RNA polymerase III (Pol III) is responsible for the production of small noncoding RNA species, including tRNAs and 5S rRNA. Pol III–dependent transcription is generally enhanced in transformed cells and tumors, but the underlying mechanisms remain not well understood. It has been demonstrated that the BRF1 subunit of TFIIIB is essential for the accurate initiation of Pol III–dependent transcription. However, it is not known whether BRF1 undergoes ubiquitin modification and whether BRF1 ubiquitination regulates Pol III–dependent transcription. Here, we show that RNF12, a RING domain-containing ubiquitin E3 ligase, physically interacts with BRF1. Via direct interaction, RNF12 catalyzes Lys27- and Lys33-linked polyubiquitination of BRF1. Furthermore, RNF12 is able to negatively regulate Pol III–dependent transcription and cell proliferation via BRF1. These findings uncover a novel mechanism for the regulation of BRF1 and reveal RNF12 as an important regulator of Pol III–dependent transcription.

As the largest of the eukaryotic DNA-dependent RNA polymerases, RNA polymerase III (Pol III) is responsible for the transcription of small noncoding RNAs, including tRNAs, 5S rRNA, and U6 snRNA, and U6 snRNA (1, 2). These Pol III transcripts control several fundamental metabolic processes such as protein translation and RNA processing, thereby dictating the growth rate of a cell. The accurate initiation of Pol III–dependent transcription requires at least two general transcription factors, TFIIIB (3, 4) and TFIIIC (5, 6). TFIIIC recognizes and binds to specific sequence elements in target gene promoters, thereafter allowing the recruitment of TFIIIB. The binding of TFIIIB to the promoter in turn precisely positions Pol III at the transcription initiation start site. The TFIIIB complex, used for transcription of both tRNAs and 5S rRNA, consists of BRF1 (B-related factor 1), TBP (TATA box-binding protein), and BDP1 (B double prime 1). In contrast, the U6 RNA gene uses a TFIIIB complex that is composed of TBP, BDP1, and BRF2, a splicing variant of BRF1 (5).

It has long been recognized that compared with normal cells, both transformed and tumor cells exhibit elevated Pol III–dependent transcription (6–8), indicating that deregulated Pol III–dependent transcription plays a critical role in tumorigenesis. Consistent with this idea, enhanced Pol III–dependent transcription is required for oncogenic transformation of normal cells (9, 10). In addition, we have previously shown that the Pol III transcription product tRNA is able to inhibit apoptosis via directly binding to cytochrome c and preventing cytochrome c-initiated caspase activation (11). Given that the evasion of apoptosis is a prominent hallmark of cancer (12), it is conceivable that increased expression of tRNA, caused by Pol III deregulation, may promote tumorigenesis via the inhibition of apoptosis. These findings suggest that enhanced Pol III–dependent transcription not only allows cancer cells to meet their high demands for protein synthesis, but it is also actively involved in tumorigenesis.

Because of the fundamental role of Pol III–dependent transcription, this cellular process is not unexpectedly subjected to intricate regulation (1). As mentioned above, TFIIIB is essential for accurate and efficient Pol III–dependent transcription. Therefore, it is not surprising that a variety of cellular factors are able to regulate Pol III–dependent transcription via targeting TFIIIB directly or indirectly. For instance, the tumor suppressors p53 and Rb directly interact with TFIIIB to inhibit its function (13–15), whereas the oncogenic protein c-Myc induces Pol III–dependent transcription by directly binding to TFIIIB and enhancing its recruitment to promoters (16). In contrast, through its ability to control P13K/Akt activity, the tumor suppressor PTEN indirectly regulates the integrity of the TFIIIB complex by modulating the association between TBP and BRF1 (17).

In addition to its regulation by oncogenes and tumor suppressors, Pol III–dependent transcription is also affected by BRF1 expression. It has been shown that reduced BRF1 expression significantly decreases Pol III–dependent transcription (18). The biallelic mis-sense mutations of BRF1 also decrease Pol III–dependent transcription activity and cause neurodevel-
opmental anomalies (19). Moreover, post-translational modification of BRF1 plays an important role in the regulation of Pol III–dependent transcription. Several protein kinases, including Ck2, ERK, and PIK1, are able to phosphorylate BRF1, thereby controlling Pol III–dependent transcription (20–22). It is well-known that in addition to phosphorylation, protein post-translational modifications also include ubiquitination (23). However, it remains unknown whether cellular BRF1 is subjected to ubiquitin modification and whether Pol III–dependent transcription is regulated by BRF1 ubiquitination.

RFN12, also known as RLIM, is an X-linked and Ring domain–containing ubiquitin E3 ligase (24). It has been demonstrated that the cellular functions of RFN12 are largely attributed to its E3 ligase activity. For example, RFN12 regulates the activities of several transcription factors by controlling the protein levels of their co-factors (25–27). By targeting TRF1 for degradation, RFN12 controls telomere length homeostasis (28). RFN12 also modulates the TGFβ superfamily signaling pathways by promoting proteasome-dependent degradation of the negative regulator Smad7 (29). Moreover, RFN12 participates in X-chromosome inactivation in part by targeting the pluripotency factor REX1 for degradation (30–32). The genetic mutations of RFN12 that disrupt its E3 ligase activity have also been associated with X-linked intellectual disability (33, 34).

In this study, we report RFN12 as a novel interaction partner of BRF1. Via direct interaction, RFN12 promotes both Lys27- and Lys33-linked polyubiquitination of BRF1. Functionally, RFN12 negatively regulates Pol III–dependent transcription and cell proliferation via BRF1. Collectively, these results reveal RFN12 as a critical regulator of BRF1 and define an important function of RFN12 in the regulation of Pol III–dependent transcription.

Results

RFN12 is a BRF1-interacting protein

To investigate whether BRF1 undergoes ubiquitin modification, we performed the ubiquitination assay with WT ubiquitin or mutant ubiquitin (Ub–KO, all lysine residues replaced by arginine residues). The results showed that exogenous BRF1 was strongly polyubiquitinated in the presence of WT ubiquitin (Fig. 1A, lane 4). However, in the presence of Ub–KO, the polyubiquitination of BRF1 was barely detected (Fig. 1A, lane 5). In addition, the polyubiquitination of BRF1 was also verified at the endogenous level (Fig. 1B). These data suggest that BRF1 indeed undergoes polyubiquitination in cells.

We next sought to determine how cellular BRF1 is polyubiquitinated. We employed an affinity purification method to identify novel BRF1-interacting proteins. HeLa cells were treated with formaldehyde to stabilize protein–protein interactions. The cell lysates were immunoprecipitated with anti-BRF1 antibody. The immunoprecipitates were analyzed by MS. RFN12, a RING domain–containing ubiquitin E3 ligase, was identified in the anti-BRF1 immunoprecipitates (Fig. 1C and Table S1). To further verify the interaction between RFN12 and BRF1, we expressed GFP–RFN12 alone or together with Flag–BRF1 in HEK293T cells. An immunoprecipitation assay indicated a specific interaction of these two proteins (Fig. 1D). A reciprocal immunoprecipitation experiment using lysates from HEK293T cells expressing Flag–RFN12 and GFP–BRF1 also confirmed the RFN12–BRF1 interaction (Fig. 1E). Using a co-immunoprecipitation assay with anti-BRF1 antibody, the interaction between endogenous RFN12 and BRF1 was readily detected (Fig. 1F). Moreover, an in vitro binding assay with purified GST–RFN12 and Flag–BRF1 proteins revealed that RFN12 directly associated with BRF1 (Fig. 1G). The immunofluorescence assay showed that ectopically expressed RFN12 and BRF1 were co-localized in the nucleus (Fig. 1H). Taken together, these results demonstrate that RFN12 is a novel binding partner for BRF1.

Structural determinants of the RFN12–BRF1 interaction

To identify the region of BRF1 that mediates the interaction with RFN12, we generated three BRF1 deletion mutants (Fig. 2A). BRF1 (aa 1–260) strongly associated with RFN12, whereas BRF1 (aa 261–520) and BRF1 (aa 521–677) exhibited no interaction with RFN12 (Fig. 2B), implying that the N-terminal region (aa 1–260) of BRF1 is responsible for its interaction with RFN12. To delineate the BRF1-biding domain in RFN12, we also generated a panel of RFN12 deletion mutants (Fig. 2C). Both RFN12 (aa 1–569) and RFN12 (aa 206–409) strongly bound to BRF1, whereas RFN12 (aa 1–205) and RFN12 (aa 410–624) showed no binding to BRF1 (Fig. 2D). These data suggest that the central region (aa 206–409) of RFN12 mediates the interaction with BRF1. To further determine whether the N-terminal region (aa 1–260) of BRF1 interacts with the central region (aa 206–409) of RFN12, we expressed GFP–RFN12 (206–409) alone or together with Flag–BRF1 (1–260) in HEK293T cells. The subsequent immunoprecipitation experiment showed that RFN12 (206–409) indeed interacted with BRF1 (1–260) (Fig. 2E). In addition, an in vitro binding assay with purified GST–RFN12 (206–409) and Flag–BRF1 (1–260) proteins revealed that RFN12 (206–409) directly associated with BRF1 (1–260) (Fig. 2F).

RFN12 catalyzes BRF1 ubiquitination

Given the interaction of RFN12 with BRF1 and the previously reported ubiquitin E3 ligase activity of RFN12 (30), we asked whether RFN12 could be responsible for BRF1 polyubiquitination. We first performed an in vivo ubiquitination assay. RFN12 was shown to promote BRF1 polyubiquitination (Fig. 3A). The RFN12-promoted BRF1 polyubiquitination also occurred under denaturing conditions (Fig. 3B). Compared with WT RFN12, the ubiquitin E3 ligase inactive mutant of RFN12 (H569A/C572A) failed to show any effect on BRF1 polyubiquitination (Fig. 3C). To further determine whether RFN12 acts as a ubiquitin E3 ligase for BRF1, an in vitro ubiquitination assay was performed with purified recombinant proteins. The results showed that WT RFN12, but not RFN12 (H569A/C572A), enhanced the polyubiquitination of endogenous BRF1 (Fig. 3, E and F). These data indicate that RFN12 is a bona fide ubiquitin E3 ligase for BRF1.

To identify the potential lysine residue(s) of BRF1 that are targeted for polyubiquitination by RFN12, we first evaluated which domain of BRF1 was ubiquitinated in cells. The results...
showed that similar to WT BRF1, the N-terminal region (aa 1–260) of BRF1 was heavily polyubiquitinated (Fig. 3G, lanes 1–4). In contrast, neither BRF1 (261–520) nor BRF1 (521–677) was polyubiquitinated (Fig. 3G, lanes 5–8). These data suggest that polyubiquitination may occur at the lysine(s) residing in the N-terminal region (aa 1–260) of BRF1. We therefore individually mutated the nine lysines (Lys65, Lys79, Lys115, Lys127, Lys165, Lys199, Lys215, Lys247, and Lys254) residing in the N terminus of BRF1 to arginine. Mutation of each single lysine to arginine did not completely abolish the polyubiquitination of BRF1 by RNF12 (Fig. 3H), indicating that RNF12-mediated BRF1 polyubiquitination may occur at multiple lysine residues.

**RNF12 catalyzes Lys\(^{27}\)– and Lys\(^{33}\)–linked polyubiquitination of BRF1**

The finding that RNF12 enhanced BRF1 polyubiquitination prompted us to ask whether RNF12 could promote BRF1 protein degradation through the ubiquitin–proteasome pathway. Surprisingly, neither knockdown nor overexpression of RNF12 had an obvious effect on BRF1 protein levels (Fig. 4A). In addition, the half-life of BRF1 was not affected by ectopic expression of RNF12.
These data suggest that RNF12 does not regulate BRF1 protein turnover. We therefore sought to determine the type of BRF1 polyubiquitin chain induced by RNF12. Polyubiquitination usually occurs at Lys48 or Lys63 of ubiquitin. It has been well-known that Lys48-linked polyubiquitination serves as a recognition signal for target protein degradation via proteasome, whereas Lys63-linked polyubiquitination acts primarily as a regulatory rather than a proteolytic signal (35, 36).

We first performed the ubiquitination assay with four ubiquitin mutants UbK48R (Lys48 replaced by Arg), UbK63R (Lys63 replaced by Arg), Ub48K (lacks all lysine residues except Lys48), and Ub63K (lacks all lysine residues except Lys63). The results showed that RNF12 greatly enhanced the polyubiquitination of BRF1 in the presence of either UbK48R or UbK63R (Fig. 4C). However, RNF12 failed to induce polyubiquitination of BRF1 in the presence of Ub48K or Ub63K (Fig. 4C). These results indicate that the polyubiquitin chains attached to BRF1 catalyzed by RNF12 are not linked via either Lys48 or Lys63 of ubiquitin.

We next performed the ubiquitination assay with four ubiquitin mutants UbK48R (Lys48 replaced by Arg), UbK63R (Lys63 replaced by Arg), Ub48K (lacks all lysine residues except Lys48), and Ub63K (lacks all lysine residues except Lys63). The results showed that RNF12 greatly enhanced the polyubiquitination of BRF1 in the presence of either UbK48R or UbK63R (Fig. 4C). However, RNF12 failed to induce polyubiquitination of BRF1 in the presence of Ub48K or Ub63K (Fig. 4C). These results indicate that the polyubiquitin chains attached to BRF1 catalyzed by RNF12 are not linked via either Lys48 or Lys63 of ubiquitin. We next performed the ubiquitination assay with five additional ubiquitin mutants: Ub6K, Ub11K, Ub27K, Ub29K, and Ub33K, in which all lysine residues were mutated to arginine residues except Lys63. In the presence of Ub6K, Ub11K, or Ub29K, RNF12 did not promote BRF1 polyubiquitination (Fig. 4D). Intriguingly, in the presence of Ub27K or Ub33K, RNF12 was shown to enhance the polyubiquitination of BRF1, although to a lesser extent than WT ubiquitin (Fig. 4D). Taken together, these findings indicate that RNF12 promotes both Lys27- and Lys33-linked polyubiquitination of BRF1. In support of this, RNF12 enhanced the polyubiquitination of BRF1 in the presence of either UbK27R (Lys27 replaced by Arg) or UbK33R (Lys33 replaced by Arg) (Fig. 4E). However, RNF12 failed to increase BRF1 polyubiquitination in the presence of UbK27R/K33R (both Lys27 and Lys33 replaced by Arg) (Fig. 4F).

RNF12 negatively regulates RNA polymerase III–dependent transcription and cell proliferation

To investigate whether RNF12 regulates Pol III–dependent transcription, we performed real-time RT–PCR analysis to examine the levels of Pol III transcripts pre-tRNA_Leu, tRNA_Tyr, and 5S rRNA. Knockdown of RNF12 greatly increased, whereas ectopic expression of RNF12 strongly decreased the levels of pre-tRNA_Leu, tRNA_Tyr, and 5S rRNA (Fig. 5, A and B), indicating that RNF12 inhibits Pol III–dependent transcription. To determine whether RNF12 exerts this function via BRF1, exogenous BRF1 was introduced into RNF12-overexpressing cells. Ectopic expression of BRF1 indeed reversed the inhibitory effect of RNF12 on pre-tRNA_Leu, tRNA_Tyr, and 5S rRNA (Fig. 5B). However, mutant BRF1 (521–677), lacking RNF12-binding ability, failed to reverse the effect of RNF12 on pre-tRNA_Leu, tRNA_Tyr, and 5S rRNA levels (Fig. 5B). Therefore, these data suggest that RNF12 negatively regu-
lates Pol III–dependent transcription through BRF1. To further determine whether RNF12 could also regulate Pol I– and Pol II–dependent transcription, we performed real-time RT–PCR analysis to examine the levels of Pol I transcripts 18S rRNA and 28S rRNA, and Pol II transcripts PRMT6 and E2F1. The results showed that the levels of these Pol I– and Pol II–specific transcripts were not affected by either overexpression or knockdown of RNF12 (Fig. 5C), indicating the specific regulatory effect of RNF12 on Pol III–dependent transcription.

Given the ability of RNF12 to inhibit Pol III–dependent transcription, we sought to evaluate whether RNF12 could regulate cell proliferation. Cell proliferation and colony formation
experiments were therefore performed. The results showed that ectopic expression of RNF12 greatly inhibited cell proliferation, as manifested by the slower growth curve and the decreased colony numbers in RNF12- overexpressing cells (Fig. 5, D and E). Intriguingly, RNF12-decreased cell proliferation was rescued by ectopic expression of BRF1, but not by mutant BRF1 (521–677) (Fig. 5F). Taken together, these data suggest that RNF12 negatively regulates Pol III–dependent transcription and cell proliferation via BRF1.

To further explore how RNF12 inhibits Pol III–dependent transcription, we first examined whether RNF12 regulates the integrity of the TFIIIB complex such as the BRF1–TBP interaction. The results showed that ectopic expression of RNF12 did not evidently affect the BRF1–TBP interaction (Fig. 5F). We next asked whether RNF12 influences the binding of BRF1 to target gene promoters. The results showed that overexpression of RNF12 decreased, whereas knockdown of RNF12 increased the binding of BRF1 to the promoters of 5S rRNA and tRNA-Leu (Fig. 5, G and H). These findings indicate that RNF12 may inhibit Pol III–dependent transcription via decreasing the binding of BRF1 to target gene promoters.

**Discussion**

BRF1 is an essential initiation factor for Pol III–dependent transcription. Therefore, investigation of mechanisms underlying the regulation of BRF1 is of great importance to understanding Pol III–dependent transcription. The current study reveals that RNF12, a RING domain-containing E3 ligase, catalyzes the polyubiquitination of BRF1. Functionally, RNF12 regulates Pol III–dependent transcription and cell proliferation via BRF1.

As the critical component of TFIIIB, BRF1 is subjected to intricate regulation in cells. For example, BRF1 is phosphorylated by multiple protein kinases, such as ERK and PKI (20–22). BRF1 phosphorylation is capable of regulating Pol III–dependent transcription. In this study, we report for the first time that cellular BRF1 also undergoes ubiquitin modification. Both *in vitro* and *in vivo* evidence demonstrates that RNF12 is a *bona fide* E3 ligase for BRF1. Protein ubiquitination is catalyzed by E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and a variety of E3 ubiquitin-ligating enzymes (37). Depending on the type of polyubiquitin chain formed on the target protein, ubiquitin modification may play distinct functions. For example, the Lys48-linked polyubiquitin chain usually serves as a protein degradation signal, whereas the Lys63-linked polyubiquitin chain primarily acts as a regulatory signal (36). Intriguingly, the polyubiquitin chains attached to BRF1 catalyzed by RNF12 are not the conventional Lys48- and Lys63-linked polyubiquitin chains. RNF12 is able to catalyze atypical Lys27- and Lys33-linked polyubiquitination chains of BRF1. It has been shown that both Lys27- and Lys33-linked ubiquitin modifications are involved in the regulation of protein recruitment (35). We therefore hypothesize that RNF12-catalyzed Lys27- and Lys33-linked polyubiquitination of BRF1 may affect the formation of the TFIIIB–TFIIC or TFIIIB–Pol III functional complexes. Here, we show that although RNF12 appears not to affect TFIIIB complex integrity, RNF12 is able to decrease the binding of BRF1 to the promoters of 5S rRNA and tRNA-Leu. These data indicate that RNF12 may inhibit Pol III–dependent transcription via decreasing the binding of BRF1 to target gene promoters. The detailed underlying mechanism needs to be further determined in the future.

It has long been recognized that Pol III transcripts are elevated in transformed and tumor cells. Enhanced Pol III–dependent transcription has been also linked to a variety of human cancers (6–8). The tumor suppressors p53, Rb, and PTEN repress, whereas the oncogenic c-Myc induces, Pol III–dependent transcription (13–17). Deregulation of these tumor suppressors and oncogenic proteins is therefore believed to contribute to enhanced Pol III–dependent transcription in cancer (10). In addition, overexpression of specific transcription factors such as TFIIIB and TFIIIC may also lead to Pol III deregulation in cancer (38–41). In this study, we show that RNF12 negatively regulates Pol III–dependent transcription. Given the recent finding of RNF12 down-regulation in hepatocellular cancer (42), our data suggest that dysregulated RNF12 expression may represent an additional mechanism of enhanced Pol III–dependent transcription in cancer. Correlated with its ability to inhibit Pol III–dependent transcription, RNF12 is also shown to negatively regulate cell proliferation. Consistent with our data, it has been recently reported that RNF12 is able to positively regulate p53 and negatively regulate c-Myc, leading to the inhibition of cell proliferation (43, 44). These findings imply that RNF12 may inhibit cell proliferation via multiple molecular mechanisms. In summary, the data presented in this study suggest that RNF12-mediated BRF1 ubiquitination plays an important role in the regulation of Pol III–dependent transcription.

**Figure 3. RNF12 acts as a ubiquitin E3 ligase for BRF1.** A. HEK293T cells were transfected with Flag–BRF1, HA–Ub, and increasing amounts of GFP–RNF12 as indicated. 24 h after transfection, the cells were treated with MG132 for an additional 6 h. The cell lysates were then immunoprecipitated with anti-Flag antibody, followed by Western blotting analysis. B. HEK293T cells were co-transfected with the indicated plasmids. 24 h later, the cells were treated with MG132 for an additional 6 h. The cell lysates were denatured before proteins conjugated to His–ubiquitin were pulled down by nickel–nitrilotriacetic acid beads. The bead-bound proteins and input were analyzed by Western blotting. C. HEK293T cells were transfected with GFP–BRF1, HA–Ub, Flag–RNF12 (WT), and Flag–RNF12 H569A/C572A (MT) in the indicated combinations. 24 h later, the cells were treated with MG132 for an additional 6 h. The cell lysates were then immunoprecipitated with anti-GFP antibody, followed by Western blotting analysis. D. purified Flag–BRF1 proteins were incubated with E1 (50 nM), E2 (UbcH5a, 500 nM), Flag–ubiquitin (200 μM), and either recombinant GST, GST–RNF12 (WT), or GST–RNF12 H569A/C572A (MT) in 20 μl of *in vitro* ubiquitination reaction buffer. The reaction mixtures were analyzed by Western blotting with anti-Flag antibody. GST–tagged proteins were also analyzed by Coomassie Blue staining. E. HEK293T cells were infected with lentiviruses expressing RNF12 ubiquitin as indicated. 48 h later, the cell lysates were immunoprecipitated with anti-Flag antibody or an isotype-matched control IgG, followed by Western blotting analysis. F. HEK293T cells were infected with lentiviruses expressing RNF12 or ubiquitin as indicated. 48 h later, the cell lysates were immunoprecipitated with anti-Flag antibody or an isotype-matched control IgG, followed by Western blotting analysis. G. HEK293T cells were transfected with the indicated Flag–BRF1 construct alone or together with HA–Ub. 24 h later, the cells were treated with MG132 for an additional 6 h. The cell lysates were then immunoprecipitated with anti-Flag antibody, followed by Western blotting analysis.
Figure 4. RNF12 promotes Lys27- and Lys33-linked polyubiquitination of BRF1. A, HeLa cells were infected with lentiviruses expressing control shRNA, RNF12 shRNA#1, RNF12 shRNA#2, control proteins, or RNF12 proteins as indicated. 48 h after infection, the cell lysates were analyzed by Western blotting with anti-BRF1 and anti-RNF12 antibodies. GAPDH was also included as a loading control. B, HeLa cells were infected with lentiviruses expressing RNF12 or control proteins. 48 h after infection, the cells were treated with 20 μg/ml cycloheximide for the indicated periods of time. The cell lysates were then subjected to Western blotting analysis. C, HEK293T cells were transfected with Flag–BRF1, GFP–RNF12, HA–Ub (WT), HA–Ub (K48R), HA–Ub (K63R), HA–Ub (48K), and HA–Ub (63K) in the indicated combinations. 24 h later, the cells were treated with MG132 for an additional 6 h. The cell lysates were then subjected to immunoprecipitation with anti-Flag antibody, followed by Western blotting analysis. D, HEK293T cells were transfected with Flag–BRF1, GFP–RNF12, HA–Ub (WT), HA–Ub (6K), HA–Ub (11K), HA–Ub (27K), HA–Ub (29K), and HA–Ub (33K) in the indicated combinations. 24 h later, the cells were treated with MG132 for an additional 6 h. The cell lysates were then subjected to immunoprecipitation, followed by Western blotting analysis. E, HEK293T cells were transfected with Flag–BRF1, GFP–RNF12, HA–Ub (K27R), and HA–Ub (K33R) in the indicated combinations. 24 h later, the cells were treated with MG132 for an additional 6 h. The cell lysates were then subjected to immunoprecipitation, followed by Western blotting analysis. F, HEK293T cells were transfected with Flag–BRF1, GFP–RNF12, HA–Ub (K27R/K33R), and HA–Ub (K27R/K33R) in the indicated combinations. 24 h later, the cells were treated with MG132 for an additional 6 h. The cell lysates were then subjected to immunoprecipitation, followed by Western blotting analysis. IB, immunoblotting; Ni-NTA, nickel–nitrilotriacetic acid.
**Experimental procedures**

**Reagents and antibodies**

The following reagents used in this study were purchased from the indicated sources: MG132 (Calbiochem, 20 µM), Hoechst 33342 (Sigma, 1 µg/ml), Lipofectamine 2000 (Invitrogen), complete EDTA free protease inhibitor mixture (Roche Applied Science), GSH beads (GE Healthcare), antibodies against GAPDH (Santa Cruz, catalog no. sc-166545, 1:5000), GFP for RNF12 catalyzes BRF1 ubiquitination.
immunoprecipitation (BD Biosciences, catalog no. 566040), GFP for Western blotting (Santa Cruz, catalog no. sc-9996, 1:1000), Flag (Sigma, catalog no. F3165, 1:4000), HA (Sigma, catalog no. H9658, 1:4000), ubiquitin (Cell Signaling, catalog no. 3936, 1:1000), BRF1 (Bethyl, catalog no. A301–228A, 1:2000), TBP (Bethyl, catalog no. A301–229A, 1:2000), and horseradish peroxidase– conjugated secondary antibodies against mouse (catalog no. 115–035-062) and rabbit (catalog no. 111–035–144) (Jackson ImmunoResearch, 1:10,000). Flag–ubiquitin, E1, UbcH5a, and Mg\textsuperscript{2+}–ATP were purchased from Sigma. Anti-RNF12 antibody was kindly provided by Dr. Ingolf Bach.

**Cell culture**

HeLa, H1299, and HEK293T cell lines were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% FBS and antibiotics (Gibco). All cell lines were routinely tested for mycoplasma contamination before they were used for experiments.

**Identification of RNF12 as a BRF1-interacting protein**

HeLa cells were cross-linked with 0.2% formaldehyde. The cross-linking reaction was quenched with 0.15 M of glycine (pH 7.4). The cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 1% sodium deoxycholates, 0.1% SDS, and 20 μM MG132) supplemented with 1× protease inhibitor mixture. After sonication, the cell lysates were precleared with protein A/G–coupled agarose beads. Lysates were then immunoprecipitated with anti-BRF1 antibody for 10 h at 4 °C. After the beads were extensively washed with RIPA buffer, the bead-bound proteins were eluted using elution buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 2.5 mM MgCl\textsubscript{2}, and 0.4% SDS) at room temperature for 30 min and analyzed by MS. The MS data were provided as Table S1.

**Real-time RT–PCR**

Total RNA was isolated using TRIzol (Invitrogen). 1 μg of RNA was used to synthesize cDNA using the PrimeScript\textsuperscript{TM} RT reagent kit (Takara, catalog no. DRR037A) according to the manufacturer’s instructions. Real-time PCR was performed using SYBR premix EX Taq (TakaRa) and analyzed with the StepOnePlus real-time PCR system (Thermo Fisher Scientific). The expression levels of the examined RNA were normalized to acidic ribosomal phosphoprotein P0. Real-time primer sequences are as follows: 5S rRNA, 5′-GGCCATACCCACCCTGAAGCC-3′ and 5′-CAGACCGCCGTTATTCCCAGG-3′; tRNA\textsubscript{Leu}, 5′-GTCCAGGATGGCCAGTGTCTAAGGC-3′; 18S rRNA, 5′-CAGGCACCTCGGAGA-3′; acidic ribosomal phosphoprotein P0, 5′-GCAGTTGCGGACATCTTC-3′ and 5′-TGAAGCTTCATCGGTGCA-3′; 28S rRNA, 5′-AGAGTTCTTTTCTTTGTGTTG-3′ and 5′-GATGCACCTTGAGACCTGCT-3′; E2F1, 5′-GCGACCTGACTCTGGCCACCATAG-3′ and 5′-CTGGCCA-TCCGGGACAAAC-3′; PRMT6, 5′-CCCTCCAACGATGTTT-3′ and 5′-TTCTCCAGGCTTGAAGCTC-3′; and GAPDH, 5′-CCATGGGGAAGTGAAGTGC-3′ and 5′-GAAGGGTCATTGATGGCAAAC-3′.

**Western blotting analysis and co-immunoprecipitation**

Western blotting analysis and co-immunoprecipitation were performed as we previously described (45). For Western blotting, the cells were harvested, boiled in 1× SDS loading buffer, and resolved on SDS-PAGE. For co-immunoprecipitation, the cells were treated with MG132 for 6 h before they were lysed in IP lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM MgCl\textsubscript{2}, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% Triton X-100, 10% glycerol, and 20 μM MG132) supplemented with 1× protease inhibitor mixture by gentle sonication. The cell lysates were precleared with protein A/G–coupled Sepharose beads for 2 h before they were immunoprecipitated with the indicated antibodies. The immunoprecipitates and input were then subjected to Western blotting analysis. To verify the interaction between exogenously expressed RNFI2 and BRF1, HEK293T cells were utilized because of the high transfection efficiency of these cells. Additionally, in these immunoprecipitation experiments, the immunoprecipitated samples were washed with 500 mM NaCl-containing IP buffer. This information has been specified in the indicated figure legends.

**Figure 5. RNF12 regulates RNA polymerase III–dependent transcription and cell proliferation.** A, H1299 cells were infected with lentiviruses expressing either control shRNA, RNFI2 shRNA#2, or RNFI2 shRNA#3. 48 h later, total RNA was subjected to real-time RT–PCR analysis to examine RNA polymerase III–dependent transcription. The data are the means ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01. The knockdown efficiency of RNFI2 was also evaluated by Western blotting analysis. B, H1299 cells were infected with lentiviruses expressing RNFI2, BRF1, BRF1 (521–677), both RNFI2 and BRF1, or both RNFI2 and BRF1 (521–677) proteins as indicated. 48 h later, total RNA was subjected to real-time RT–PCR analysis to examine RNA polymerase III–dependent transcription. The data are the means ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01. N.S., no significance. The successful overexpression of RNFI2, BRF1, and BRF1 (521–677) was also confirmed by Western blotting analysis. C, H1299 cells were infected with lentiviruses expressing control shRNA, RNFI2 shRNA#2, control proteins, or RNFI2 proteins as indicated. 48 h later, total RNA was subjected to real-time RT–PCR analysis to examine expression levels of PolI transcripts 18S rRNA and 28S rRNA, and Pol II transcripts PRMT6 and E2F1. D, H1299 cells were infected with lentiviruses expressing either control or RNFI2 proteins. 48 h later, the cells were plated (day 1), and cell numbers were counted at the indicated time points. The data are the means ± S.D. of three independent experiments. The successful overexpression of RNFI2 was also confirmed by Western blotting analysis. E, H1299 cells were infected with lentiviruses expressing RNFI2, BRF1, BRF1 (521–677), both RNFI2 and BRF1, or both RNFI2 and BRF1 (521–677) proteins as indicated. 48 h after infection, 200 cells were plated and cultured for an additional 10 days. The colonies were then stained with crystal violet. The images are representative of three independent experiments. The data are the means ± S.D. (n = 3), **, p < 0.01; ***, p < 0.001. N.S., no significance. F, H1299 cells were transfected with GFP–RNFI2 and Flag–BRF1 in the indicated combinations. 24 h later, the cell lysates were immunoprecipitated with anti-Flag antibody, followed by Western blotting analysis with anti-TBP antibody. G, lysates from H1299 cells expressing RNFI2 or control proteins were subjected to a ChIP assay using anti-BRF1 antibody or an isotype-matched control IgG. ChIP products were amplified by PCR. H, lysates from H1299 cells expressing control shRNA or RNFI2 shRNA#2 were subjected to a ChIP assay using anti-BRF1 antibody or an isotype-matched control IgG. ChIP products were amplified by PCR, ctrl, control. PCDH is a lentivirus-expressing vector.
RNAi

RNAi was performed as we previously described (46). To generate lentiviruses expressing the indicated shRNAs, HEK293T cells grown on a 6-cm dish were transfected with 2 µg of shRNA (cloned in PLKO.1) or control vector, 2 µg of pREV, 2 µg of pGag/Pol/Pre, and 1 µg of pVSVG. 12 h after transfection, the cells were cultured with DMEM medium containing 20% FBS for an additional 24 h. The culture medium containing lentivirus particles was filtered through a 0.45-µm PVDF filter (Millipore) and incubated with HeLa or H1299 cells supplemented with 8 µg/ml Polybrene (Sigma) for 24 h, followed by selection with 2 µg/ml puromycin for another 24 h. The knockdown efficiency was evaluated by Western blotting analysis.

Protein expression and purification

The DNA sequences encoding RNF12 and RNF12 (H569A/C572A) were individually cloned into the pGEX-6P-1 vector. Protein expression and purification were performed according to the procedure we described previously (47). To purify Flag–BRF1 proteins, a Flag–BRF1 expressing construct was transfected into HEK293T cells. The cell lysates were immunoprecipitated with anti-Flag M2 affinity beads (Sigma). To remove nonspecific binding proteins, the beads were subjected to sequential washes with lysis buffer containing 0.25%, 0.5%, and 1 M KCl as previously described (47). The bead-bound Flag–BRF1 proteins were eluted with 3× Flag peptide (Sigma).

In vivo and in vitro ubiquitination assay

HEK293T cells were transfected with the indicated plasmids. 24 h later, the cells were treated with 20 µM MG132 for an additional 6 h. The in vivo ubiquitination assay was then performed according to the procedure we described previously (48). Briefly, the cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 1% sodium deoxycholates, 0.1% SDS, and 20 µM MG132) supplemented with 1× protease inhibitor mixture. The cell lysates were incubated with anti-Flag M2 affinity beads at 4 °C for 4 h. The immunoprecipitates and input were then subjected to Western blotting analysis to examine BRF1 ubiquitination. Alternatively, the cells were lysed in denaturing buffer (6 M guanidine HCl, 0.1 M Na2HPO4/NaH2PO4, 10 mM imidazole, pH 8.0). The cell lysates were incubated with nickel–nitrilotriacetic acid–agarose beads to pulldown proteins conjugated to His–ubiquitin. Bead-bound proteins were then analyzed by Western blotting.

Statistical analysis

Statistical analysis was carried out using Microsoft Excel software and GraphPad Prism to assess differences between experimental groups. Statistical significance was analyzed by Student’s t test and expressed as a p value. p values lower than 0.05 were considered statistically significant (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Acknowledgment—We thank Dr. Ingolf Bach for kindly providing us anti-RNF12 antibody.

References

1. Turowski, T. W., and Tolliver, D. (2016) Transcription by RNA polymerase III: insights into mechanism and regulation. Biochim Soc Trans 44, 1367–1375
2. Dieci, G., Conti, A., Pagano, A., and Carnevali, D. (2013) Identification of RNA polymerase III-transcribed genes in eukaryotic genomes. Biochim Biophys Acta 1829, 296–305
3. Vannini, A., and Cramer, P. (2012) Conservation between the RNA polymerase I, II, and III transcription initiation machineries. Mol Cell 45, 439–446
4. White, R. J. (2011) Transcription by RNA polymerase III: more complex than we thought. Nat Rev Genet 12, 459–463
5. Schramm, L., and Hernandez, N. (2002) Recruitment of RNA polymerase III to its target promoters. Genes Dev. 16, 2593–2620
6. Pavon-Eternod, M., Gomes, S., Guelain, R., Dai, Q., Rosner, M. R., and Pan, T. (2009) tRNA over-expression in breast cancer and functional consequences. Nucleic Acids Res. 37, 7268–7280
RNF12 catalyzes BRF1 ubiquitination

7. Marshall, L., and White, R. J. (2008) Non-coding RNA production by RNA polymerase III is implicated in cancer. *Nat Rev Cancer* 8, 911–914

8. Bywater, M. I., Pearson, R. B., McArthur, G. A., and Hannan, R. D. (2013) Dysregulation of the basal RNA polymerase transcription apparatus in cancer. *Nat Rev Cancer* 13, 299–314

9. Johnson, S. A., Dubeau, L., and Johnson, D. L. (2008) Enhanced RNA polymerase III–dependent transcription is required for oncogenic transformation. *J. Biol. Chem.* 283, 19184–19191

10. Marshall, L. (2008) Elevated RNA polymerase III transcription drives proliferation and oncogenic transformation. *Cell Cycle* 7, 3327–3329

11. Mei, Y., Yong, J., Liu, H., Shi, Y., Meinkoth, J., Dreyfuss, G., and Yang, X. (2010) tRNA binds to cytochrome c and inhibits caspase activation. *Mol Cell* 37, 668–678

12. Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646–674

13. Crighton, D., Woiwode, A., Zhang, C., Mandavida, N., Morton, J. P., Warnock, L. J., Müller, I., White, R. J., and Johnson, D. L. (2003) p53 regulates RNA polymerase III transcription by targeting TBP and inhibiting promoter occupancy by TFIIB. *EMBO J.* 22, 2810–2820

14. Sutcliffe, J. E., Brown, T. R., Allison, S. J., Scott, P. H., and White, R. J. (2000) The mitogen-activated protein (MAP) kinase pathway: distinct mechanisms for repression of RNA polymerase III transcription. *Cell* 102, 552–565

15. Nathan, J. A., Kim, H. T., Ting, L., Gygi, S. P., and Goldberg, A. L. (2013) The histone deacetylase inhibitor valproic acid selectively induces chromosome inactivation by targeting REX1 for degradation. *Nature* 485, 386–390

16. Sutcliffe, J. E., Brown, T. R., Allison, S. J., Scott, P. H., and White, R. J. (2000) Distinct mechanisms for repression of RNA polymerase III transcription by the retinoblastoma tumor suppressor protein. *Mol. Cell. Biol.* 24, 5989–5999

17. Hirschi, H. A., Jawedkar, G. W., Lee, K. A., Gu, L., and Henry, R. W. (2004) Distinct mechanisms for repression of RNA polymerase III transcription by the retinoblastoma tumor suppressor protein. *Mol. Cell. Biol.* 24, 5989–5999

18. Gomez-Roman, N., Grandori, C., Eisenman, R. N., and White, R. J. (2003) Direct activation of RNA polymerase III transcription by c-Myc. *Nature* 421, 290–294

19. Woiwode, A., Johnson, S. A., Zhang, S., Zhang, C., Roeder, R. G., Teichmann, M., and Johnson, D. L. (2008) PTEN represses RNA polymerase III–dependent transcription by targeting the TFIIB complex. *Mol. Cell Biol.* 28, 4204–4214

20. Zhang, Q., Zhong, Q., Evans, A. G., Levy, D., and Zhong, S. (2011) Phosphorylation of Bdp1 executes cell cycle-specific RNA polymerase III transcription repression. *Cell Cycle* 10, 299–314

21. Borck, G., Hog, F., Dentici, M. L., Tan, P. L., Sowada, N., Medeira, A., Johnsen, S. A., Gungor, C., Prenzel, T., Riethdorf, S., Riethdorf, L., Tani-Neurodevelopmental anomalies. *Mol. Psychiatry* 10, 299–314

22. Frints, S. G. M., Ozanturk, A., Rodriguez Criado, G., Grasshoff, U., de Gruijl, C., and Kalscheuer, V. M. (2018) Pathogenic variants in E3 ubiquitin ligase RNF12 cause an X-linked dose-dependent activator of X chromosome inactivation. *Cell* 139, 999–1011

23. Huang, Y., Nie, M., Li, C., Zhao, Y., Li, J., Zhou, L., and Wang, L. (2017) A RNF12 controls embryonic stem cell fate and morphogenesis in zebrafish embryos by targeting Smad5 for degradation. *Mol Cell* 46, 650–661

24. Felton-Edkins, Z. A., and White, R. J. (2002) Multiple mechanisms control the encoded X-linked dose-dependent activator of X chromosome inactivation. *Cell Cycle* 23, 1599–1611

25. Barakat, T. S., Sheppard, K. A., Mickanin, C., Porter, J. A., Vertegal, A. C., van Dam, H., Gribnau, J., Lu, C. X., and ten Dijke, P. (2012) RNF12 controls embryonic stem cell fate and morphogenesis in zebrafish embryos by targeting Smad5 for degradation. *Mol Cell* 46, 650–661

26. Johnson, S. A., Dubeau, L., and Johnson, D. L. (2008) Enhanced RNA polymerase III transcription drives proliferation and oncogenic transformation. *Cell Cycle* 7, 3327–3329

27. Marshall, L., and White, R. J. (2008) Non-coding RNA production by RNA polymerase III is implicated in cancer. *Nat Rev Cancer* 8, 911–914

28. Bywater, M. I., Pearson, R. B., McArthur, G. A., and Hannan, R. D. (2013) Dysregulation of the basal RNA polymerase transcription apparatus in cancer. *Nat Rev Cancer* 13, 299–314

29. Johnson, S. A., Dubeau, L., and Johnson, D. L. (2008) Enhanced RNA polymerase III–dependent transcription is required for oncogenic transformation. *J. Biol. Chem.* 283, 19184–19191

30. Marshall, L. (2008) Elevated RNA polymerase III transcription drives proliferation and oncogenic transformation. *Cell Cycle* 7, 3327–3329

31. Mei, Y., Yong, J., Liu, H., Shi, Y., Meinkoth, J., Dreyfuss, G., and Yang, X. (2010) tRNA binds to cytochrome c and inhibits caspase activation. *Mol Cell* 37, 668–678

32. Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646–674

33. Crighton, D., Woiwode, A., Zhang, C., Mandavida, N., Morton, J. P., Warnock, L. J., Müller, I., White, R. J., and Johnson, D. L. (2003) p53 regulates RNA polymerase III transcription by targeting TBP and inhibiting promoter occupancy by TFIIB. *EMBO J.* 22, 2810–2820

34. Sutcliffe, J. E., Brown, T. R., Allison, S. J., Scott, P. H., and White, R. J. (2000) Distinct mechanisms for repression of RNA polymerase III transcription by the retinoblastoma tumor suppressor protein. *Mol. Cell. Biol.* 24, 5989–5999

35. Gomez-Roman, N., Grandori, C., Eisenman, R. N., and White, R. J. (2003) Direct activation of RNA polymerase III transcription by c-Myc. *Nature* 421, 290–294

36. Feng, Z., Yi, Y., Shi, G., Li, S., Chen, S., Lin, Y., Li, Z., He, Z., Li, W., and Zhong, S. (2017) Role of Brf1 interaction with ERAlpha and significance of its overexpression, in human breast cancer. *Mol Oncol* 11, 1752–1767

37. Huang, Y., Nie, M., Li, C., Zhao, Y., Li, Z., Hou, L., and Wang, L. (2017) RNF12 suppresses hepatocellular carcinomaogenesis by up-regulating p15 and p21. *Oncotarget* 8, 83075–83087

38. Gao, K., Wang, C., Jin, X., Xiao, J., Zhang, E., Yang, X., Wang, D., Huang, H., Yu, L., and Zhang, P. (2016) RNF12 promotes p53-dependent cell proliferation.
growth suppression and apoptosis by targeting MDM2 for destruction. 
*Cancer Lett* **375**, 133–141

44. Gao, R., Wang, L., Cai, H., Zhu, J., and Yu, L. (2016) E3 Ubiquitin Ligase 
RLIM Negatively Regulates c-Myc Transcriptional Activity and Restraints 
Cell Proliferation. *PLoS ONE* **11**, e0164086

45. Wang, X., Zha, M., Zhao, X., Jiang, P., Du, W., Tam, A. Y., Mei, Y., and Wu, 
M. (2013) Siva1 inhibits p53 function by acting as an ARF E3 ubiquitin 
ligase. *Nat Commun* **4**, 1551

46. Yang, F., Zhang, H., Mei, Y., and Wu, M. (2014) Reciprocal regulation of 
HIF-1alpha and lincRNA-p21 modulates the Warburg effect. *Mol Cell* **53**, 
88–100

47. Huang, X., Wu, Z., Mei, Y., and Wu, M. (2013) XIAP inhibits autophagy 
via XIAP-Mdm2-p53 signalling. *EMBO J.* **32**, 2204–2216

48. Zhao, K., Yang, Y., Zhang, G., Wang, C., Wang, D., Wu, M., and Mei, Y. 
(2018) Regulation of the Mdm2-p53 pathway by the ubiquitin E3 ligase 
MARCH7. *EMBO Rep* **19**, 305–319