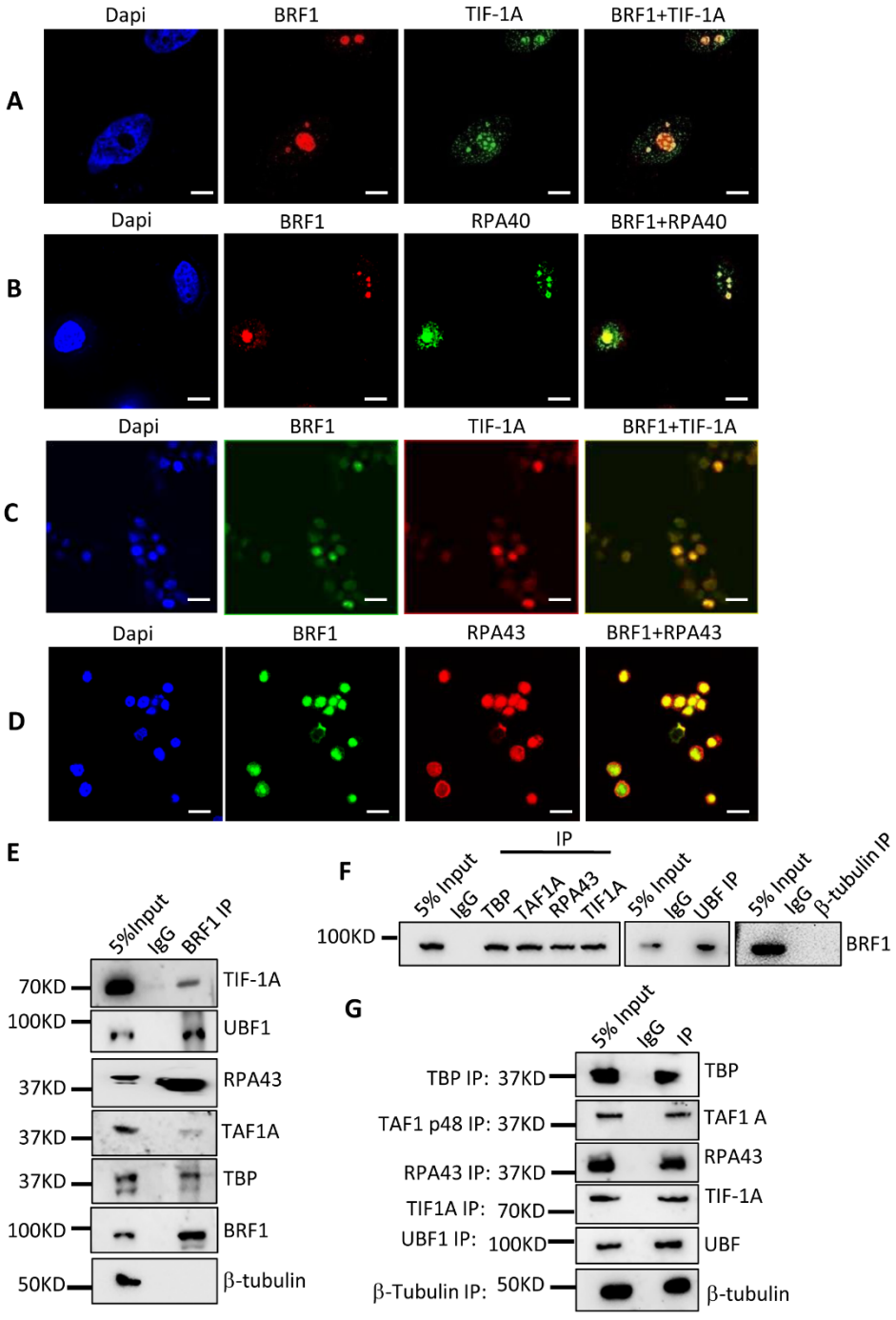
Figure 5. BRF1 knockdown inhibited tumour growth *in vivo*.



(**A**) Comparison of volumes between tumours expressing BRF1 shRNA and tumours expressing control shRNA during tumour formation (n=10). (**B**) An image showing the difference in the tumour size between tumour samples expressing BRF1 shRNA and tumour samples expressing control shRNA. Five weeks after tumour cell injection, the tumour tissues were removed from the mice and photographed with a digital camera (Canon). (**C**) Statistical analysis of tumour samples shown in B (**D**) Representative images showing H&E staining of tumour, cervix, and skin tissues from nude mice. The scale bars in the images represent 100 μm. (**E**) Immunohistochemical staining of tumour tissues expressing BRF1 shRNA or control shRNA using an anti-BRF1 antibody. The scale bars in the images represent 100 μm. (**F**) Immunoblot analysis of

tissue samples expressing BRF1 shRNA or control shRNA. The intensity of the Western blot bands (upper panel) was analysed with ImageJ software (bottom panel). (**G**) Analysis of Pol I products in tumour tissues expressing BRF1 shRNA or control shRNA. *, $P \leqq 0.05$; **, $P \leqq 0.01$; P values were obtained by Student's t test performed with data for the control and treated samples.

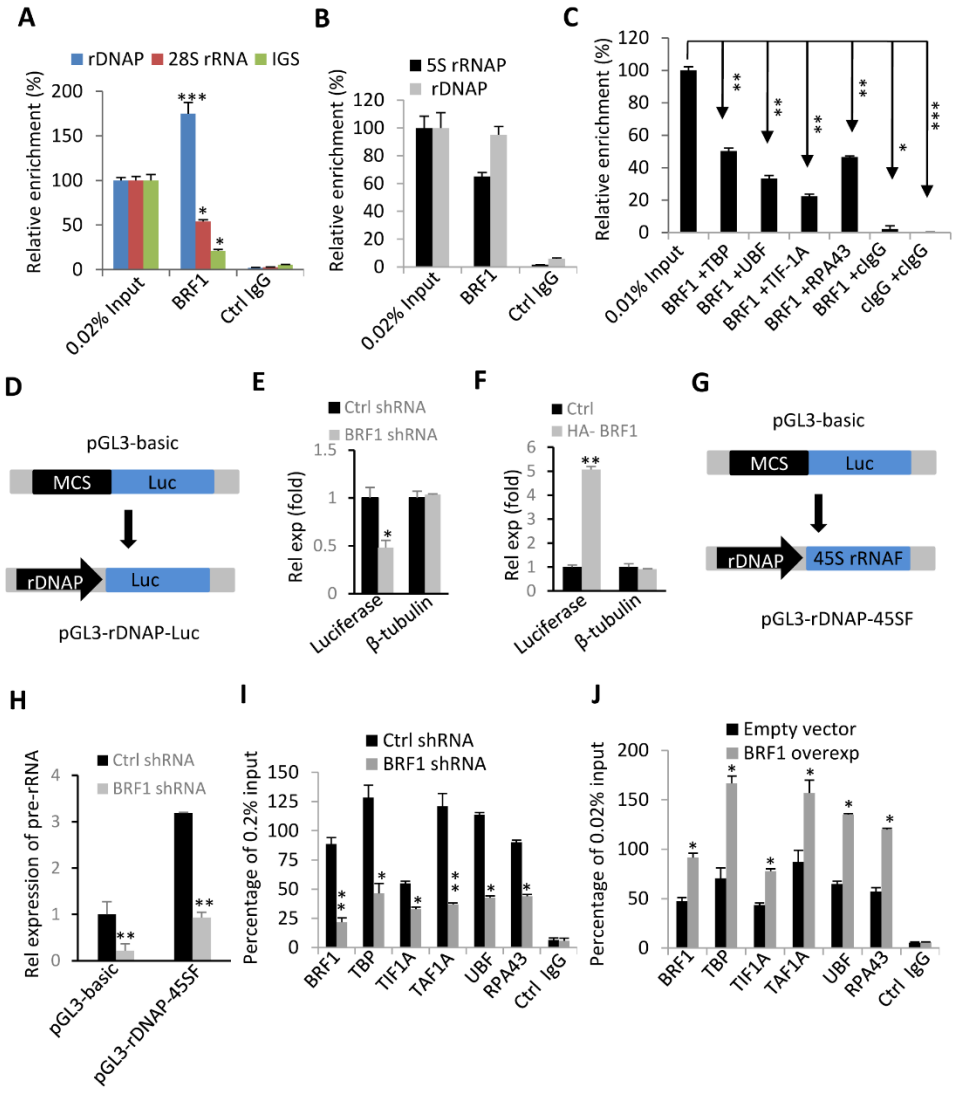
Figure 6. BRF1 interacts with components of the Pol I transcription machinery.



(**A**) BRF1 and TIF-1A were colocalized in the nucleoli of HeLa cells. IF staining was performed using anti-BRF1 and anti-TIF-1A antibodies. The scale bar in each image represents 2.5 μm. (**B**) BRF1 and RPA40 were colocalized in the nucleoli of HeLa cells. IF staining was performed using anti-BRF1 and anti-RPA40 antibodies. The scale bar

in each image represents 2.5 μm. (**C**) Colocalization analysis of BRF1 and TIF-1A using nucleolar particles purified from HeLa cells. The scale bar in each image represents 2.5 μm. (**D**) Colocalization analysis of BRF1 and RPA43 using nucleoli purified from HeLa cells. The scale bar in each image represents 5 μm. (**E**) Components of the Pol I transcription machinery were precipitated by the anti-BRF1 antibody. Nuclear extracts from HeLa cells were used for BRF1 IP assays. The IP samples were analysed by Western blotting using the indicated antibodies. (**F**, **G**) BRF1 was precipitated by the indicated antibodies. IP assays were performed with antibodies against TBP, TIF-1A, TAF1A, UBF, and RPA43. BRF1 (F) and protein antigens (G) were detected by Western blotting.

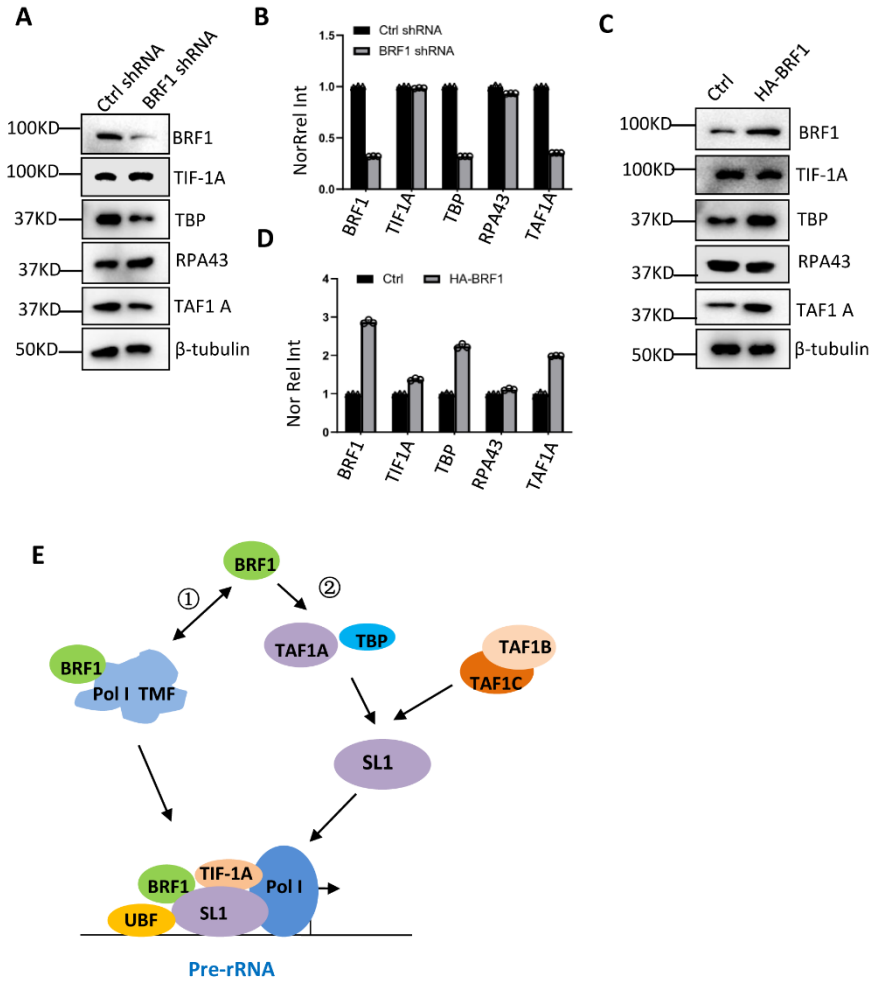
Figure 7. BRF1 modulates Pol I-mediated transcription by controlling Pol I transcription machinery assembly at the rDNA promoter.



(**A**) A ChIP–qPCR result showing BRF1 binding to the rDNA promoter (rDNAP), coding region (28S rRNA), and intergenic spacer (IGS) region. ChIP assays were performed using HeLa cells and an anti-BRF1 antibody or control IgG. Relative

enrichment was determined by comparing the quantity of promoter DNA in a 1 μL ChIP sample to that in 0.02% of the input DNA (1 ng of genomic DNA). (**B**) Comparison of BRF1 binding to the rDNA promoter (rDNAP) and the 5S rRNA promoter (5S rRNAP). A ChIP assay was performed as described in (A). (**C**) Sequential ChIP assays of BRF1 and the components of the Pol I transcription machinery. One microlitre of each DNA sample (40 μL) recovered from the sequential ChIP assay or 0.5 ng of genomic DNA (0.01% of the input) was used for qPCR. Relative enrichment was determined as described in A. (**D**) A scheme showing the construction of the reporter vector pGL3-rDNAP-Luc. rDNAP: rDNA promoter, MCS: multiple cloning site. Luc: *luciferase* gene. (**E**) BRF1 knockdown inhibited rDNA promoter activity. HeLa cells were transfected with the rDNA promoter-driven reporter vectors obtained as described in C. Reporter gene expression was analysed by RT–qPCR. (**F**) BRF1 overexpression enhanced rDNA promoter activity. HeLa cells were transfected with the rDNA promoter-driven reporter vectors obtained as described in C. Reporter gene expression was detected by RT–qPCR. (**G**) A scheme showing the construction of the vector pGL3-rDNA-45SF. 45SF: 45S rRNA fragment. (**H**) BRF knockdown inhibited the expression of the rRNA fragment driven by rDNAP. HeLa cells were transfected with the vector pGL3-rDNAP-45SF. The expression of 45S rRNA was determined by RT–qPCR using rRNA gene-targeted primers. (**I**) BRF1 knockdown inhibited the recruitment of the Pol I transcription machinery components to the rDNA promoter. ChIP assays were performed using HeLa cell lines and the antibodies indicated in the graph. Quantitative PCR was performed as described in (A). The percentage of the 0.02% input value (relative enrichment) was determined by comparing the quantity of promoter DNA in 1/40 of ChIP DNA sample to that in the 0.02% input DNA sample. (**J**) BRF1 overexpression enhanced the recruitment of the Pol I transcription machinery components to the rDNA promoter. ChIP assays were performed with HeLa cell lines and the indicated antibodies. Quantitative PCR and data analysis were performed as described in (A) and (I). *, $P \leqq 0.05$; **, $P \leqq 0.01$; *P* values were obtained by Student's *t* test performed with data from the two groups as indicated.

Figure 8. BRF1 regulates the expression of TBP and TAF1A.



(**A**) The effect of BRF1 silencing on the expression of the Pol I transcription machinery components. HeLa cells stably expressing BRF1 shRNA and the corresponding control cells were cultured and harvested for Western blot analysis using the indicated antibodies. (**B**) Quantitative analysis of the Western blot results in A. The normalized relative intensity was determined by comparing the intensity of a target protein in the Ctrl shRNA samples to that in the BRF1 shRNA samples after normalization to the corresponding β-tubulin intensity. Western blot band intensities were obtained with ImageJ software. (**C**) The effect of BRF1 overexpression on the expression of the Pol I transcription machinery components. HeLa cells stably expressing HA-BRF1 and the corresponding control cells were cultured and harvested for Western blot analysis using the indicated antibodies. (**D**) Quantitative analysis of the Western blot results in C. The normalized relative intensity was determined by comparing the intensity of a target protein in the Ctrl samples to that in the HA-BRF1 samples after normalization to the corresponding β-tubulin intensity. *, $P \leqq 0.05$; **, $P \leqq 0.01$; *P* values in B and D were obtained by Student's *t* test performed with data from the two groups as indicated. (**E**) A model showing how BRF1 regulates Pol I-directed transcription. ①, BRF1 interacts with the Pol I transcription machinery components (Pol I TMC) to regulate Pol I transcription machinery assembly at the rDNA promoter

and thus Pol I-dependent transcription. ② BRF1 regulates the expression of TBP and TAF1A, which subsequently controls the recruitment of the Pol I transcription machinery components to the rDNA promoter and thus Pol I-dependent transcription.

Abbreviations

BRF1: TFIIB-related factor 1; Pol I: RNA polymerase I; Pol III: RNA polymerase III; rDNA: DNA encoding the 45S ribosomal RNA; FLNA: Filamin A, NPM1: Nucleophosmin 1; FBL: Fibrillarin; TBP: TATA-binding protein; TIF-1A: transcription initiator factor 1A; RPA40: DNA-directed RNA polymerase I and III 40 kDa polypeptide; RPA43: DNA-directed RNA polymerase I subunit F; UBF: upstream binding factor; SL1: Selectivity factor 1; DAPI: 4′,6-diamidino-2-phenylindole; RT–qPCR, reverse transcription coupled quantitative polymerase chain reaction; Ctrl IgG: Control immunoglobulin G; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IF: immunofluorescence; IP: immunoprecipitation; ChIP: chromatin immunoprecipitation; HA: Haemagglutinin; GSK3B: glycogen synthase kinase 3 beta; BDP1: B Double Prime 1; TFIIIB: transcription factor III B; TFIIIC, transcription factor III C; TAF1A: TATA box binding protein associated factor, RNA polymerase I subunit A; TAF1B: TATA box binding protein associated factor, RNA polymerase I subunit B; ICE: internal core element; A (Fig. 8E): Box A element; C (Fig. 8E): Box C element; RNA: ribonucleic acid; DNA: deoxyribonucleic acid; Pre-rRNA: ribosomal ribonucleic acid precursor; Ub: ubiquitin; EU:, 5-ethynyl uridine; EdU: 5-ethynyl-2'-deoxyuridine; CCK-8: Cell Counting Kit-8.