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ORIGINAL ARTICLE

The deubiquitinating enzyme USP37 regulates the oncogenic fusion protein PLZF/RARA stability

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Acute promyelocytic leukemia (APL) is predominantly characterized by chromosomal translocations between the retinoic acid receptor, alpha (*RARA*) gene and the promyelocytic leukemia (*PML*) or promyelocytic leukemia zinc finger (*PLZF*) gene. In APL cells with PML/RARA fusions, arsenic trioxide and all-trans retinoic acid treatments specifically target the fusion protein for proteasome-dependent degradation, thereby promoting cellular differentiation and clinical remission of disease. In contrast, APL cells expressing PLZF/RARA fusion proteins are largely resistant to similar treatments and prognosis for patients with this translocation is poor. Understanding the molecular mechanisms regulating PLZF/RARA protein stability would provide novel therapeutic targets for PLZF/RARA-associated APL. Toward this end, we have performed an RNAi-based screen to identify factors affecting PLZF/RARA stability. Among the factors identified was the ubiquitin-specific peptidase 37 (USP37). We showed that USP37 interacted with PLZF/RARA through the PLZF moiety and sustained PLZF/RARA steady state levels. Domain mapping study revealed that N-terminal domain of USP37 is required for the PLZF/RARA interaction and protein regulation. Furthermore, overexpression or depletion of USP37 caused an increase or decrease of PLZF/RARA protein half-life, correlating with down- or upregulation of PLZF/RARA poly-ubiquitination, respectively. By PLZF/RARA-transduced primary mouse hematopoietic progenitor cells, we demonstrated that *Usp37* knockdown alleviated PLZF/RARA-mediated target gene suppression and cell transformation potential. Altogether, our findings of USP37-modulating PLZF/RARA stability and cell transformation suggest that USP37 is a potential therapeutic target for PLZF/RARA-associated APL.

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Keywords: acute promyelocytic leukemia; deubiquitinating enzyme; USP37; PLZF; RARA

INTRODUCTION

Acute promyelocytic leukemia (APL) is a rare disease characterized by the chromosomal translocations between the retinoic acid receptor, alpha (*RARA*) gene and its counterpart gene (X), resulting in an aberrant fusion protein X-RARA, such as promyelocytic leukemia (PML)/RARA, promyelocytic leukemia zinc finger (PLZF)/RARA, NPM/RARA, NuMA/RARA or STAT5b/RARA.[1] In APL patients, abnormal accumulation of undifferentiated promyelocytes is generally observed in bone marrow, which is because of the blockage of cellular differentiation in myeloid lineage. Such differentiation arrest is in part resulted from dysregulation of key transcriptional regulators such as CEBPα involved in myeloid differentiation by those X-RARA proteins.[2,3] *All-trans* retinoic acid (ATRA) treatment has been introduced for APL cells expressing those X-RARA fusions by promoting cellular differentiation and clinical remission of disease. Although APL cells expressing PLZF/RARA fusion are responsive to ATRA treatment for cell differentiation,[4] PLZF/RARA-associated APL patients, distinct from other X-RARA types of APL patients, are resistant to ATRA therapy.[2] In addition to ATRA, arsenic trioxide has been successfully used for clinical remission of APL patients carrying PML/RARA fusions specifically.[5,6] Arsenic trioxide treatment specifically triggers the poly-SUMO chain formation of PML/RARA, subsequently targeting PML/RARA fusions for poly-ubiquitination and protein degradation.[7,8] Such therapy-triggered degradation of an oncoprotein could potentially serve as a general strategy to eliminate cancer cells.

Ubiquitination-proteasome-dependent proteolysis is a major cellular pathway to control protein stability.[9] Protein ubiquitination is a cascade reaction involving a group of specialized protein family called ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3. Conversely, this biological process can be reversed by deubiquitinating enzymes (DUBs), which are proteases functioning by removing conjugated ubiquitin from substrates.[10] In general, the ubiquitination level of a substrate is regulated by its associated E3 ubiquitin ligase and/or DUB, correlating with the regulation of substrate protein stability. Thus, E3 ubiquitin ligases and DUBs are considered as potential targets for regulation of disease-associated protein stability. By sequence comparison, about 100 DUB genes have been annotated in human genome.[11,12] According to the protein secondary structure of DUBs, these DUBs can be divided into five different subclasses, including four cysteine protease DUBs, ubiquitin-specific protease (USP), Machado–Joseph disease protease, Otubain protease (OTU), and ubiquitin C-terminal hydrolase, and one metalloprotease DUB, JAB1/MPN/Mov34 metalloenzyme.[12] By global proteomic approach, DUBs have been shown to participate in several cellular functions, including DNA damage and repair, protein quality control and degradation, RNA transcription and processing, and signal transductions.[13]

In present study, we have identified USP37-regulating PLZF/RARA protein stability by RNAi screening. USP37 could bind and deubiquitinate PLZF/RARA fusion, thereby enhancing PLZF/RARA protein stability. Knockdown of Usp37 in mouse primary

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hematopoietic progenitor cells could attenuate PLZF/RARA-mediated target gene suppression and cell transformation. These results suggest that USP37 is an important factor in modulating oncogenesis induced by PLZF/RARA fusion in APL cells.

RESULTS

RNAi screening identifies DUBs modulating PLZF/RARA protein level

To monitor PLZF/RARA protein level in cells, we have generated the U937 myeloid leukemia cell line carrying a cassette in which both EGFP-fused PLZF/RARA and tRFP driven by an internal ribosome entry site were induced to express by addition of doxycycline (Figure 1a). In this screening, EGFP intensity was measured to represent PLZF/RARA protein expression level, while tRFP intensity was served as an internal control for the normalization of EGFP intensity. Lentiviruses carrying shRNA clones targeting to 83 human DUBs were used to infect U937 cells (Supplementary Table S1). Following the puromycin selection for cells expressing shRNA and doxycycline induction for expression of the PLZF/RARA cassette, the cells were subjected to 96-well flow cytometry analysis for measuring both EGFP and tRFP intensities (Figure 1b). The *P*-values were calculated and adjusted on the basis of comparing EGFP/tRFP ratio between knockdown and control clones. To avoid the possible off-target effects of certain shRNA clones, DUB targeted by at least three shRNA clones showing significant effect (P-value < 0.0001) on decreasing EGFP/tRFP ratio was chosen as a candidate for further study. Eight candidate DUBs, including MPND, OTUD5, OTUD6A, OTUD6B, OTUD7B, USP2, USP29 and USP37 were initially identified (Supplementary Table S2). To further validate whether these candidate DUBs regulate PLZF/RARA protein expression, each candidate DUB was ectopically expressed with PLZF/RARA in HEK-293T cells. Only OTUD6A, OTUD7B, USP2, USP29 and USP37 could enhance PLZF/RARA protein expression in a dosage-dependent manner (Figure 1c), suggesting that these are potential DUBs to regulate PLZF/RARA protein level. The discrepancy between knockdown and overexpression of these MPND, OTUD5 and OTUD6B in affecting PLZF/RARA protein steady-state level is currently unclear. It is possible that distinct cellular contexts of HEK-293T and U937 cells may contribute to such a discrepancy.

USP37 modulates PLZF/RARA protein level through PLZF moiety

Because PLZF/RARA proteins are mainly localized in the nuclear compartment, we next examined the subcellular localization of these five candidate DUBs for possible direct interaction and regulation of PLZF/RARA. The results of immunofluorescence and western analyses demonstrated that those candidate DUBs, including USP29, USP37, OTUD6A and OTUD7B, were present in the nuclear compartment (Supplementary Figures S1a and b). Of note, OTUD6A was distributed in the peri-nuclear region (Supplementary Figure S1a). These results implicated possible involvement of USP29, USP37 and OTUD7B in PLZF/RARA regulation via a direct association.

Because PLZF/RARA is a fusion protein resulted from reciprocal chromosomal translocations between *PLZF* and *RARA* genes, it is possible that USP29, USP37 or OTUD7B could modulate PLZF/RARA protein level through the moiety of PLZF or RARA, or both portions. To test these possibilities, HA-tagged PLZF or RARA was transiently co-expressed with Flag-tagged USP29, USP37 or OTUD7B in HEK-293T cells. Of note, PLZF protein but not RARA protein was elevated by USP37 in a dose-dependent manner (Figure 2a), while expression of USP29 or OTUD7B enhanced RARA protein but not PLZF protein (Supplementary Figures S2a and b). Furthermore, increasing USP37 expression was unable to alter the protein level of the reciprocal chromosomal translocation product, RARA/PLZF (Figure 2b), neither affected the protein level of another abnormal fusion protein PML/RARA (Figure 2c). In contrast, expression of USP29 but not OTUD7B could increase PML/RARA protein level (Supplementary Figure S2c). These data suggest that USP37 may stabilize PLZF/RARA through the PLZF moiety, whereas USP29 may exert similar function via the RARA moiety in cells. The nature of OTUD7B affecting the levels of PLZF/RARA and RARA, but not the levels of PLZF and PML/RARA, is currently unclear. We further substantiated the specificity of USP37 targeting to the PLZF moiety by USP37 knockdown experiments. USP37 depletion reduced endogenous PLZF level but not RARA level in HL60 cells (Figure 2d, left panel). In contrast, USP37 knockdown failed to significantly alter the protein level of endogenous PML/RARA and RARA in NB4 cells (right panel), a cell line derived from long-term cultures of human APL. Altogether, the findings that USP37 conferred the specific regulation on PLZF/RARA, but not on PML/RARA level, led us to focus on the study of USP37 in regulating PLZF/RARA.

We next examined whether USP37-regulated PLZF/RARA protein level is relevant to its protease catalytic activity. USP37 catalytically inactive mutant, converting Cys350 to Ala (CA), was generated to examine for PLZF/RARA regulation. As expected, the USP37 CA mutant was impaired to enhance PLZF/RARA protein level as compared with WT (Figure 2e), indicating the importance of USP37 catalytic activity for PLZF/RARA regulation. Noted that overexpression of USP37 CA mutant rendered USP37 multiple-band shifts. These slowly migrating bands were K48-linkage ubiquitinated USP37 proteins, as evidenced by western blot analysis with antibodies against specific ubiquitin linkage (Supplementary Figure S3). In addition, PLZF/RARA mRNA level was not altered by USP37 overexpression (Supplementary Figure S4), suggesting that the regulation of PLZF/RARA by USP37 was not at the mRNA level.

USP37 regulates PLZF/RARA protein level via a direct protein interaction

Given that USP37 regulates PLZF/RARA protein steady-state level, we further assessed whether USP37-regulated PLZF/RARA protein level is through a protein interaction between USP37 and PLZF/RARA. To this end, we first demonstrated that USP37 and PLZF/RARA can form complexes in cells. The results of co-immunoprecipitation experiments revealed that overexpressed Flag-tagged USP37 could be detected in the immunocomplex of HA-PLZF/RARA (Figure 3a), and the interaction of USP37 and PLZF/RARA was further confirmed by reciprocal immunoprecipitation experiments (Figure 3a). We further substantiated the complex formation of PLZF/RARA with endogenous USP37, using U937 cells conditionally expressing Flag-tagged PLZF/RARA by adding doxycycline. As expected, endogenous USP37 could form complexes with PLZF/RARA in cells by the immunoprecipitation with either anti-USP37 or anti-Flag antibody (Figure 3b). To further test whether USP37 binding to PLZF/RARA is through a direct interaction, *in vitro* GST pull-down assay was performed using GST-fused PLZF/RARA and MBP-fused USP37 recombinant proteins. Notably, MBP-USP37 could be pulled down by GST-PLZF/RARA but not by GST (Figure 3c), suggesting that USP37 can directly interact with PLZF/RARA.

To demonstrate the importance of USP37-PLZF/RARA interaction for PLZF/RARA protein regulation, we performed the domain mapping study. In line with the result that USP37 enhanced PLZF but not RARA protein steady-state level (Figure 2a), Flag-tagged USP37 could precipitate HA-tagged PLZF but not HA-tagged RARA (Figure 3d). On the front of USP37, we generated and tested N- and C-terminal deletion mutants of USP37 for PLZF/RARA interaction (Figure 3e). The results of co-immunoprecipitation experiments showed that N-terminal deletion mutant (301–979), although it contains the entire USP domain, significantly reduced

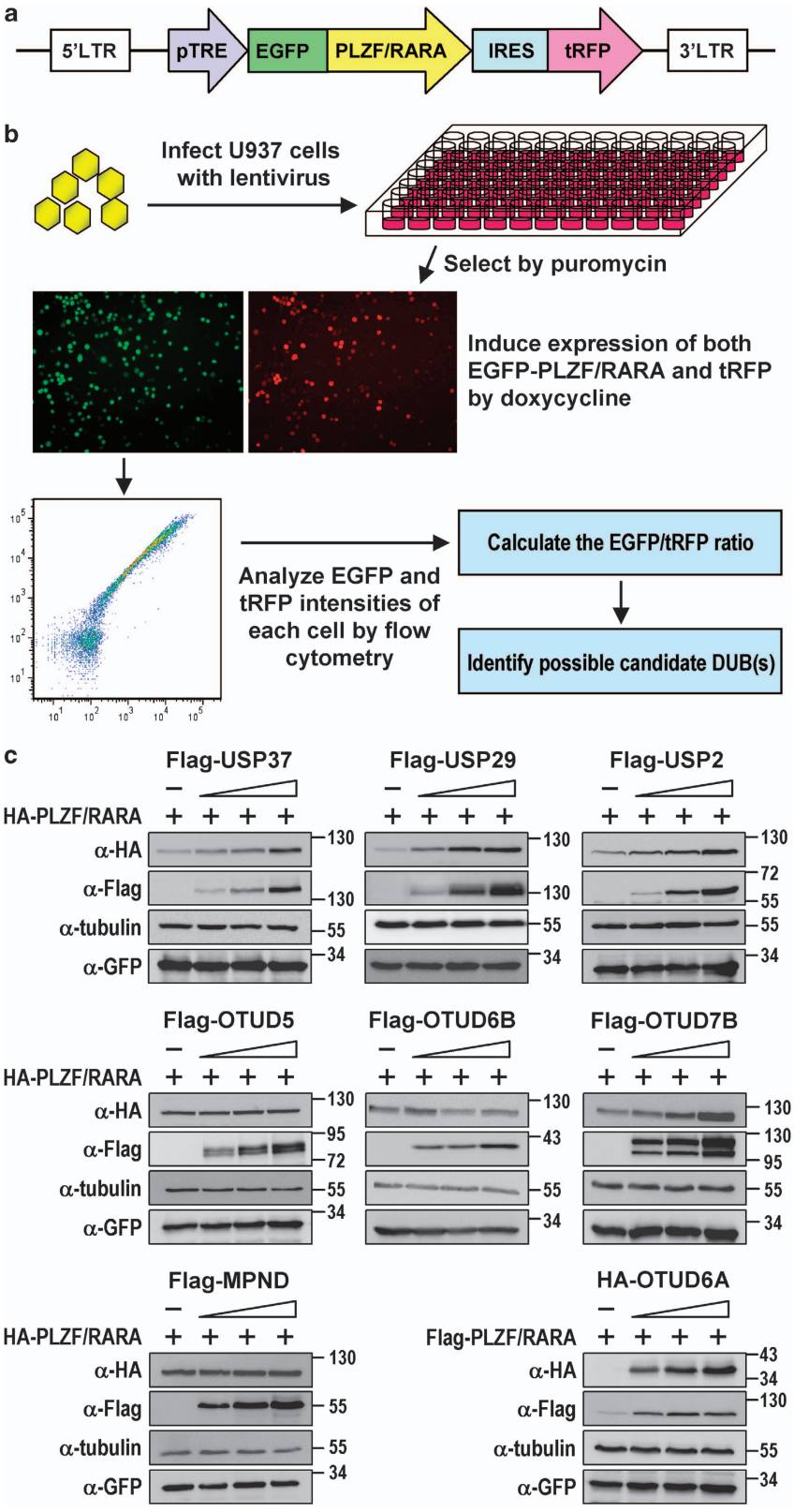

Figure 1. Identification of candidate DUBs in regulating PLZF/RARA protein level. (**a**) A schematic diagram of the cassette construct expressing EGFP-PLZF/RARA and tRFP. (**b**) Flowchart of functional RNAi screening for regulating PLZF/RARA level. (**c**) Western blots show the PLZF/RARA levels in HEK-293T cells cotransfected with indicated DUB constructs along with CMV-EGFP. EGFP was used as cotransfection control.

the PLZF/RARA interaction (Figure 3e, lane 4), while USP37 C-terminal deletion mutant (1–700) bound to PLZF/RARA with the extent slightly lesser to WT (lanes 2 and 3). Of note, the protein steady-state level of USP37 (301–979) fragment was not significantly affected by USP37, compared with (1–700) fragment or WT (Figure 3f). Altogether, these results provide a nice

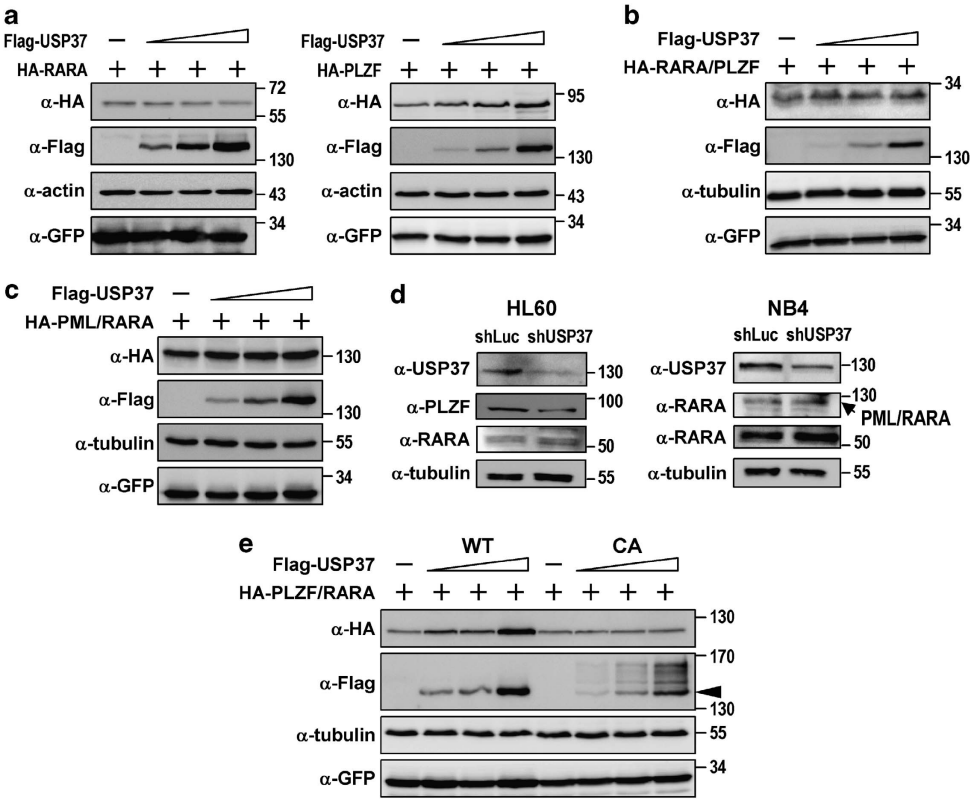

Figure 2. USP37 regulates PLZF/RARA expression level. (**a**-**c**) Western blots of HEK-293T cells transfected with Flag-tagged USP37 and EGFP along with HA-tagged PLZF or RARA (**a**), or HA-tagged RARA/PLZF (**b**) or HA-tagged PML/RARA (**c**). (**d**) Immnoblotting shows endogenous level of USP37, PLZF, RARA and PML/RARA in indicated cells with shUSP37 or shLuc. Arrow indicates PML/RARA. (**e**) Immunoblots show PLZF/RARA levels in HEK-293T cells cotransfected with USP37 WT or catalytic mutant C350A (CA). Arrowhead indicates non-modified band of USP37 CA mutant.

correlation between the binding and regulation of PLZF/RARA protein by USP37.

USP37 regulates the protein stability and ubiquitination of PLZF/RARA

We next examined the effect of USP37 on PLZF/RARA protein half-life. cycloheximide (CHX)-chase experiments showed that USP37 WT, but not CA mutant, prolonged PLZF/RARA protein half-life in HEK-293T cells (Supplementary Figures S5a and b). Likewise, knockdown of USP37 by shRNA clones identified from initial RNAi screening (Supplementary Figure S5c and Supplementary Table S2) showed a reduction of PLZF/RARA protein half-life in U937 cells (Supplementary Figures S5d and e). These results clearly demonstrate that USP37 enhances PLZF/RARA protein stability.

In addition, the findings that USP37 catalytic activity is required for increasing PLZF/RARA protein stability led us to test whether USP37 modulates PLZF/RARA protein ubiquitination level. Overexpression of USP37 WT, but not CA mutant, significantly decreased the ubiquitination of PLZF/RARA in HEK-293T cells (Figure 4a). It should be noted that effects of USP37 on PLZF/RARA deubiquitination were specific because global ubiquitination profile was not significantly changed by USP37 overexpression (Supplementary Figure S6). Accordingly, knockdown of USP37 in U937 cells increased the ubiquitination level of PLZF/RARA (Figure 4b). We further demonstrated that the recombinant USP37 WT but not CA mutant could deubiquitinate PLZF/RARA *in vitro* (Figure 4c). Along with above binding study results, these data strongly suggest that USP37 enhances PLZF/RARA protein stability by deubiquitinating PLZF/RARA.

USP37 modulates the cell transformation potential of PLZF/RARA

PLZF/RARA is able to transform hematopoietic progenitor cells by increasing capacity of cell self-renew and proliferation, and blocking the differentiation of myeloid cell lineage.[14] Because USP37 regulates PLZF/RARA protein stability, it is conceivable that USP37 affects PLZF/RARA-mediated cell transformation of primary hematopoietic progenitor cells. To test this possible scenario, we first established the PLZF/RARA-mediated transformation using mouse primary hematopoietic progenitor cells transduced by retrovirus-expressing PLZF/RARA. As a control, PLZF/RARA-transduced hematopoietic progenitor cells showed decreased expression levels of CCAAT/enhancer-binding protein family members including *Cebpa*, *Cebpb* and *Cebpe*, compared with cells infected with retrovirus carrying an empty vector (Figure 5a). These results are consistent with previous reports showing suppression of CCAAT/enhancer-binding protein transcriptional factors involved in APL cells.[3,15]

We next accessed the effect of Usp37 on PLZF/RARA-mediated transformation by knockdown experiments. We infected the PLZF/RARA-transduced cells with lentivirus-expressing Usp37 shRNAs. Two different Usp37 shRNAs were tested for depletion efficiency. The shUsp37#2 showed a better efficiency in downregulating Usp37 than the shUsp37#1 (Figure 5b, left panel). As expected, depletion of *Usp37* expression alleviated PLZF/RARA-associated suppression of CCAAT/enhancer-binding protein family gene expression (Figure 5b, right panel). Consistent with the depletion efficiency, we observed that shUsp37#2-treated cells yielded higher levels of the CCAAT/enhancer-binding protein family gene expression than shUsp37#1-treated cells. These results suggest that Usp37 is important for PLZF/RARA-mediated cell transformation.

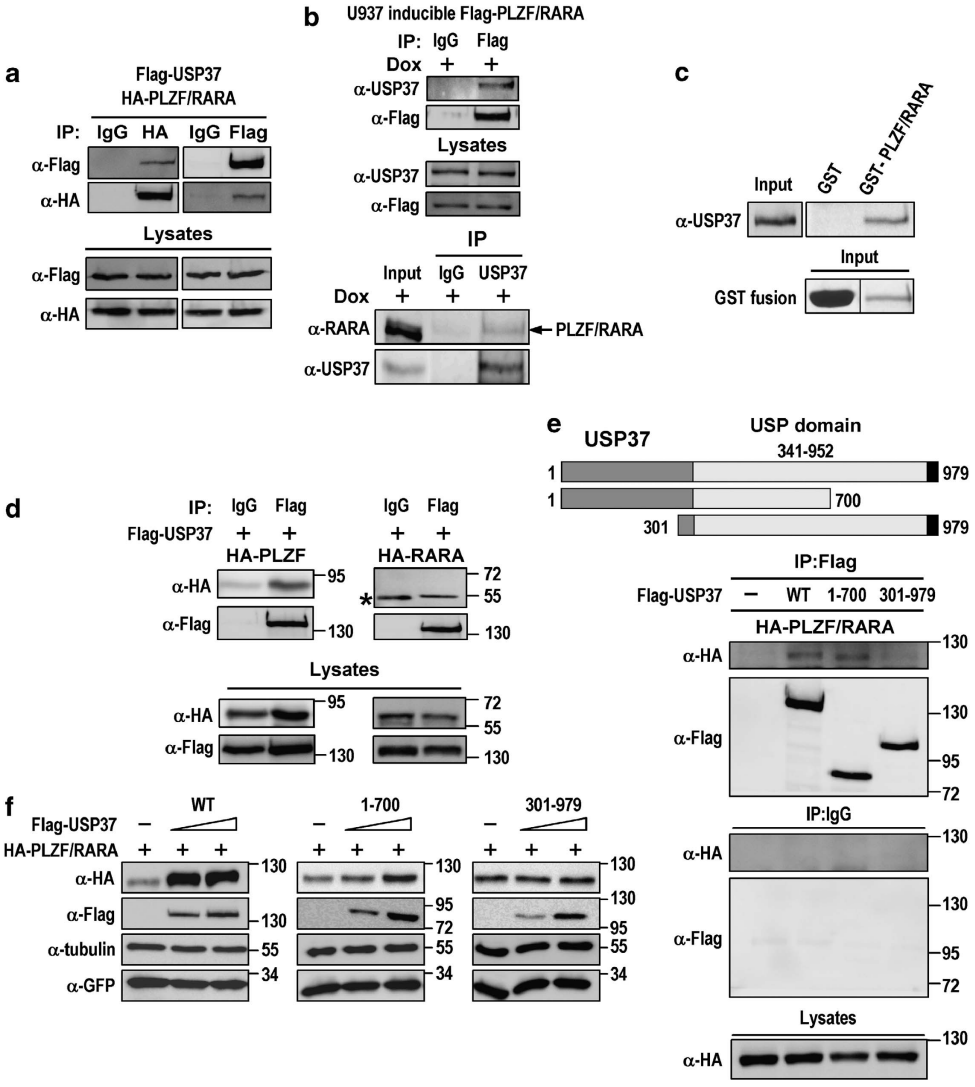

Figure 3. USP37 interacts with PLZF/RARA. (**a**) Western blots show the complex formation of Flag-tagged USP37 and HA-tagged PLZF/RARA in HEK-293T cells transfected with indicated constructs. (**b**) Western blots show the interaction of endogenous USP37 and Flag-tagged PLZF/RARA in TetOn-U937 cells with or without 0.5 μg/ml doxycycline induction for 16 h and precipitated by anti-USP37 or anti-Flag antibody. (**c**) Immunoblotting shows USP37 pulled down by GST-PLZF/RARA. Input represents the 10% amount of recombinant USP37 protein subjected to binding assays. Coomassie blue staining shows GST fusion proteins used for each binding reaction. (**d**) Western blots show the complex formation of USP37 with PLZF but not with RARA in HEK-293T cells transfected with indicated constructs. Asterisk indicates IgG heavy chain from immunoprecipitation. (**e**) Diagram view of wild-type and deleted mutants of USP37. The USP domain is indicated. Western blotting analysis of immunoprecipitated complex from HEK-293T cells transfected with indicated constructs. (**f**) Western blots show the PLZF/RARA levels in HEK-293T cells cotransfected with USP37 WT or deletion mutants.

To demonstrate the role of Usp37 in PLZF/RARA-elicited transformation, we further performed the methylcellulose culture of mouse primary hematopoietic progenitor cells transduced by PLZF/RARA along with or without Usp37 knockdown.[16] As a control, hematopoietic progenitor cells infected by retrovirus carrying MSCV empty vector showed a loss of colony-formation ability after the second passage in methylcellulose culture (Figure 5c, lanes 1 and 2). Notably, these cells expressing either shLuc or shUsp37#2 gave comparable colony number (Figure 5c, lanes 1–4), indicating Usp37 depletion did not alter the characteristics of hematopoietic progenitors in methylcellulose culture. By contrast, PLZF/RARA-transduced cells conferred a significant increase of colony number after the second replating (lane 6). Remarkably, such PLZF/RARA-mediated colony formation was significantly reduced in Usp37-depeleted cells (lane 8) to an extent close to that of cells infected with control vector (lanes 2 and 4). Of note, Usp37 depletion failed to reduce PML/RARA-elicited hematopoietic cells grown in methylcellulose (Supplementary Figure S7). These data suggest that the effect of Usp37 knockdown on the reduction of PLZF/RARA-induced clonogenicity is the consequence of PLZF/RARA loss.

In line with the colony-formation number, we observed that PLZF/RARA expression induced large and compact colony formation on methylcellulose culture (Figure 5d, panels i and ii). Usp37 knockdown changed PLZF/RARA-elicited colony-formation pattern from compact and large to diffuse and smaller in size (panel ii versus iv). Furthermore, Giemsa staining showed that PLZF/RARA-transduced hematopoietic progenitor cells yielded immature cells with a feature of large nucleus and scant cytoplasm as compared with control cells (Figure 5e, panels i and ii). Of note, such PLZF/RARA-elicited immature cell phenotype was significantly decreased in Usp37 knockdown cells (panel iv). Accordingly,

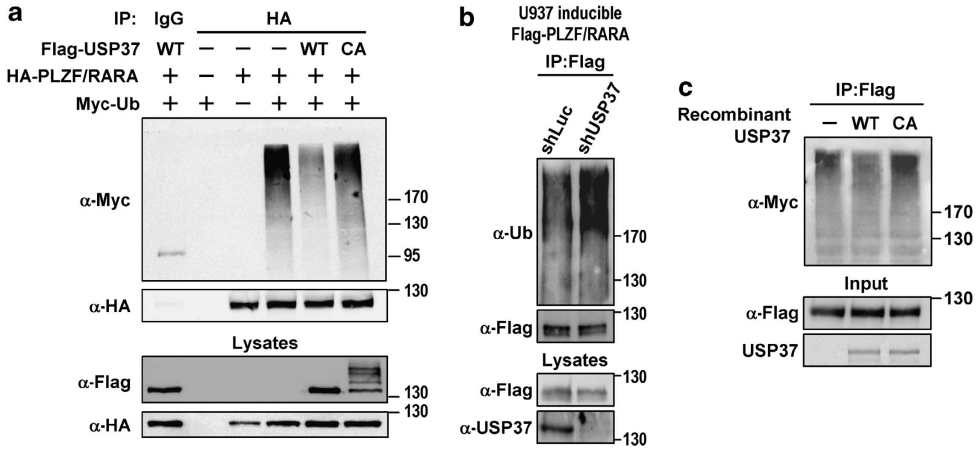

Figure 4. USP37 deubiquitinates PLZF/RARA. (**a**) Western blots show PLZF/RARA deubiquitinated by USP37 in HEK-293T cells transfected with indicated constructs and treated with 20 μM MG132 for 4 h before harvest. (**b**) Immunoblotting shows PLZF/RARA ubiquitination in TetOn-U937 cells with USP37 knockdown. TetOn-U937 cells expressing FLAG-PLZF/RARA were infected with lentivirus carrying shLuc or shUSP37#1 construct and treated with 10 μM MG132 for 4 h before harvest. (**c**) Immunoblotting shows *in vitro* deubiquitination of poly-ubiquitinated PLZF/RARA immunoprecipitated from HEK-293T cell lysates incubated with purified USP37 WT or CA mutant protein for 2 h. *Input* represents the 10% amount of immunoprecipitated proteins subjected to deubiquitination assays. Coomassie blue staining shows USP37 proteins used for each deubiquitinating reaction.

FACS analyses revealed that PLZF/RARA-transduced hematopoietic progenitor cells rendered a marked increase of the c-kit progenitor cell marker together with a reduction of myeloid differentiation markers Gr-1 and Mac-1 (Figure 5f, panels ii and vi), compared with control cells (panels i and v). Such poor differentiation phenotypes were significantly attenuated by Usp37 knockdown (panels iv and viii). Noted that Usp37 knockdown did not significantly change the myeloid cell differentiation profiles (panels iii and vii), implying that Usp37 itself is not associated with normal hematopoietic progenitor cell differentiation into myeloid lineage. Altogether, our data suggest that USP37 has an important role in regulating the protein stability and transformation capacity of PLZF/RARA in myeloid cell lineage.

DISCUSSION

While the substrates and biological functions of most DUBs are unclear, accumulating evidences indicate that DUBs are potential important targets for the treatment of human diseases, such as cancer. For example, USP7 has been shown to stabilize Mdm2, causing tumor suppressor p53 degradation.[17] USP9X was reported to deubiquitinate and stabilize MCL1 in varied human cancer cells, thus maintaining tumor cell survival.[18] USP28 was shown to stabilize MYC oncoprotein, which is highly expressed in human colon and breast carcinomas.[19] In this study, we have identified USP37 as a DUB for PLZF/RARA by RNAi screening. We demonstrate that USP37 physically interacts and modulates PLZF/RARA protein stability and further show an important role of USP37 in PLZF/RARA-mediated transformation of hematopoietic progenitor cells. Thus, our findings not only uncover a new substrate and function of USP37 but also provide a strategy in antagonizing PLZF/RARA-elicited APL. Small molecules acting as ubiquitin protease inhibitors against specific DUBs have successfully been identified and proved to effectively alter cellular functions.[20,21] Thus, development of small molecules inhibiting USP37 deubiquitinating activity may provide more effective and reliable clinical therapy for PLZF/RARA-associated APL.

A recent study has reported that USP37 promotes the G1-S transition via regulating cyclin A stability.[22] USP37 deubiquitinates cyclin A, causing an increased level of cyclin A to further augment cyclin A-CDK2 complex formation. Cyclin A-CDK2 then phosphorylates USP37 and enhances its deubiquitinating activity toward cyclin A, forming a positive feedback loop to promote S phase entry. Interestingly, PLZF/RARA was also shown to activate the expression of cyclin A1,[23] an alternative CDK2 associated A-type cyclin, in human hematopoietic progenitor cells.[24] Since cyclin A1 could form complex with CDK2 for substrate phosphorylation and also contribute to G1-S cell cycle progression in somatic cells,[25] it is possible that PLZF/RARA-mediated transformation of hematopoietic progenitor cells is in part via activation of cyclin A1-CDK2/USP37 positive loop. In addition to causing PLZF/RARA protein destabilization, USP37 knockdown may also destroy this positive feedback loop, thus significantly reducing PLZF/RARA-mediated transformation (Figure 5).

Besides the involvement of USP37 in PLZF/RARA-mediated cell transformation, our results that USP37 targeting to PLZF/RARA is through its N-terminal domain and PLZF moiety also implicate that USP37 may play a role in modulating PLZF protein level in a physiological context. Recent studies reported that PLZF participates in several biological functions, including a negative role in myeloid cell differentiation[26–28] and a role in male germ cells for stem cell-renewal.[29,30] We found that USP37 knockdown could enhance HL60 granulocytic differentiation induced by ATRA, similar to the extent of HL60 cells with PLZF depletion (unpublished data). This observation further supports UPS37 in the PLZF regulation. In addition, USP37 expression was found to be elevated in germ cells according to the microarray database (http://www.ebi.ac.uk/arrayexpress/). This also raises a possibility that USP37 may be involved in stabilizing PLZF protein in germ cells for spermatogonial stem cell maintenance.

In addition to USP37, at least, four other DUBs, including USP2, USP29, OTUD6A, and OTUD7B, are capable of regulating PLZF/RARA level in cells (Figure 1c). The regulation of PLZF/RARA level by these four DUBs could directly or indirectly target PLZF/RARA protein for ubiquitination. Although the distribution of USP2 and OTUD7B is not mainly localized in the nuclear compartment, we cannot exclude the possibility that both factors also play important roles for modulating PLZF/RARA protein level. Similarly, we showed that USP29 modulates PLZF/RARA protein level via RARA portion. This finding also creates an opportunity in fine-tuning PLZF/RARA level in APL cells, in additional to USP37.

In summary, we demonstrate that USP37 is required for the protein stabilization and cell transformation of PLZF/RARA, thus

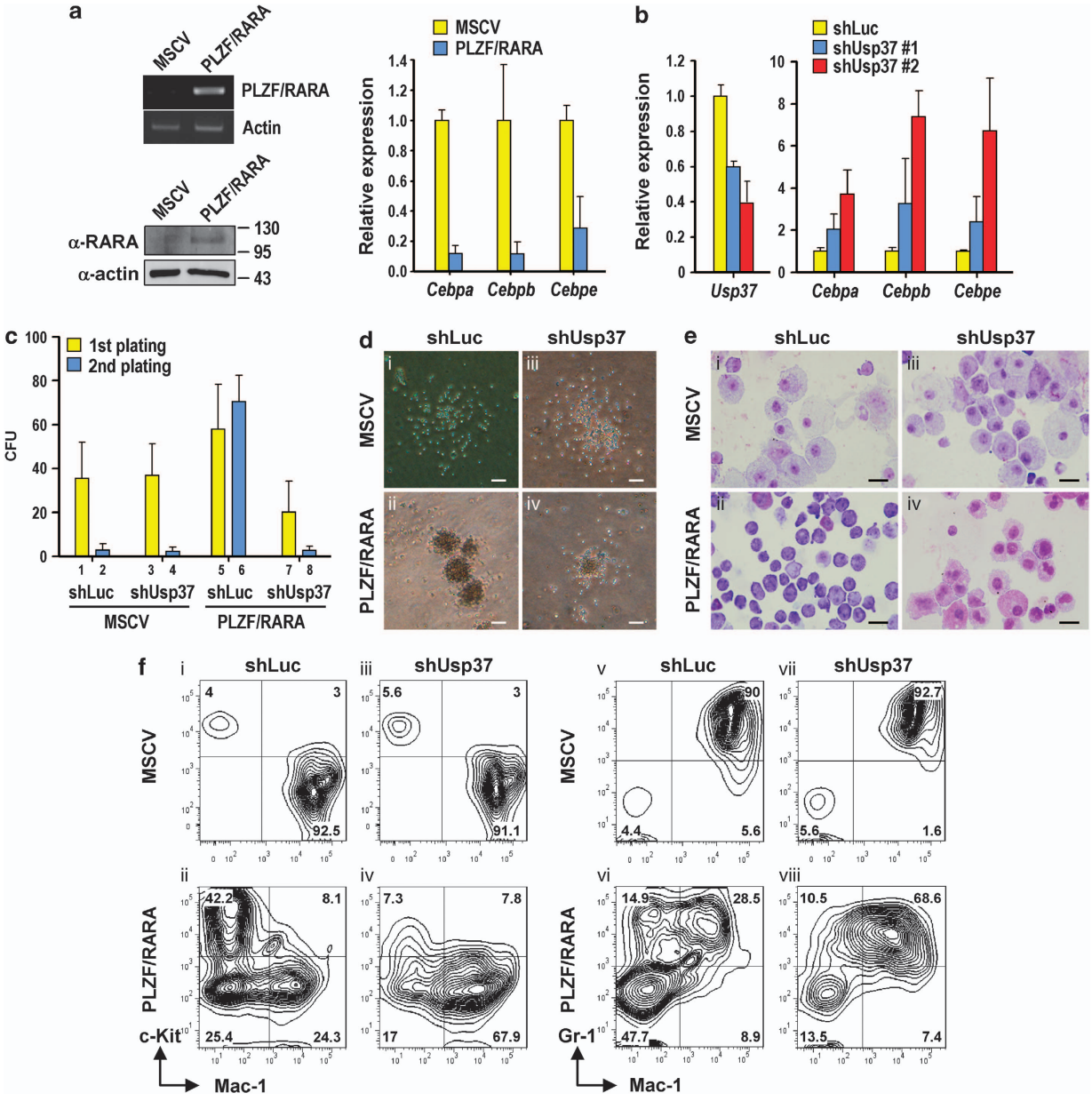

Figure 5. Knockdown of Usp37 attenuates PLZF/RARA-mediated gene suppression and cell transformation. (**a**) Real-time qPCR analyses of endogenous *Cebpa, Cebpb* and *Cebpe* expression in mouse hematopoietic progenitor cells expressing PLZF/RARA or empty vector MSCV. Gel image and western blots show PLZF/RARA expression in retrovirus-infected mouse hematopoietic progenitor cells. Data represent the relative expression of indicated genes. Error bars are mean ± s.d. from three experiments performed in duplicate. (**b**) Real-time qPCR analyses of endogenous *Usp37, Cebpa, Cebpb* and *Cebpe* in PLZF/RARA-transduced mouse hematopoietic progenitor cells expressing indicated shRNAs. Error bars are mean ± s.d. from three experiments performed in duplicate. (**c**) Bar graph represents the colony number formed from methylcellulose medium culture of mouse hematopoietic progenitor cells transduced with MSCV vector or MSCV-PLZF/RARA in combination with shLuc or shUsp37#2. Error bars indicate s.d. from three independent experiments. CFU: colony-formation unit. (**d**) Representative images of colonies formation from transduced mouse hematopoietic progenitor cells in methylcellulose medium at second-round replating. Bar, 200 μm. (**e**) Giemsa staining of indicated transduced mouse hematopoietic progenitor cells from second-round replating of methylcellulose culture. Bar, 20 μm. (**f**) FACS analysis of surface marker expression of indicated transduced mouse hematopoietic progenitor cells from second-round replating. Data are representative of three independent experiments.

providing USP37 as a potential target for the development of specific inhibitor in treatment of PLZF/RARA-associated APL.

MATERIALS AND METHODS

Antibodies and plasmid constructs

The following primary antibodies were used: anti-USP37 (Bethyl Laboratories, Montgomery, TX, USA), anti-PLZF (Merck, Whitehouse Station, NJ, USA), anti-Flag (Sigma-Aldrich, St Louis, MO, USA), anti-HA (Covance, Princeton, NJ, USA), anti-tubulin (Epitomics, Burlingame, CA, USA), anti-actin (Millipore, Billerica, MA, USA), anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Myc (LTK BioLaboratories, Taoyuan, Taiwan), anti-Ub (a gift from Dr. Sheng-Chung Lee), and Anti-Flag and anti-HA beads (Sigma-Aldrich) for Western blotting and immunoprecipitation as where indicated. All of the shRNA constructs against DUBs and luciferase were obtained from the RNAi consortium at Academia Sinica. The pLKO-AS3-TetOn-Neo construct (the RNAi consortium, Academia Sinica) was used to generate stable TetOn-U937 cells by lentiviral infection. For the functional RNAi screening, the complementary DNA (cDNA) coding EGFP-PLZF/RARA was cloned into pLKO-AS3W-Tet-TRE-tRFP vector (the RNAi

consortium, Academia Sinica) and was expressed in TetOn-U937 cells by lentiviral infection. PLZF/RARA cDNA was separately constructed into pLKO-AS4.1W-Tet-Hyg (the RNAi consortium, Academia Sinica) and pMSCVneo for lentivirus-expressing Flag-tagged PLZF/RARA in TetOn-U937 and for retrovirus-expressing PLZF/RARA in mouse hematopoietic progenitor cells, respectively. The pLKO.1-mouse *Usp37* shRNA lentiviral constructs expressing target sequences are (#1): 5′-CGCCTAATGTTGACTT TACAA-3′, and (#2): 5′-GCAGAAGATGATATTCCAGAA-3′. The cDNA constructs of USP29, OTUD5, MPND and OTUD6A were purchased from Open Biosystems, while USP37, OTUD6B, OTUD7B and USP2 cDNAs were kindly gifted from Dr J Wade Harper.[13] The cDNAs of USP2, USP29, USP37, OTUD5, OTUD6B, OTUD7B, MPND and PLZF/RARA were cloned into pCMV-3xFLAG vector for Flag-tagged protein expression. The cDNAs of OTUD6A and PLZF/RARA were inserted into pcDNA3-HA vector for HA-tagged protein expression. Ubiquitin cDNA was cloned into pCMV-3xMyc vector. USP37 and PLZF/RARA cDNAs were cloned into pMAL-c2X and pGEX-4T-2 for MBP and GST fusion protein production, respectively. The catalytically inactive USP37 (USP37 C350A) mutant was generated by site-directed mutagenesis, as described previously.[31] The USP37 fragment cDNAs encoding 1–700, 301–979 and 1–300 amino-acid residues were cloned into pCMV-3xFLAG vector.

Cell culture, transfection, immunoprecipitation and western analyses

HEK-293T and GP2-293 cells were maintained in DMEM with 10% FBS (Gibco, Life Technologies, Grand Island, NY, USA). U937, HL60 and NB4 cells were maintained in RPMI1640 with 10% FBS (Gibco). OP9 cells were maintained in α-MEM with 20% FBS (Hyclone, Thermo Scientific, Austin, TX, USA) and 60 μM 2-mercaptoethanol, and were served as feeder layer after 4-hour treatment with 10 μg/ml mitomycin C (Sigma-Aldrich). Mouse bone marrow cells were obtained from femurs of combined two male C57BL/6 mice at 8 weeks of age. Mouse hematopoietic progenitor cells were purified from bone marrow cells by negative selection with magnetic beads, according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, USA) and short-term expanded by co-culture with OP9 feeder layer in IMDM containing 10% FBS (Hyclone), 60 μM 2-mercaptoethanol, 20 ng/ml murine Scf, 20 ng/ml murine Tpo and 20 ng/ml murine Flt-3 ligand. All cytokines were purchased from Peprotech (Rocky Hill, NJ, USA). Calcium phosphate method was performed for transient transfection of HEK-293T cells. Immunoprecipitation and western analyses were performed, as described previously.[32] In brief, transfected HEK-293T cells were harvested in NP40 lysis buffer (50 mM Tris–HCl pH 7.5, 5 mM EDTA, 1% NP40 and 150 mM NaCl) supplemented with 5 mM NEM and protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were immunoprecipitated with agarose beads conjugated with anti-Flag or anti-HA antibody for 2 h at 4 °C. For co-immunoprecipitation experiments of endogenous USP37 with PLZF/RARA, TetOn-U937 cells conditionally expressing Flag-tagged PLZF/RARA were harvested by NP40 lysis buffer, incubated with anti-USP37 or anti-Flag antibody for 16 h at 4 °C and followed by adding magnetic protein G Sepharose beads (GE Healthcare, Waukesha, WI, USA) for additional 1 h. Both resulting beads were washed and subjected to western analysis with .specific antibody

GST pull-down and deubiquitination assays

GST pull-down assay was performed, as described.[32] Two μg of recombinant GST or GST-PLZF/RARA was incubated with 2 μg of MBP-USP37 and immobilized glutathione beads (Thermo Scientific) in binding buffer (10 mM HEPES pH 7.5, 0.5 mM DTT, 0.5 mM EDTA, 0.1% NP-40, and 50 mM NaCl) for 4 h at 4 °C. After washing three times, the samples were then subjected to western analysis. For *in vitro* deubiquitination assay, poly-ubiquitinated PLZF/RARA proteins immunoprecipitated from HEK-293T cells expressing Flag-tagged PLZF/RARA and Myc-tagged Ub were incubated with recombinant USP37 protein in 100 μl of deubiquitination buffer (50 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$ and 1 mM DTT) for 2 h at 37 °C. The deubiquitination reaction was stopped by adding SDS sampling buffer and bound proteins were extracted from beads for western analysis.

Viral infection and replating assay

Lentiviral supernatants were prepared, as described previously.[31] In brief, 12 μg lentiviral DNA construct, 3 μg pMD2.G (addgene, Cambridge, MA, USA) and 9 μg psPAX2 (addgene) were cotransfected in HEK-293T cells with 60% confluency in 10-cm dish. Likewise, retroviral supernatants were prepared from 60% confluent GP2-293 cells in 10-cm dish cotransfected with 9 μg pVSV-G and 12 μg retroviral DNA constructs. Both lentivirus and retrovirus were concentrated by ultra-centrifugation with 20 000 rpm for 2 h at 4 °C. Mouse hematopoietic progenitor cells co-cultured with OP9 feeder were infected by retrovirus carrying PLZF/RARA construct with addition of 0.8 μg/ml polybrene (Sigma-Aldrich). After 16 h infection, culture medium was replaced by fresh differentiation culture medium, which is IMDM supplemented with 10% FBS, 20 ng/ml SCF, 10 ng/ml of each IL3, IL6 and GM-CSF. For serial infection, the resulting cells were further infected with lentivirus-expressing indicated shRNA for additional 16 h and replaced with fresh differentiation culture medium. After selection by G418 (500 μg/ml) and puromycin (1 μg/ml), cells were subjected for quantitative real time PCR analyses. For replating assay, transduced mouse hematopoietic progenitor cells (10 000 cells/ml) were then plated to MethoCult (STEMCELL Technology, Vancouver, BC, Canada) medium supplemented with 20 ng/ml SCF, 10 ng/ml of each IL3, IL6, and GM-SCF, and 1 mg/ml G418 and 2 μg/ml puromycin for selection. After 7 days, cells were replated for additional 7 days and then the number of colony in each condition was counted under light microscope. Cellular morphology was analyzed by Giemsa staining of cytospin from the second-round replating cells. The expression levels of cell surface marker were analyzed by FACS with fluorochrome-conjugated antibodies (c-kit/CD117, clone 2B8; Mac-1/CD11b, clone M1/70; Gr-1/Ly-6G, clone RB6-8C5; all purchased from BioLegend, San Diego, CA, USA).

Quantification of PLZF/RARA-regulated gene level

Total cellular RNAs were extracted by TRIzol reagent (Invitrogen, Life Technologies, Grand Island, NY, USA), and RNA of each sample was subsequently reverse transcribed using ThermoScript reverse transcription-PCR system (Invitrogen, Life Technologies), according to the manufacturer's instruction. Reverse transcription PCR product was used for quantitative real-time PCR analyses (Applied Biosystems 7500 Life Technologies, Grand Island, NY, USA) with specific primers, as following: Cebpa forward 5′-AGGAACTTGAAGCACAAT-3′ and reverse 5′-ACACAGA GACCAGATACA-3′; Cebpb forward 5′-CGGGGTTGTTGATGTTTT-3′ and reverse 5′-CATACGCCTCTTTTCTCATAG-3′; Cebpe forward 5′-CAAGAAGG CAGTGAACAA-3′ and reverse 5′- GCTGAGTCTCCATAATGC-3′; Usp37 forward 5′-CTCATCAGTGTTGTCAGT-3′ and reverse 5′-TCCAGGTCATTG TAAGTG-3′; Hprt forward 5′-GATTAGCGATGATGAACCAGGTT-3′ and reverse 5′-CCTCCCATCTCCTTCATGACA-3′. Hprt gene expression was used as an internal control for normalization. The RT-PCR product was used for semiquantitative PCR analyses with specific primers as following: PLZF/RARA forward 5′-TGAAGACGTACGGGTGCGAG-3′ and reverse 5′-TGTA GATGCGGGGTAGAGGG-3′; actin forward 5′-CCTAGAAGCATTTGCGGTGG-3′ and reverse 5′-GAGCTACGAGCTGCCTGACG-3′. The PCR products were then resolved by 1.5% agarose gel containing ethidium bromide.

Statistical analysis

Statistical analyses were carried out by using SAS 9.1.2 (SAS institute Inc, Cary, NC, USA) with two-tailed student *t* test. Two-tailed student *t* test was used here to calculate the EGFP/tRFP intensity ratio between experimental and control groups. To eliminate false-positive rate occurred from multiple testing, we introduced Bonferroni's adjustment to correct *P*-values obtained from *t*-test, therefore, data with P-value < 0.0001 was considered as statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)