RNF12 catalyzes BRF1 ubiquitination and regulates RNA polymerase III–dependent transcription

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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 Lys^{27}- and Lys^{33}-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)[4] is responsible for the transcription of small noncoding RNAs, including tRNAs, 5S rRNA, 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 and TFIIIC (3, 4). 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 transcripti1on 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 PI3K/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-

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This article contains Table S1.

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[4] The abbreviations used are: Pol III, polymerase III; KO, knockout; aa, amino acid(s); RIPA, radioimmune precipitation assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IP, immunoprecipitation; Ub, ubiquitin.

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 PlK1, 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.

RNF12, 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 RNF12 are largely attributed to its E3 ligase activity. For example, RNF12 regulates the activities of several transcription factors by controlling the protein levels of their co-factors (25–27). By targeting TRF1 for degradation, RNF12 controls telomere length homeostasis (28). RNF12 also modulates the TGFβ superfamily signaling pathways by promoting proteasome-dependent degradation of the negative regulator Smad7 (29). Moreover, RNF12 participates in X-chromosome inactivation in part by targeting the pluripotency factor REX1 for degradation (30–32). The genetic mutations of RNF12 that disrupt its E3 ligase activity have also been associated with X-linked intellectual disability (33, 34).

In this study, we report RNF12 as a novel interaction partner of BRF1. Via direct interaction, RNF12 promotes both Lys^{27}- and Lys^{33}-linked polyubiquitination of BRF1. Functionally, RNF12 negatively regulates Pol III–dependent transcription and cell proliferation via BRF1. Collectively, these results reveal RNF12 as a critical regulator of BRF1 and define an important function of RNF12 in the regulation of Pol III–dependent transcription.

Results

RNF12 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. 1*A*, *lane 4*). However, in the presence of Ub–KO, the polyubiquitination of BRF1 was barely detected (Fig. 1*A*, *lane 5*). In addition, the polyubiquitination of BRF1 was also verified at the endogenous level (Fig. 1*B*). 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. RNF12, a RING domain–containing ubiquitin E3 ligase, was identified in the anti-BRF1 immunoprecipitates (Fig. 1*C* and Table S1). To further verify the interaction between RNF12 and BRF1, we expressed GFP–RNF12 alone or together with Flag–BRF1 in HEK293T cells. An immunoprecipitation assay indicated a specific interaction of these two proteins (Fig. 1*D*). A reciprocal immunoprecipitation experiment using lysates from HEK293T cells expressing Flag–RNF12 and GFP–BRF1 also confirmed the RNF12–BRF1 interaction (Fig. 1*E*). Using a co-immunoprecipitation assay with anti-BRF1 antibody, the interaction between endogenous RNF12 and BRF1 was readily detected (Fig. 1*F*). Moreover, an *in vitro* binding assay with purified GST–RNF12 and Flag–BRF1 proteins revealed that RNF12 directly associated with BRF1 (Fig. 1*G*). The immunofluorescence assay showed that ectopically expressed RNF12 and BRF1 were co-localized in the nucleus (Fig. 1*H*). Taken together, these results demonstrate that RNF12 is a novel binding partner for BRF1.

Structural determinants of the RNF12–BRF1 interaction

To identify the region of BRF1 that mediates the interaction with RNF12, we generated three BRF1 deletion mutants (Fig. 2*A*). BRF1 (aa 1–260) strongly associated with RNF12, whereas BRF1 (aa 261–520) and BRF1 (aa 521–677) exhibited no interaction with RNF12 (Fig. 2*B*), implying that the N-terminal region (aa 1–260) of BRF1 is responsible for its interaction with RNF12. To delineate the BRF1-binding domain in RNF12, we also generated a panel of RNF12 deletion mutants (Fig. 2*C*). Both RNF12 (aa 1–569) and RNF12 (aa 206–409) strongly bound to BRF1, whereas RNF12 (aa 1–205) and RNF12 (aa 410–624) showed no binding to BRF1 (Fig. 2*D*). These data suggest that the central region (aa 206–409) of RNF12 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 RNF12, we expressed GFP–RNF12 (206–409) alone or together with Flag–BRF1 (1–260) in HEK293T cells. The subsequent immunoprecipitation experiment showed that RNF12 (206–409) indeed interacted with BRF1 (1–260) (Fig. 2*E*). In addition, an *in vitro* binding assay with purified GST–RNF12 (206–409) and Flag–BRF1 (1–260) proteins revealed that RNF12 (206–409) directly associated with BRF1 (1–260) (Fig. 2*F*).

RNF12 is a ubiquitin E3 ligase for BRF1

Given the interaction of RNF12 with BRF1 and the previously reported ubiquitin E3 ligase activity of RNF12 (30), we asked whether RNF12 could be responsible for BRF1 polyubiquitination. We first performed an *in vivo* ubiquitination assay. RNF12 was shown to promote BRF1 polyubiquitination (Fig. 3*A*). The RNF12-promoted BRF1 polyubiquitination also occurred under denaturing conditions (Fig. 3*B*). Compared with WT RNF12, the ubiquitin E3 ligase-inactive mutant of RNF12 (H569A/C572A) failed to show any effect on BRF1 polyubiquitination (Fig. 3*C*). To further determine whether RNF12 acts as a ubiquitin E3 ligase for BRF1, an *in vitro* ubiquitination assay was performed with purified recombinant proteins. The results showed that WT RNF12, but not RNF12 (H569A/C572A), enhanced the polyubiquitination of BRF1 *in vitro* (Fig. 3*D*). Moreover, ectopic expression of RNF12 increased, whereas knockdown of RNF12 decreased the polyubiquitination of endogenous BRF1 (Fig. 3, *E* and *F*). These data indicate that RNF12 is a *bona fide* ubiquitin E3 ligase for BRF1.

To identify the potential lysine residue(s) of BRF1 that are targeted for polyubiquitination by RNF12, we first evaluated which domain of BRF1 was ubiquitinated in cells. The results

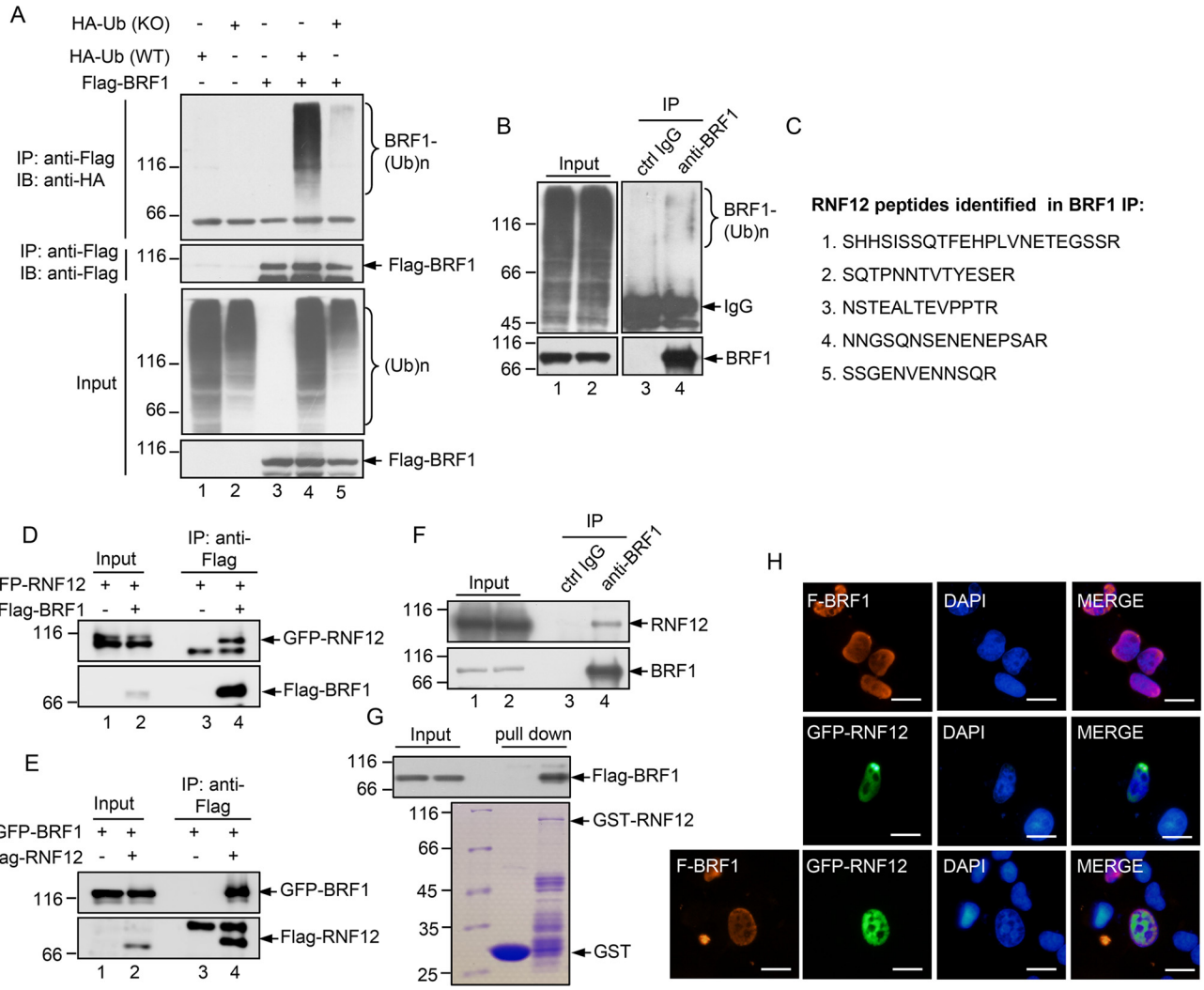


Figure 1. RNF12 interacts with BRF1 both *in vitro* and *in vivo*. *A*, HEK293T cells were transfected with Flag–BRF1, HA–Ub (WT), and HA–Ub (KO) in the indicated combinations. 24 h after transfection, the cells were treated with 20 μM MG132 for an additional 6 h. The cell lysates were then subjected to immunoprecipitation with anti-Flag antibody. The immunoprecipitates and input were analyzed by Western blotting. Molecular mass standards (in kDa) are shown on the *left*. *B*, HeLa cells were treated with MG132 for 6 h before they were lysed in the lysis buffer. The cell lysates were immunoprecipitated with anti-BRF1 antibody, followed by Western blotting analysis with anti-ubiquitin antibody. *C*, lysates from HeLa cells were immunoprecipitated with anti-BRF1 antibody. The immunoprecipitated proteins were characterized by MS analysis. RNF12 was identified in BRF1 precipitates, and the RNF12 peptide sequences obtained by MS are shown. *D*, HEK293T cells were transfected with either GFP–RNF12 alone or together with Flag–BRF1. 24 h later, the cell lysates were immunoprecipitated with anti-Flag antibody, followed by Western blotting analysis. In these immunoprecipitation experiments, the immunoprecipitated samples were washed with 500 mM NaCl-containing IP buffer. *E*, HEK293T cells were transfected with either GFP–BRF1 alone or together with Flag–RNF12. 24 h later, the cell lysates were immunoprecipitated with anti-Flag antibody, followed by Western blotting analysis. In these immunoprecipitation experiments, the immunoprecipitated samples were washed with 500 mM NaCl-containing IP buffer. *F*, lysates from HeLa cells were immunoprecipitated with anti-BRF1 antibody or an isotype-matched control IgG. The immunoprecipitates and input were analyzed by Western blotting. *G*, purified GST or GST–RNF12 proteins immobilized on GSH beads were incubated with purified Flag–BRF1. Input and bead-bound proteins were analyzed by Western blotting with anti-Flag antibody. GST and GST–RNF12 were also visualized by Coomassie Blue staining. *H*, HEK293T cells were transfected with Flag–BRF1, GFP–RNF12, or both Flag–BRF1 and GFP–RNF12. 24 h later, the cells were subjected to immunofluorescence analysis. The images were taken with a fluorescence microscope (Olympus IX73). The *scale bars* indicate 10 μm. *ctrl*, control; *DAPI*, 4′,6′-diamino-2-phenylindole; *IB*, immunoblot.

showed that similar to WT BRF1, the N-terminal region (aa 1–260) of BRF1 was heavily polyubiquitinated (Fig. 3*G*, *lanes 1–4*). In contrast, neither BRF1 (261–520) nor BRF1 (521–677) was polyubiquitinated (Fig. 3*G*, *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 (Lys^{65}, Lys^{79}, Lys^{115}, Lys^{127}, Lys^{165}, Lys^{199}, Lys^{215}, Lys^{247}, and Lys^{254}) 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. 3*H*), 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. 4*A*). In addition, the half-life of BRF1 was not affected by ectopic expression

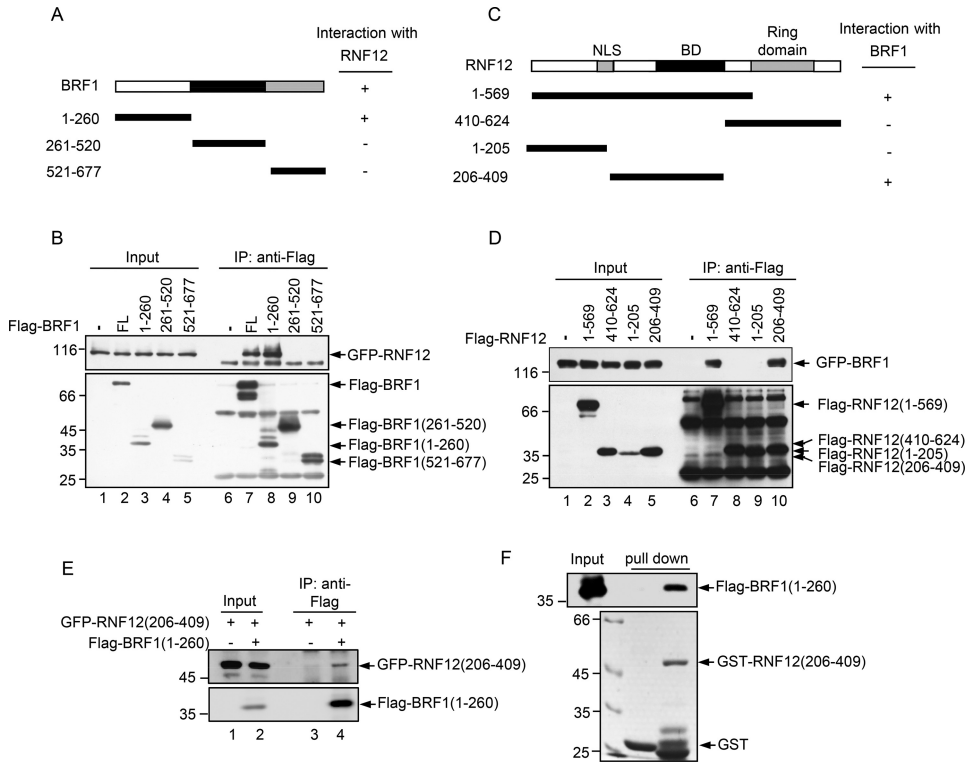


Figure 2. Mapping of the interaction regions between RNF12 and BRF1. *A*, schematic representation of WT BRF1 and the indicated deletion mutants. *B*, HEK293T cells were transfected with GFP–RNF12 alone or together with the indicated Flag–BRF1 constructs. 24 h later, the cell lysates were immunoprecipitated with anti-Flag antibody, followed by Western blotting analysis. *C*, schematic representation of WT RNF12 and the indicated deletion mutants. *D*, HEK293T cells were transfected with GFP–BRF1 alone or together with the indicated Flag–RNF12 constructs. 24 h later, the cell lysates were immunoprecipitated with anti-Flag antibody, followed by Western blotting analysis. *E*, HEK293T cells were transfected with GFP–BRF1 (206–409) alone or together with Flag–RNF12 (1–260). 24 h later, the cell lysates were immunoprecipitated with anti-Flag antibody, followed by Western blotting analysis. *F*, purified GST or GST–RNF12 (206–409) proteins immobilized on GSH beads were incubated with purified Flag–BRF1 (1–260). Input and bead-bound proteins were analyzed by Western blotting with anti-Flag antibody. GST and GST–RNF12 were also visualized by Coomassie Blue staining. *FL*, full length.

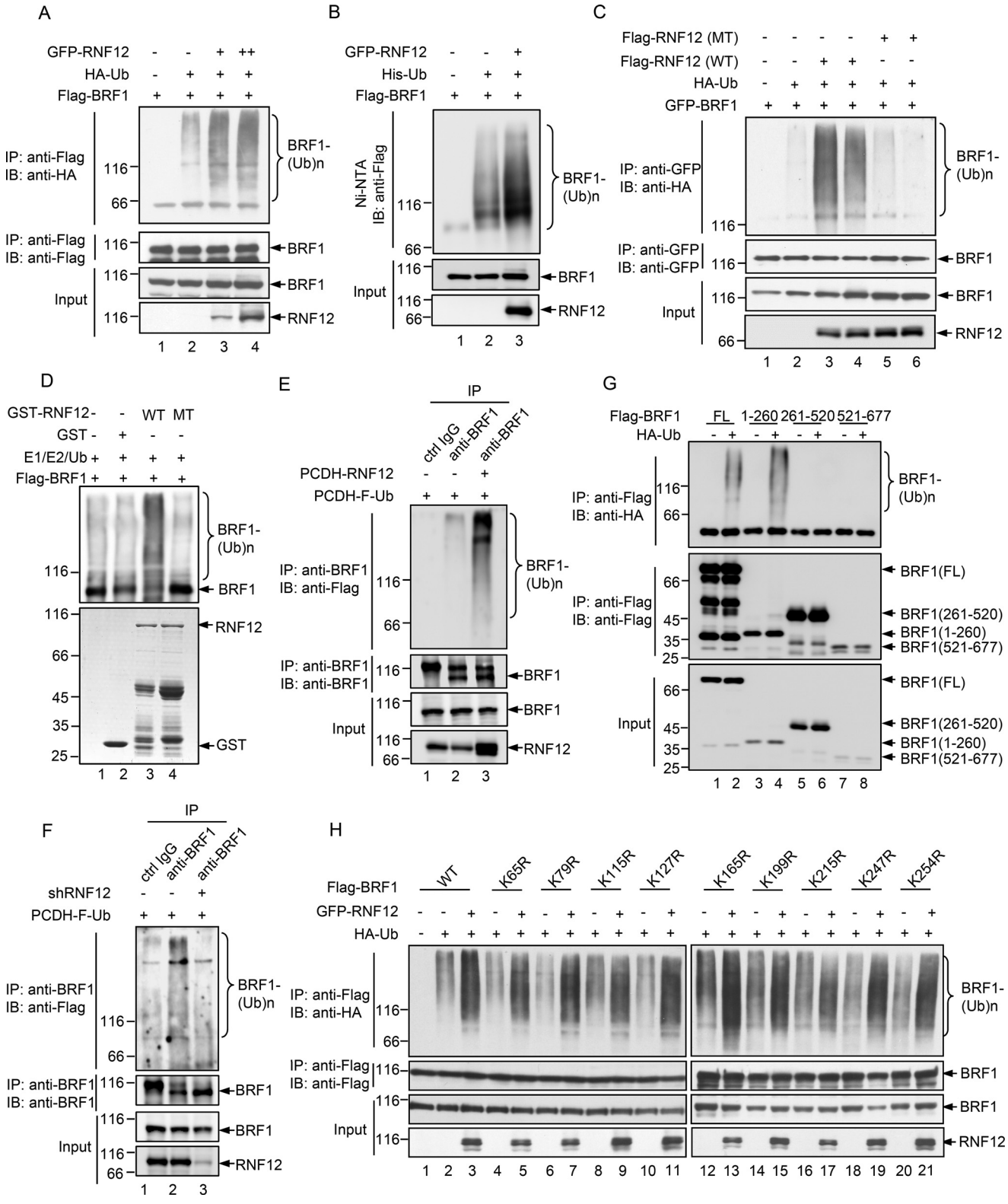
of RNF12 (Fig. 4*B*). These data suggest that RNF12 does not regulate BRF1 protein turnover. We therefore sought to determine the type of BRF1 polyubiquitin chain induced by RNF12. Polyubiquitination usually occurs at Lys^{48} or Lys^{63} of ubiquitin. It has been well-known that Lys^{48}-linked polyubiquitination serves as a recognition signal for target protein degradation via proteasome, whereas Lys^{63}-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 (Lys^{48} replaced by Arg), UbK63R (Lys^{63} replaced by Arg), Ub48K (lacks all lysine residues except Lys^{48}), and Ub63K (lacks all lysine residues except Lys^{63}). The results showed that RNF12 greatly enhanced the polyubiquitination of BRF1 in the presence of either UbK48R or UbK63R (Fig. 4*C*). However, RNF12 failed to induce polyubiquitination of BRF1 in the presence of Ub48K or Ub63K (Fig. 4*C*). These results indicate that the polyubiquitin chains attached to BRF1 catalyzed by RNF12 are not linked via either Lys^{48} or Lys^{63} 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 Lys^{6}, Lys^{11}, Lys^{27}, Lys^{29}, and Lys^{33}, respectively. In the presence of Ub6K, Ub11K, or Ub29K, RNF12 did not promote BRF1 polyubiquitination (Fig. 4*D*). 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. 4*D*). Taken together, these findings indicate that RNF12 promotes both Lys^{27}- and Lys^{33}-linked polyubiquitination of BRF1. In support of this, RNF12 enhanced the polyubiquitination of BRF1 in the presence of either UbK27R (Lys^{27} replaced by Arg) or UbK33R (Lys^{33} replaced by Arg) (Fig. 4*E*). However, RNF12 failed to increase BRF1 polyubiquitination in the presence of UbK27R/K33R (both Lys^{27} and Lys^{33} replaced by Arg) (Fig. 4*F*).

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 the expression levels of pre-$tRNA^{Leu}$, $tRNA^{Tyr}$, and 5S rRNA (Fig. 5*B*). 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. 5*B*). 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. 5*C*), 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. 5*E*). 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. 5*F*). 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 PlK1 (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 Lys^{48}-linked polyubiquitin chain usually serves as a protein degradation signal, whereas the Lys^{63}-linked polyubiquitin chain primarily acts as a regulatory signal (36). Intriguingly, the polyubiquitin chains attached to BRF1 catalyzed by RNF12 are not the conventional Lys^{48}- and Lys^{63}-linked polyubiquitin chains. RNF12 is able to catalyze atypical Lys^{27}- and Lys^{33}-linked polyubiquitination chains of BRF1. It has been shown that both Lys^{27}- and Lys^{33}-linked ubiquitin modifications are involved in the regulation of protein recruitment (35). We therefore hypothesize that RNF12-catalyzed Lys^{27}- and Lys^{33}-linked polyubiquitination of BRF1 may affect the formation of the TFIIIB–TFIIIC 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-BRF1 antibody. GST-tagged proteins were also analyzed by Coomassie Blue staining. *E*, HeLa cells were infected with lentiviruses expressing RNF12 or ubiquitin as indicated. 48 h later, the cell lysates were immunoprecipitated with anti-BRF1 antibody or an isotype-matched control IgG, followed by Western blotting analysis. *F*, HeLa cells were infected with lentiviruses expressing RNF12 shRNA or ubiquitin as indicated. 48 h later, the cell lysates were immunoprecipitated with anti-BRF1 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. *H*, HEK293T cells were transfected with GFP–RNF12, HA–Ub, Flag–BRF1, Flag–BRF1(K65R), Flag–BRF1(K79R), Flag–BRF1(K115R), Flag–BRF1(K127R), Flag–BRF1(K165R), Flag–BRF1(K199R), Flag–BRF1(K215R), Flag–BRF1(K247R), and Flag–BRF1(K254R) 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-Flag antibody, followed by Western blotting analysis.

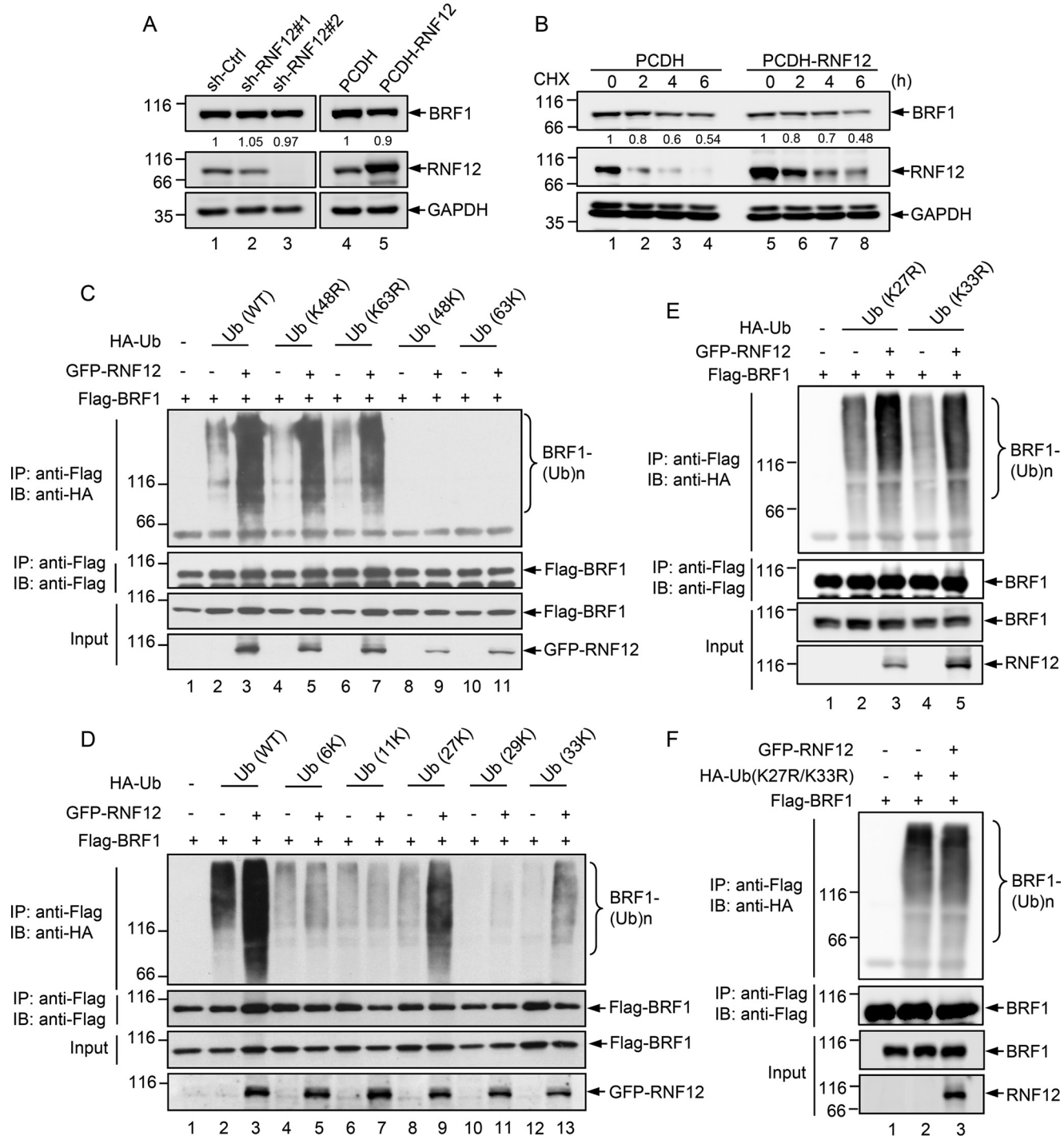
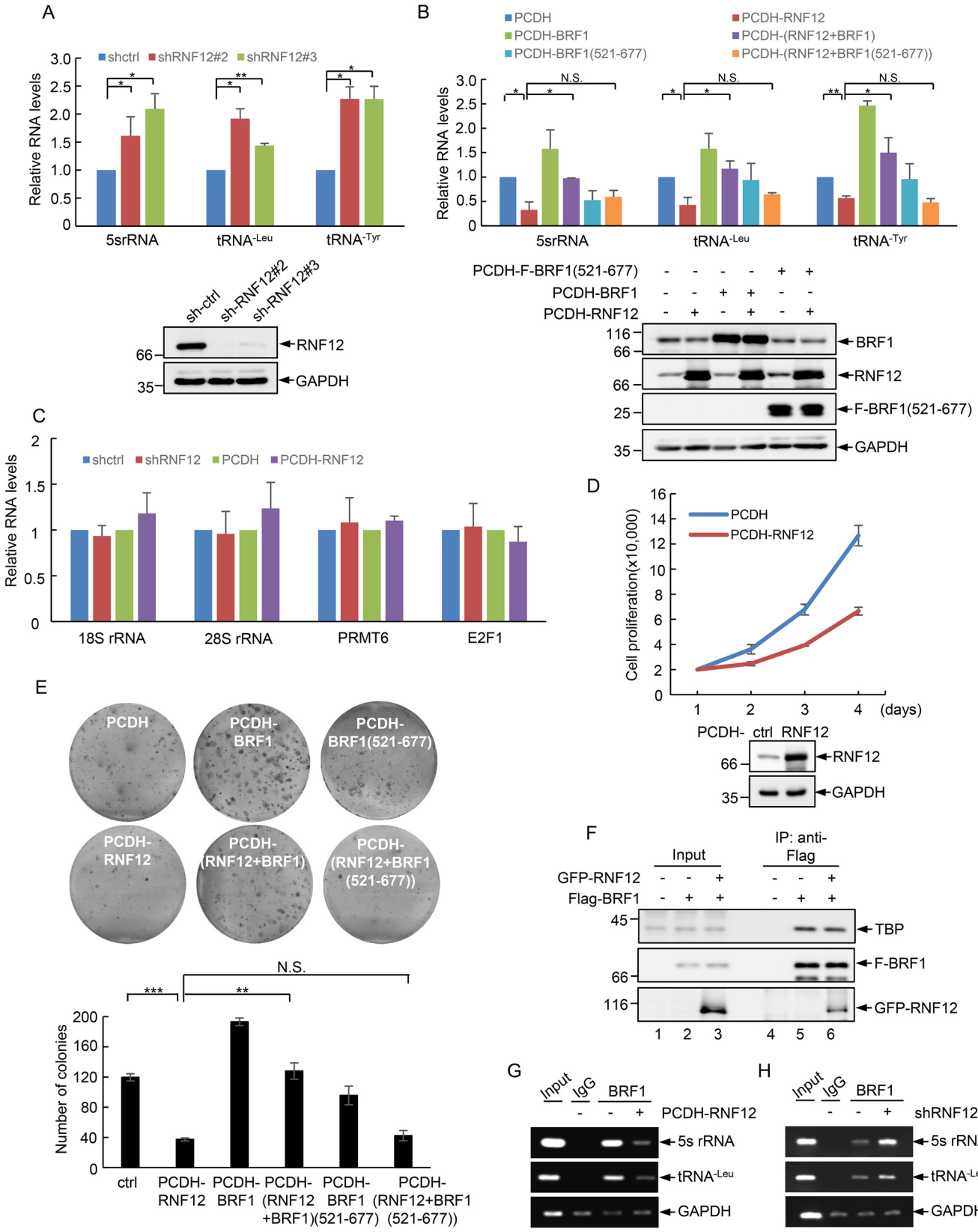


Figure 4. RNF12 promotes Lys^{27}- and Lys^{33}-linked polyubiquitination of BRF1. *A*, HeLa cells were infected with lentiviruses expressing control shRNA, RNF12 shRNA#1, RNF12 shRNA#2, control proteins, or RNF12 proteins as indicated. 48 h after infection, the cell lysates were analyzed by Western 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, 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



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^{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 $MgCl_2$, 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™ 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′-GGCCATACCACCCTGAACGC-3′ and 5′-CAGCACCCGGTATTCCCAGG-3′; $tRNA^{Leu}$, 5′-GTCAGGATGGCCGAGTGGTCTAAGGCGCC-3′ and 5′-CCACGCCTCCATACGGAGACCAGAAGACCC-3′; $tRNA^{Tyr}$, 5′-CCTTCGATAGCTCAGCTGGTAGAGCGGAGG-3′ and 5′-CGGAATTGAACCAGCGACCTAAGGATGTCC-3′; acidic ribosomal phosphoprotein P0, 5′-GCACTGGAAGTCCAACTACTTC-3′ and 5′-TGAGGTCCTCCTTGGTGAACAC-3′; 18S rRNA, 5′-CGGCGACGACCCATTCGAAC-3′ and 5′-GAATCGAACCCTGATTCCCCGTC-3′; 28S rRNA, 5′-GAGAGTTCTCTTTTCTTTGTG-3′ and 5′-GTTCACCTTGGAGACCTGCT-3′; E2F1, 5′-GCCACTGACTCTGCCACCATAG-3′ and 5′-CTGCCCATCCGGGACAAC-3′; PRMT6, 5′-CCCCTCAACAACGGATACAG-3′ and 5′-TTCTCCCAGCTTCAGACTT-3′; and GAPDH, 5′-CCATGGGGAAGGTGAAGGTC-3′ and 5′-GAAGGGGTCATTGATGGCAAC-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 $MgCl_2$, 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 RNF12 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, RNF12 shRNA#2, or RNF12 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 RNF12 was also evaluated by Western blotting analysis. *B*, H1299 cells were infected with lentiviruses expressing RNF12, BRF1, BRF1 (521–677), both RNF12 and BRF1, or both RNF12 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 RNF12, BRF1, and BRF1 (521–677) was also confirmed by Western blotting analysis. *C*, H1299 cells were infected with lentiviruses expressing control shRNA, RNF12 shRNA#2, control proteins, or RNF12 proteins as indicated. 48 h later, total RNA was subjected to real-time RT–PCR analysis to examine expression levels of Pol I transcripts 18S rRNA and 28S rRNA, and Pol II transcripts PRMT6 and E2F1. *D*, H1299 cells were infected with lentiviruses expressing either control or RNF12 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 RNF12 was also confirmed by Western blotting analysis. *E*, H1299 cells were infected with lentiviruses expressing RNF12, BRF1, BRF1 (521–677), both RNF12 and BRF1, or both RNF12 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–RNF12 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 RNF12 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 RNF12 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.

ChIP assay

H1299 cells expressing RNF12 or control proteins were cross-linked with 1% formaldehyde for 10 min. The ChIP assay was performed by using anti-BRF1 antibody and the ChIP assay kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. Normal rabbit IgG was used as a negative control. The bound DNA fragments were subjected to PCR analysis using the specific primers. The primer sequences are as follows: tRNA-Leu, 5′-GAGGACAACGGGGACAGTAA-3′ and 5′-TCCACCAGAAAAACTCCAGC-3′; 5S rRNA, 5′-GGCCATACCACCCTGAACGC-3′ and 5′-CAGCACCCGGTATTCCCAGG-3′; and GAPDH, 5′-TACTAGCGGTTTTACGGGCG-3′ and 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′.

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. The shRNA target sequences used in this study are as follows: sh-control, CCTAAGGTTAAGTCGCCCTCG; sh-RNF12-#1, GCTGATATAGTGATGGGCAAA; sh-RNF12-#2, GCTCAGTCTCAAATCGAAATA; and sh-RNF12-#3, GCATCCAATGAGTGAAATTCC.

Protein expression and purification

The DNA sequences encoding RNF12 and RNF12 (H569A/C572A) were individually cloned into the pGEX-6P-1 vector. The constructs were transformed into *Escherichia coli* Rosetta2 (DE3) cells. The cells were cultured at 37 °C until the $A_{600\ nm}$ reached 0.6 and were then induced with 0.2 mM isopropyl β-D-thiogalactoside (Promega) for 16 h at 25 °C. The cells were suspended in 50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl, 1 mM DTT (Promega), and 1 mg/ml lysozyme, incubated on ice for 30 min, and sonicated. After spinning at 13,000 × *g* for 15 min at 4 °C, the supernatant was incubated with GSH Sepharose beads for 2 h. After extensive washing, the bead-bound GST–RNF12 or GST–RNF12 (H569A/C572A) proteins were used for the indicated experiments.

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 Na_2HPO_4/NaH_2PO_4, 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.

For the *in vitro* ubiquitination assay, the purified Flag–BRF1 proteins were incubated with E1 (50 nM), E2 (UbcH5a, 500 nM), Flag–ubiquitin (200 μM), and either GST, GST–RNF12, or GST–RNF12 (H569A/C572A) proteins in 20 μl of *in vitro* ubiquitination reaction buffer (40 mM Tris-HCl, pH 7.6, 2.5 mM Mg^{2+}-ATP, and 1 mM DTT). After 2 h of incubation at 30 °C, the reaction mixtures were analyzed by Western blotting with anti-BRF1 antibody.

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 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

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