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Deubiquitylase USP7 regulates human terminal erythroid differentiation by stabilizing GATA1

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ABSTRACT

Ubiquitination is an enzymatic post-translational modification that affects protein fate. The ubiquitin-proteasome system (UPS) was first discovered in reticulocytes where it plays important roles in reticulocyte maturation. Recent studies have revealed that ubiquitination is a dynamic and reversible process and that deubiquitylases are capable of removing ubiquitin from their protein substrates. Given the fact that the UPS is highly active in reticulocytes, it is speculated that deubiquitylases may play important roles in erythropoiesis. Yet, the role of deubiquitylases in erythropoiesis remains largely unexplored. In the present study, we found that the expression of deubiquitylase USP7 is significantly increased during human terminal erythroid differentiation. We further showed that interfering with USP7 function, either by short hairpin RNA-mediated knockdown or USP7-specific inhibitors, impaired human terminal erythroid differentiation due to decreased GATA1 level and that restoration of GATA1 levels rescued the differentiation defect. Mechanistically, USP7 deficiency led to a decreased GATA1 protein level that could be reversed by proteasome inhibitors. Furthermore, USP7 interacts directly with GATA1 and catalyzes the removal of K48-linked polyubiquitylation chains conjugated onto GATA1, thereby stabilizing GATA1 protein. Collectively, our findings have identified an important role of a deubiquitylase in human terminal erythroid differentiation by stabilizing GATA1, the master regulator of erythropoiesis.

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Introduction

Red blood cells, the most abundant of all circulating blood cells, facilitate gas exchange in the lungs and transporting oxygen to tissues. More than two million red blood cells are generated per second in a healthy adult through a process termed erythropoiesis. Mature red blood cells are produced from hematopoietic stem cells, which commit to erythroid progenitors followed by terminal erythroid differentiation. Terminal erythroid differentiation, driven by the glycoprotein hormone erythropoietin, begins with proerythroblasts, which sequentially divide into basophilic, polychromatic and orthochromatic erythroblasts that enucleate to generate reticulocytes.[1,2] Erythropoiesis is a tightly regulated process. Previous studies were primarily focused on the regulation of erythropoiesis by transcription factors and cytokines.[3,4]

In contrast, the regulation of erythropoiesis by other mechanisms has been less well studied. Notably, our knowledge on post-translational regulation of erythropoiesis is limited.

Ubiquitination is an enzymatic post-translational modification. Ubiquitinated proteins are degraded by the ubiquitin-proteasome system (UPS). The UPS controls the degradation of most intracellular proteins and plays important roles in many cellular processes.[5] Although the UPS was first discovered in reticulocytes over 40 years ago,[6] to date there are only limited studies on the roles of the UPS in erythropoiesis. These include the reported role of CUL4A-mediated degradation of p27 in cell proliferation in the early stages of erythropoiesis and cell cycle exit at a later stage of erythropoiesis.[7,8] A recent, exciting study demonstrated that UBE2O remodels the proteome during terminal erythroid differentiation, underscoring the importance of the UPS in erythropoiesis.[9]

Ubiquitination is a dynamic and reversible process.[10] It has been reported that deubiquitylases are capable of removing ubiquitin from their protein substrates and allow proteins to be salvaged from proteasomal degradation.[11] USP7 is a deubiquitylase that belongs to the ubiquitin-specific protease (USP) family, which constitutes the largest subgroup of deubiquitylases. Accumulated evidence has shown that USP7 plays diverse roles in genome stability, epigenetic regulation, the cell cycle, apoptosis, viral infection, immunity and stem cell maintenance.[12-17] Recently, USP7 was reported to be an important regulator of osteogenic differentiation and adipogenesis.[18,19] Our RNA-sequencing analyses revealed high-level expression of genes/pathways (including USP7) involved in the ubiquitin system during late stages of terminal erythroid differentiation.[2] Nevertheless, the function of USP7 in human erythropoiesis remains unexplored.

GATA1 is the key transcription factor for erythropoiesis, controlling the expression of a large series of erythroid genes, including erythropoietin receptor, globins and several membrane proteins.[20] GATA1-deficient mice die *in utero* due to severe anemia at embryonic day 10.5-11.5,[21] and chimeric mice lacking GATA1 fail to produce mature red blood cells, although the formation of cells of other hematopoietic lineages is normal.[22] In contrast, overexpression of GATA1 in erythroid cells inhibits their differentiation, leading to fatal anemia in mice.[23] GATA1 stability is finely regulated by multiple mechanisms,[24] since changes in its protein levels will exert a great influence on erythropoiesis. Although GATA1 degradation by the ubiquitin-proteasome pathway has been characterized,[24] how GATA1 recycles from the UPS is yet to be defined.

In this study, we demonstrated that USP7 deficiency impairs human terminal erythroid differentiation due to a decreased level of GATA1 protein. We further showed that USP7 interacts directly with GATA1 and catalyzes the removal of poly-ubiquitylation chains on GATA1, thus stabilizing GATA1. Our findings have thus not only documented the role of a deubiquitylase in erythropoiesis, but also enabled the identification of a novel mechanism by which deubiquitylases regulate GATA1 protein stability.

Methods

Reagents and antibodies

P5091 (S7132) and MG132 (S2619) were obtained from Selleckchem (TX, USA); P22077 (HY-13865) from MCE (NJ, USA); and cycloheximide was purchased from Sigma-Aldrich (MO, USA). Antibodies used for western blot, immunoprecipitation and immunofluorescence studies are detailed in the *Online Supplementary Methods*. The antibodies used for flow cytometry analysis were glycoprotein A (GPA)-PE-Cy7, GPA-APC, and α4-integrin (CD49d)-PE from BD Pharmingen (NJ, USA). Band 3-APC and 4.1R antibodies were used as previously described.[25]

Cell culture

Human cord blood samples were obtained from Xiangya Hospital of Central South University or New York Blood Center under Institutional Review Board approval and in accordance with the Declaration of Helsinki. The detailed composition of the culture medium and the cell culture protocol has been described previously.[25] HEK293T cells (American Type Culture Collection: CRL-11268) were cultured in Dulbecco modified Eagle medium (Gibco, MA, USA) supplemented with 10% fetal bovine serum (Gibco).

Lentivirus packaging and infection

USP7-specific short hairpin (sh)RNA was purchased from GenePharma (Shanghai, China) (shRNA #1: 5'-AGTCGTTCAGTCGTCGTAT-3' and #2: 5'-TGGATTTGTG-GTTACGTTACTC-3', constructed in pGLV3-H1-GFP or pGLV2-U6 vector). GATA1 overexpression (HMD-GATA1-IRES-GFP) and control plasmids have been described previously.[26] Lentiviruses were packaged in HEK293T cells according to the manufacturer's protocol (Invitrogen, MA, USA). A total of $30x10^7$ lentiviral particles were infected using polybrane with $0.5x10^7$ $CD34^+$ cells on day 3 or 4. Puromycin (1 μg/mL) was used for selection of transduced cells.

GATA1 rescue assay

For rescue experiments, erythroid cells were infected with USP7 shRNA or control shRNA lentiviruses for 3 days. On day 7 of culture, erythroid cells were transduced with the control or GATA1 lentivirus. Double-transduced cells were identified following puromycin (1 μg/mL) selection and GFP expression from the HMD vector. The extent of terminal erythroid differentiation was monitored beginning on day 9.

RNA isolation, quantitative real-time polymerase chain reaction and western blot analysis.

Standard protocols were used for RNA and protein isolation, polymerase chain reaction (PCR) and western blot analysis. Details are given in the *Online Supplementary Methods*. The GATA1 primer sequences were described previously.[27] USP7 primer sequences were: forward: 5'-AGCGTGGCATCACCATAATC-3' and reverse: 5'-CGAGGCAACCTTTCAGTTCA-3'.

Immunoprecipitation and glutathione-S-transferase pull-down

Immunoprecipitation studies were performed using M2/Flag or protein A/G-agarose beads. For the glutathione-S-transferase (GST) pull-down assay, purified Flag-USP7 and bacterial expressed GST or GST-GATA1 were used. The methods are described in detail in the *Online Supplementary Methods*.

In vivo ubiquitylation and deubiquitylation assays

For cell-based deubiquitylation assays, Flag-GATA1 and HA-ubiquitin were co-transfected with an empty vector or a vector expressing USP7 (WT or CS) for 48 h. For USP7 knockdown, the cells were infected with the lentiviruses for 48 h. Additional details of the methods are given in the *Online Supplementary Methods*.

In vitro deubiquitylation assays

In vitro ubiquitylation assays were performed as previously described[28] and additional details are provided in the *Online Supplement*.

Statistical analysis

All data are presented as mean ± standard deviation (SD), and the results were analyzed using the SPSS 18.0 software package. Significant differences between groups were determined using analysis of variance and the Tukey range test.

Results

Deficiency of USP7 impairs human terminal erythroid differentiation

To explore the roles of deubiquitylases during erythropoiesis, we first analyzed the expression patterns of deubiquitylases in human erythroblasts at different stages of differentiation from our RNA-sequencing data.[2] Figure 1A shows the expression patterns of USP family members and reveals that the expression levels of USP7 are significantly increased during erythropoiesis. Based on the previously identified important role of USP7 in cell differentiation in other cellular systems,[17-19] in the present study we focused our attention on the role of USP7 in erythroid differentiation. We confirmed the increased expression of USP7 during late stages of erythroid differentiation by both real-time PCR (Figure 1B) and by western blot analysis (Figure 1C). To examine the effect of USP7 on erythropoiesis, we employed a shRNA-mediated knockdown approach in human CD34$^+$ cells.[27,29,30] As shown in Figure 1D, USP7 knockdown impaired the terminal erythroid differentiation as demonstrated by the decreased surface expression of the erythroid marker GPA, delayed loss of α4-integrin expression in association with decreased surface expression of band 3. There was also a marked decreased in the extent of enucleation. USP7 knockdown also inhibited the expression of hemoglobin (Figure 1E). The significant impairment of the growth of late-stage erythroblasts caused by USP7 knockdown was accompanied by increased apoptosis (*Online Supplementary Figure S1A, B*). Similar to USP7 knockdown, USP7-specific inhibitors P5091 and P22077[31,32] also impaired human terminal erythroid differentiation, inhibited hemoglobin expression (Figure 1F, G) and cell proliferation (*Online Supplementary Figure S1C, D*). These results imply that USP7 plays an important role in human terminal erythroid differentiation.

USP7 regulates erythroid differentiation by modulating GATA1 protein levels

We subsequently explored the molecular mechanism(s) of the altered erythropoiesis due to USP7 deficiency. Given the fact that USP7 functions in the nucleus,[33,34] we hypothesized that USP7 might affect erythropoiesis by regulating erythroid differentiation-related transcription factors. As shown in Figure 2A and *Online Supplementary Figure S2*, GATA1 was the transcription factor most significantly decreased after knockdown of USP7, although KLF1 levels also decreased. Since KLF1 expression is regulated by GATA1,[35] we suggest that the decreased expression of KLF1 is a consequence of GATA1 downregulation. Interestingly, mRNA levels of GATA1 were not affected by USP7 knockdown on day 9 (Figure 2B), suggesting that the observed decrease in GATA1 levels is at the post-transcriptional level. We noted decreased levels of GATA1 mRNA levels on days 11 and 13 following USP7 knockdown (*Online Supplementary Figure S3A, B*), likely due to the fact that GATA1 mediates its own regulation at the late stages of erythropoiesis.[36,37] Similarly, inhibition of USP7 activity by the USP7-specific inhibitors P5091 and P22077 also resulted in significant decreases in GATA1 protein levels in a dose-dependent manner (Figure 2C, D), with no obvious effects on other transcription factors beside a slightly decreased expression of KLF1 (*Online Supplementary Figure S4*), implying that USP7-mediated regulation of GATA1 depends on the enzymatic activity of USP7. To further confirm that USP7 deficiency-induced defective erythropoiesis is due to downregulation of GATA1, we performed rescue experiments by ectopically expressing GATA1 in USP7 knockdown cells. Figure 2E shows that the delayed erythroid differentiation as well as impaired erythroblast enucleation could be rescued by restoring GATA1 levels. Furthermore, the expression of GATA1 target genes such as 4.1R and HBG were also rescued (Figure 2F). These results imply that USP7 regulates erythroid differentiation through GATA1.

USP7 regulates the stability of the GATA1 protein

GATA1 protein levels are regulated by several proteins, including HSP70, a GATA1 chaperone[38-40] and RPS19, necessary for GATA1 translation.[26,41] To examine whether HSP70 and RPS19 are involved in the regulation of GATA1 mediated by USP7, we analyzed the effect of USP7 knockdown on their expression levels. As shown in *Online Supplementary Figure S5A, B*, USP7 knockdown or inhibition had no effects on HSP70 or RPS19 protein levels. Moreover, USP7 knockdown did not affect the translocation of HSP70 into the nucleus (*Online Supplementary Figure S5C, D*). The above findings strongly suggest that USP7 regulates the stability of GATA1 protein directly. We performed several additional studies to confirm this hypothesis and to define the underlying mechanisms. First, co-expression of wildtype USP7 (USP7-WT) with GATA1 increased the GATA1 level (Figure 3A). Importantly, catalytically inactive mutant USP7 (USP7-CS, C233S) did not increase GATA1 protein levels (Figure 3B). Second, downregulation of GATA1 by USP7 knockdown or the USP7 inhibitors P5091 and P22077 was reversed by the proteasome inhibitor MG132 (Figure 3C-E), implying that USP7 maintains the steady-state levels of GATA1 by blocking its proteasomal degradation. To further examine the relationship between USP7 and GATA1, we measured the half-life of intracellular GATA1 after cells had been treated with cycloheximide to inhibit protein biosynthesis. As shown in Figure 3F, knockdown of USP7 significantly shortened the half-life of the GATA1 protein. Conversely, overexpression of USP7-WT, but not USP7-CS, prolonged the half-life of GATA1 (Figure 3G). Taken together, our data demonstrate that USP7 stabilizes GATA1 by preventing its proteasomal degradation.

USP7 interacts directly with GATA1

Having demonstrated that USP7 stabilizes GATA1, we then examined whether this effect is through their direct interaction by performing co-immunoprecipitation experiments. USP7 or GATA1 was separately immunoprecipitated from cultured primary erythroblasts and the reciprocal protein was detected by western blot analysis. As

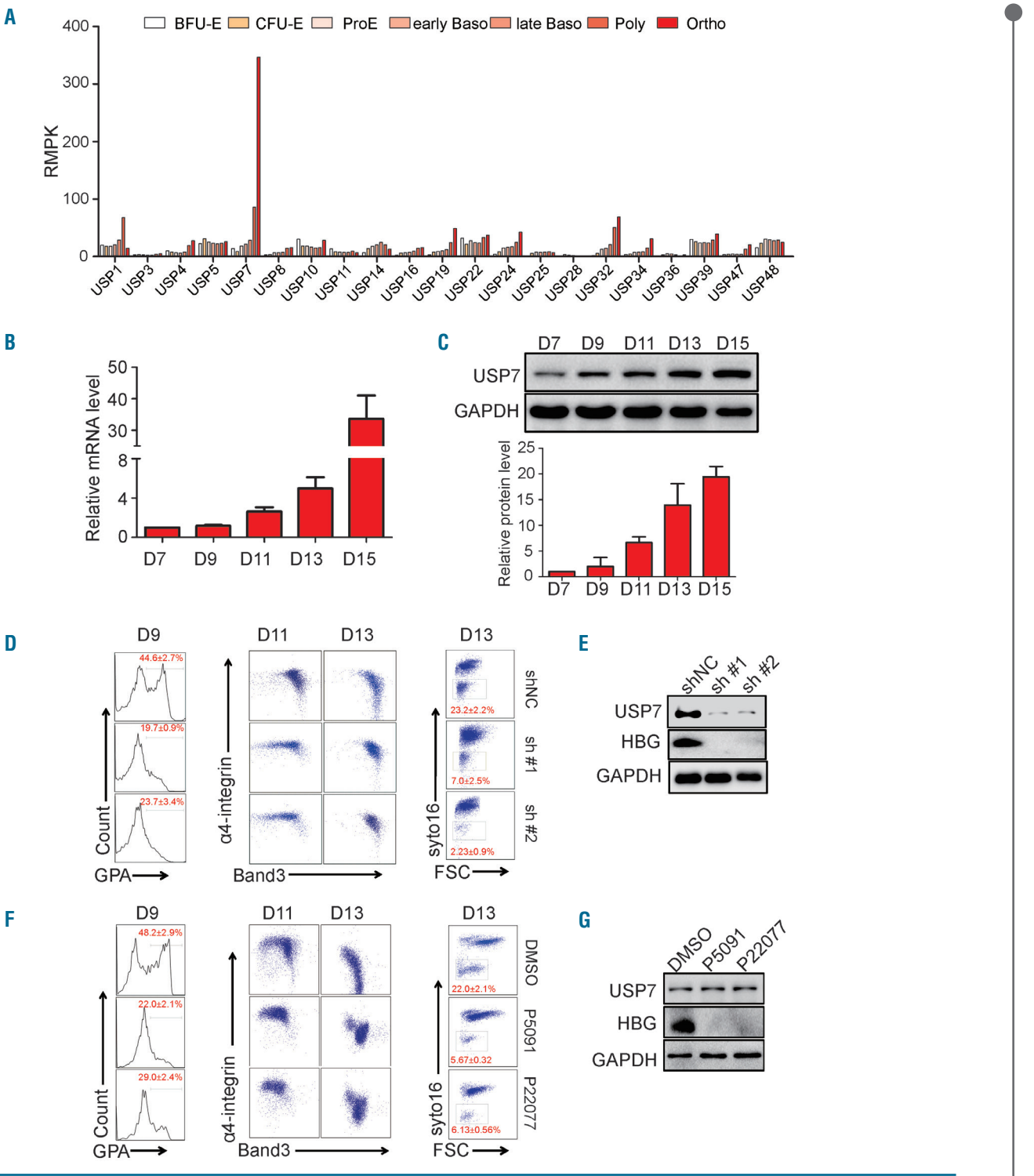

Figure 1. Deficiency of USP7 impairs human terminal erythroid differentiation. (A) RNA-sequencing data showing the expression of USP family members (fragments per kilobase of transcript per million) at each distinct stage of human terminal erythroid differentiation. (B) Real-time quantitative polymerase chain reaction results showing the expression of USP7 mRNA on the indicated days of human erythroid terminal differentiation. (C) Representative western blot analysis of the protein level of USP7 on the indicated days of human terminal erythroid differentiation. Quantitative analysis of data from three independent experiments of protein expression levels are shown (lower panel). (D) Left, representative profiles of flow cytometry-based detection of glycophorin A (GPA) expression in erythroblasts infected with the control or USP7 shRNA on day 9. Middle, representative profiles of Band3/α4-integrin levels of GPA-positive erythroblasts transfected with the control or USP7 shRNA lentviruses on days 11 and 13. Right, representative profiles of flow cytometry-based detection of enucleation by syto16 staining on day 13. Quantification from three independent experiments is indicated. (E) Representative western blot showing the protein level of γ-hemoglobin (HBG) and USP7 in erythroblasts transfected with either the control or USP7 shRNA on day 9. (F) Left, representative profiles of flow cytometry-based detection of GPA expression in erythroblasts treated with dimethylsulfoxide (DMSO) or USP7 inhibitor P5091 (5 μM) or P22077 (7.5 μM) on day 9. Middle, representative profiles of Band3/α4-integrin levels of GPA-positive erythroblasts treated with DMSO or USP7 inhibitor P5091 or P22077 on day 11 and day 13. Right, representative profiles of flow cytometry-based detection of enucleation by syto16 staining on day 13. Quantification from three independent experiments is shown. (G) Representative western blot showing the protein level of HBG and USP7 in erythroblasts treated with DMSO or USP7 inhibitors (P5091 or P22077) on day 9. For all western blot analyses, GAPDH was used as the loading control. BFU-E: burst-forming unit – erythroid; CFU-E: colony-forming unit – erythroid; ProE: proerythroblast; early Baso: early basophilic erythroblast; late Baso: late basophilic erythroblast; Poly: polychromatic erythroblast; Ortho: orthochromatic erythroblast; D: day; GPA: glycophorin A; FSC: forward scatter; DMSO: dimethylsulfoxide.

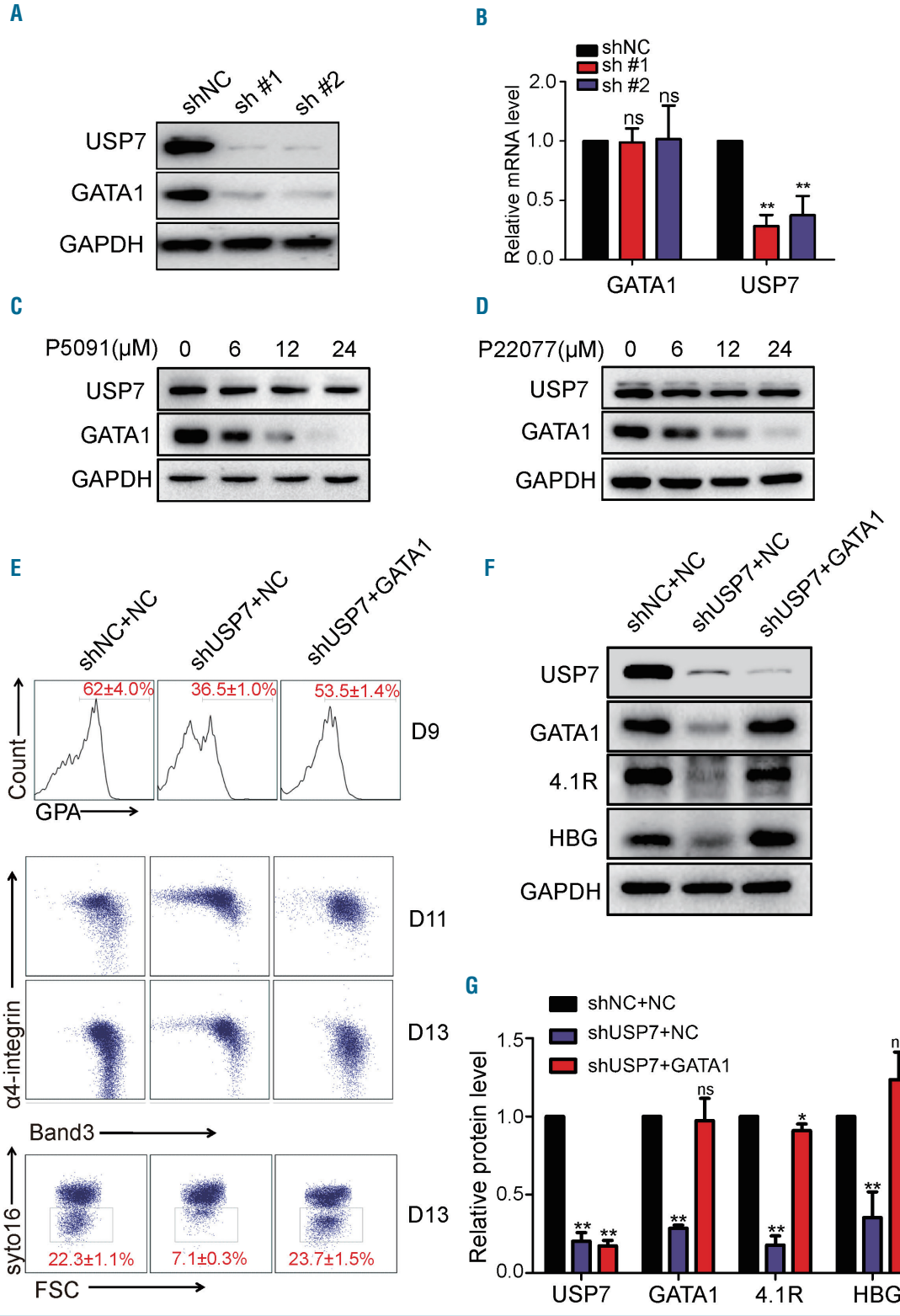

Figure 2. USP7 regulates erythroid differentiation by affecting GATA1 protein levels. (A) Representative western blot analysis of erythroblasts transfected with control or USP7 short hairpin (sh)RNA on day 9. GAPDH was used as a loading control. (B) Bar graph presentation of USP7 and GATA1 mRNA levels as determined by real-time quantitative polymerase chain reaction analysis of erythroblasts transfected with the negative control shRNA or USP7 shRNA #1 or #2, which were harvested on day 9. (C) Representative western blot analysis of the erythroblasts after treatment with indicated doses of P5091 on day 9. GAPDH was used as the loading control. (D) Representative western blot analysis of the erythroblasts after treatment with different doses of P22077 on day 9. GAPDH was used as the loading control. (E) The upper panel shows the representative profiles of flow cytometry analysis of GPA expression on day 9 in erythroblasts transfected with control shRNA and NC (HMD empty vector), USP7 shRNA and NC, or USP7 shRNA and GATA1 (GATA1-HMD). The middle panel shows the representative profiles of flow cytometry analysis of Band3/α4-integrin expression of the GPA-positive cells in the same groups on days 11 and 13. The bottom panel shows the representative profiles of flow cytometry-based detection of enucleation by syto16 staining on day 13. Quantification from three independent experiments is indicated. (F) Representative western blot analysis of erythroblasts transfected with control shRNA and NC (HMD empty vector), USP7 shRNA and NC, or USP7 shRNA and GATA1 (GATA1-HMD) on day 9. (G) Bar diagram presenting the quantitative analysis of protein expression data from (F). The plot was generated from three independent experiments and shows the means ± standard deviations (**P<0.01, *P<0.05).

shown in Figure 4A, endogenous USP7 was immunoprecipitated by anti-GATA1 antibodies but not by control IgG. Conversely, GATA1 was immunoprecipitated by anti-USP7 antibodies but not by control IgG (Figure 4B). To determine whether USP7 and GATA1 interact directly with each other, we performed GST pull-down assays under a cell-free condition by using purified recombinant GST-GATA1 and Flag-USP7 proteins. As shown in Figure 4C, the purified GST-GATA1 but not the control GST was able to pull down USP7. Furthermore, we mapped the detailed binding region of GATA1 and USP7 in HEK293T cells, a non-erythroblast environment.[38,42,43] Truncation mutants of GFP-USP7 and Flag-GATA1 were co-transfected into HEK293T cells and co-immunoprecipitation analyses revealed that the N-terminal TRAF-like domain (1-208) of USP7 was critical for the interaction between GATA1 and USP7 (Figure 4D). Conversely, mapping the region of GATA1 required for USP7 binding showed that the DNA binding domain (200-290) of GATA1 was responsible for its interaction with USP7 (Figure 4E). Collectively, these results show that USP7 interacts with GATA1 directly.

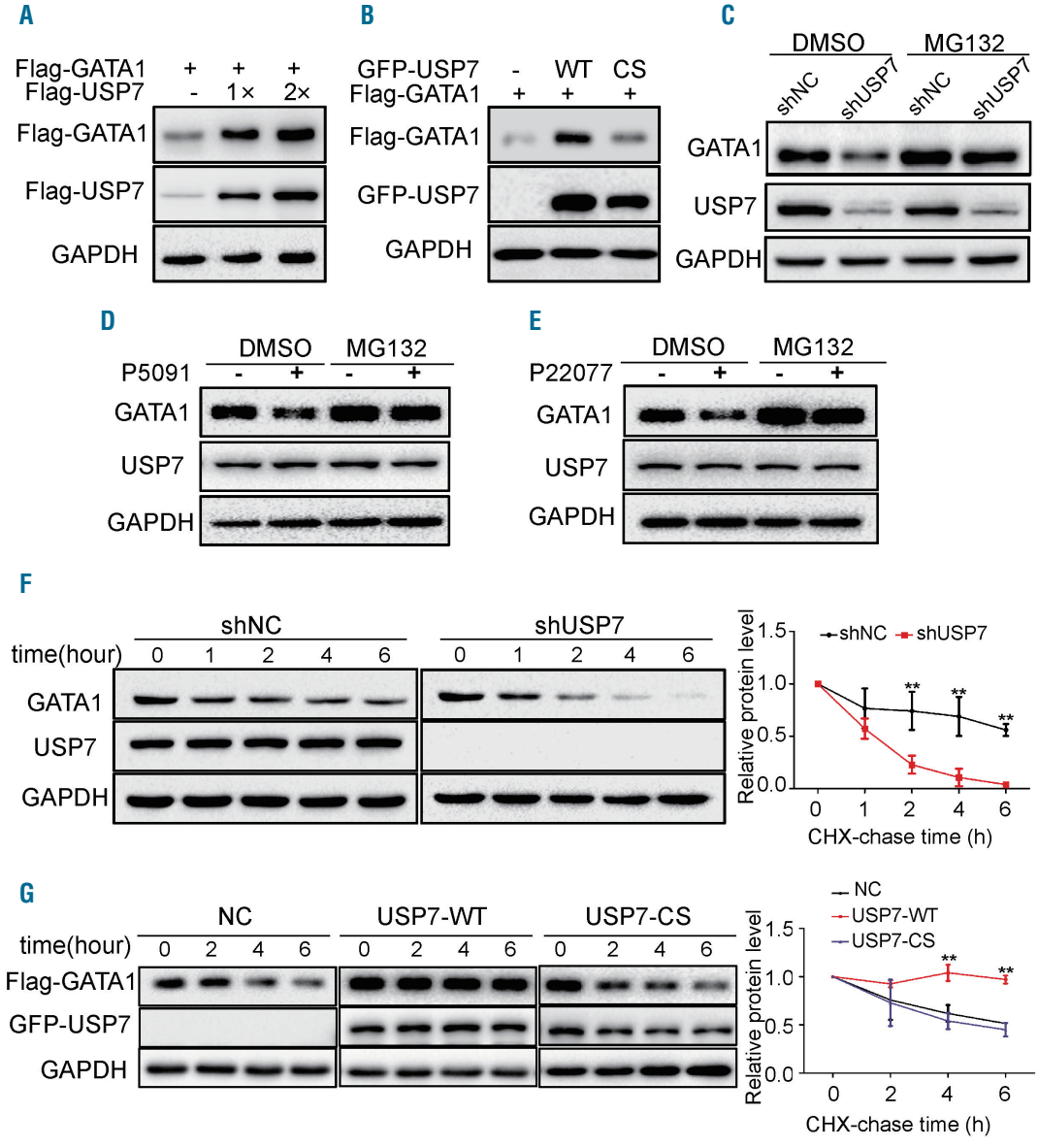

Figure 3. USP7 regulates the stability of GATA1. (A) Representative western blot analysis of HEK293T cells transfected with a GATA1-expressing plasmid and either an empty vector or increasing amounts of a GFP-USP7-expressing vector. (B) Representative western blot analysis of HEK293T cells that were transfected with a construct expressing Flag-GATA1 and an empty vector (-), a construct expressing wildtype USP7 (USP7-WT) or one expressing the USP7 (Cys223Ser) mutant (USP7-CS). (C) Representative western blot analysis of erythroblasts that were transfected with control (shNC) or USP7 shRNA treated with or without the proteasome inhibitor MG132 (20 μM, 6 h). (D) Representative western blot analysis of erythroblasts that were treated for 6 h with dimethylsulfoxide (DMSO) (-) and/or 15 μM P5091 (+) or with 15 μM P5091 and 20 μM MG132. (E) Representative western blot for the expression of GATA1 in erythroblasts treated for 6 h with DMSO (-) and/or 15 μM P22077 (+) or with 15 μM P22077 and 20 μM MG132 on day 9. (F) Erythroblasts transfected with control or USP7 shRNA were treated with cycloheximide (CHX) (150 μg/mL), and collected at the indicated times for western blot. Results are shown as mean ± standard deviation (SD) (**P<0.01). (G) Representative western blot analysis of HEK293T cells that were transfected with a vector expressing Flag-GATA1 and an empty vector (NC), one expressing GFP-USP7-WT or one expressing GFP-USP7-CS, after treatment with CHX (150 μg/mL) for the indicated amounts of time. Results are shown as mean ± standard deviation (**P<0.01). For all western blot analyses, GAPDH was used as the loading control.

USP7 stabilizes GATA1 protein through K48 deubiquitylation

Since USP7 is a deubiquitylase, it is reasonable to speculate that USP7 regulates the stability of GATA1 via deubiquitylation. To test this hypothesis, we investigated the effect of USP7 on the poly-ubiquitylation of GATA1. As expected, knockdown of USP7 resulted in a significant increase in the poly-ubiquitylation of GATA1 (Figure 5A). In contrast, ectopic expression of USP7-WT but not the catalytic inactive mutant USP7-CS reduced the level of poly-ubiquitylation of GATA1 (Figure 5B and *Online Supplementary Figure S6A*). Moreover, USP7-mediated decrease of GATA1 poly-ubiquitylation was blocked by the USP7 inhibitors P5091 and P22077 (Figure 5C and *Online Supplementary Figure S6B*), demonstrating that the enzymatic activity of USP7 is essential for the USP7-mediated deubiquitylation of GATA1. To verify that GATA1 is a direct substrate of USP7, the purified USP7 protein was incubated with ubiquitylated GATA1 in a cell-free system. As shown in Figure 5D, GATA1 poly-ubiquitylation was decreased in the presence USP7, indicating that USP7 deubiquitylates GATA1 directly. To determine which poly-ubiquitin chain on GATA1 is removed by USP7, we employed a series of ubiquitin mutants that contain only

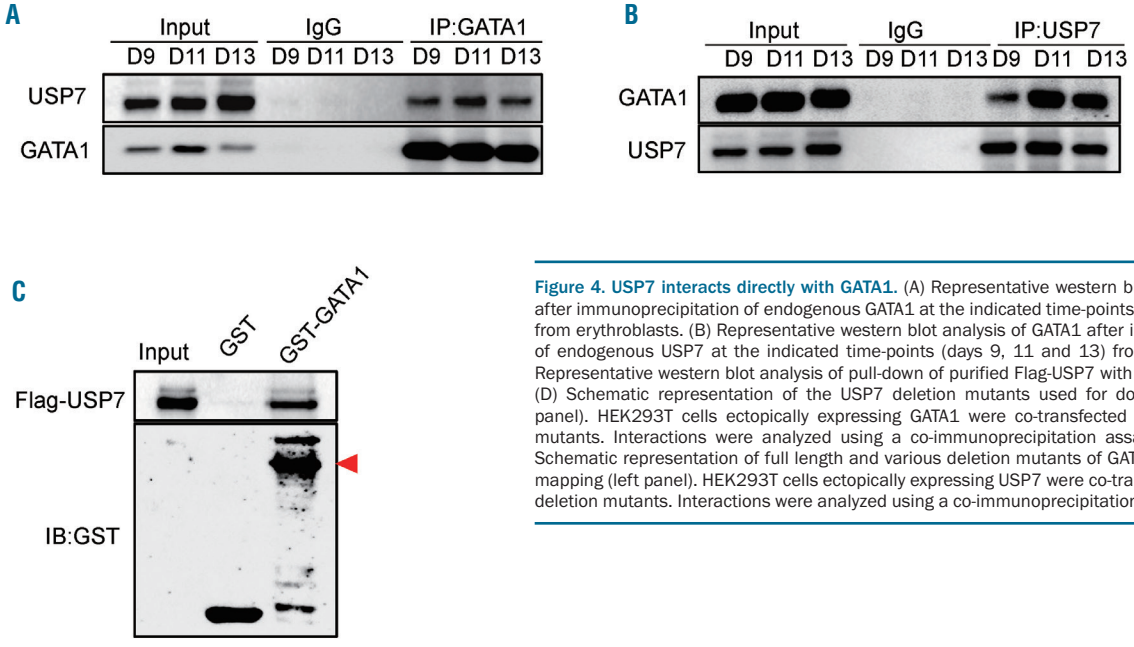

Figure 4. USP7 interacts directly with GATA1. (A) Representative western blot analysis of USP7 after immunoprecipitation of endogenous GATA1 at the indicated time-points (days 9, 11 and 13) from erythroblasts. (B) Representative western blot analysis of GATA1 after immunoprecipitation of endogenous USP7 at the indicated time-points (days 9, 11 and 13) from erythroblasts. (C) Representative western blot analysis of pull-down of purified Flag-USP7 with purified GST-GATA1. (D) Schematic representation of the USP7 deletion mutants used for domain mapping (left panel). HEK293T cells ectopically expressing GATA1 were co-transfected with USP7 deletion mutants. Interactions were analyzed using a co-immunoprecipitation assay (right panel). (E) Schematic representation of full length and various deletion mutants of GATA1 used for domain mapping (left panel). HEK293T cells ectopically expressing USP7 were co-transfected with GATA1 deletion mutants. Interactions were analyzed using a co-immunoprecipitation assay (right panel).

one lysine (K6, K11, K27, K29, K33, K48 or K63). As shown in *Online Supplementary Figure S7*, USP7 significantly decreased only the K48-linked poly-ubiquitin chain but not any other lysine isopeptide-linked poly-ubiquitin chains (K6, K11, K27, K29, K33 or K63). To further confirm that K48-linked poly-ubiquitin is removed by USP7, we replaced K48 or K63 lysine by arginine (R) and, as shown in Figure 5E, mutation of K48 but not K63 significantly impaired USP7-mediated deubiquitylation. Collectively, these results confirm that USP7 stabilizes GATA1 by removing the K48-linked poly-ubiquitin.

Discussion

Erythropoiesis is a process by which hematopoietic stem cells proliferate and differentiate to eventually produce mature red blood cells. Many cellular and molecular changes occur during this process. Morphological changes include a progressive decrease in cell size, increase in chromatin condensation and enucleation. At the molecular level, high-throughput analyses revealed dramatic changes in both the transcriptome and the proteome.[2,44] In contrast to extensive studies on transcriptional networks, very little

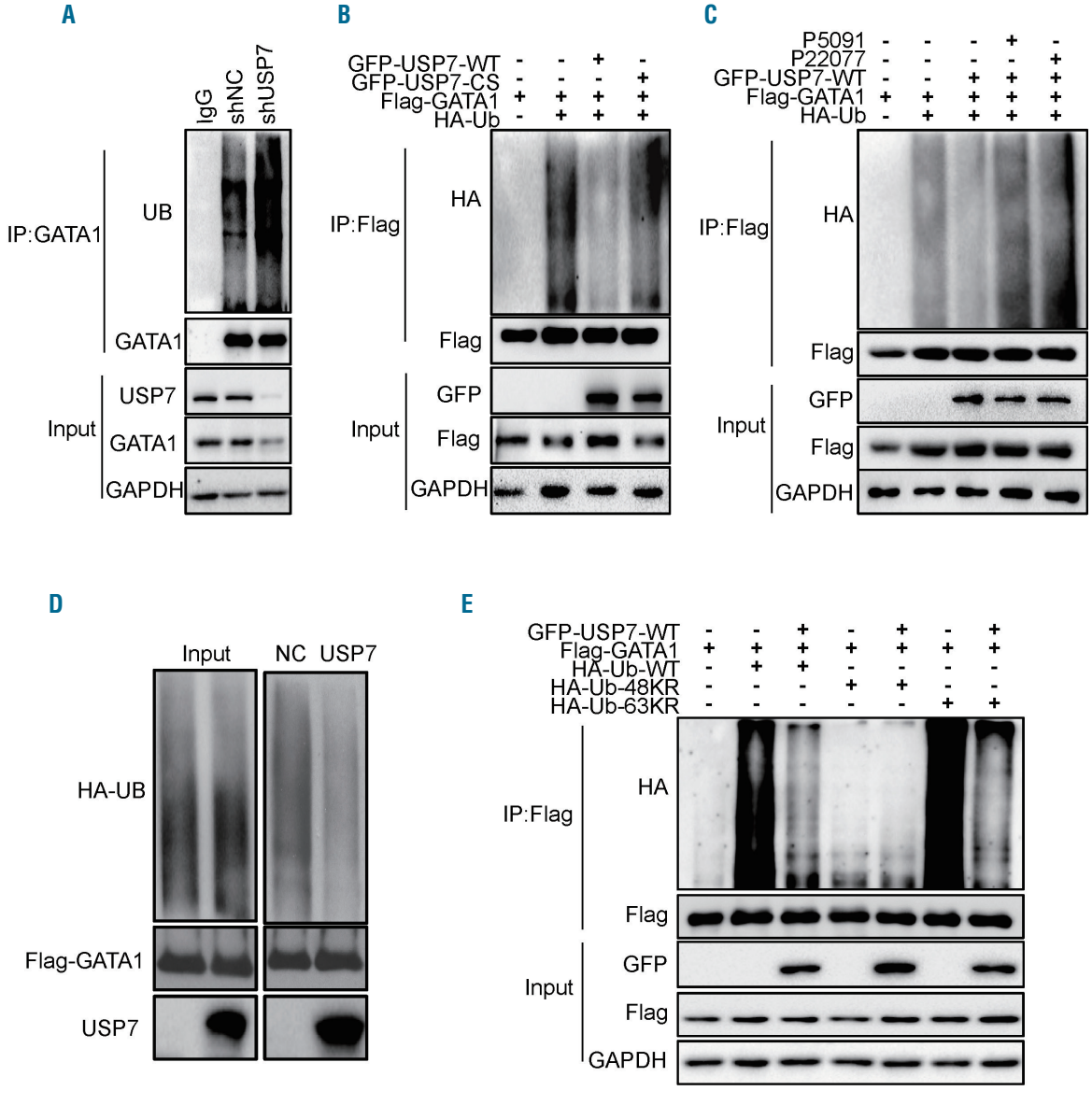

Figure 5. USP7 stabilizes GATA1 protein through deubiquitination. (A) Erythroblasts at day 7 transfected with control or USP7 shRNA (#1) lentivirus. GATA1 was immunoprecipitated with anti-GATA1 anyibody and immunoblotted with anti-ubiquitin on day 9. (B) Representative western blot analysis of ubiquitin after incubation of anti-Flag-coated beads with lysates from HEK293T cells that were transfected with empty vectors (-) or those expressing Flag-GATA1 either alone or in combination with vectors expressing USP7-WT or USP7-CS, and HA-ubiquitin. (C) Representative western blot analysis for ubiquitin after anti-Flag immunoprecipitation of HEK293T cells ectopically expressing Flag-GATA1 either alone or in combination with USP7-WT. Cells expressing both Flag-GATA1 and USP7-WT were treated with 20 μM P5091 or P22077 for 8 h before being harvested. (D) Representative western blot for the cell-free deubiquitylation assay. Ubiquitylated GATA1 was incubated with bacterial-expressed and purified USP7-WT for 2 h at 37 °C, followed by western blot with anti-HA antibody (right panel). The left panel is the input. (E) Representative western blot analysis for ubiquitin after anti-Flag immunoprecipitation of HEK293T cells ectopically expressing Flag-GATA1 either alone or in combination with USP7-WT, and ubiquitin WT or mutant (K48R or K63R).

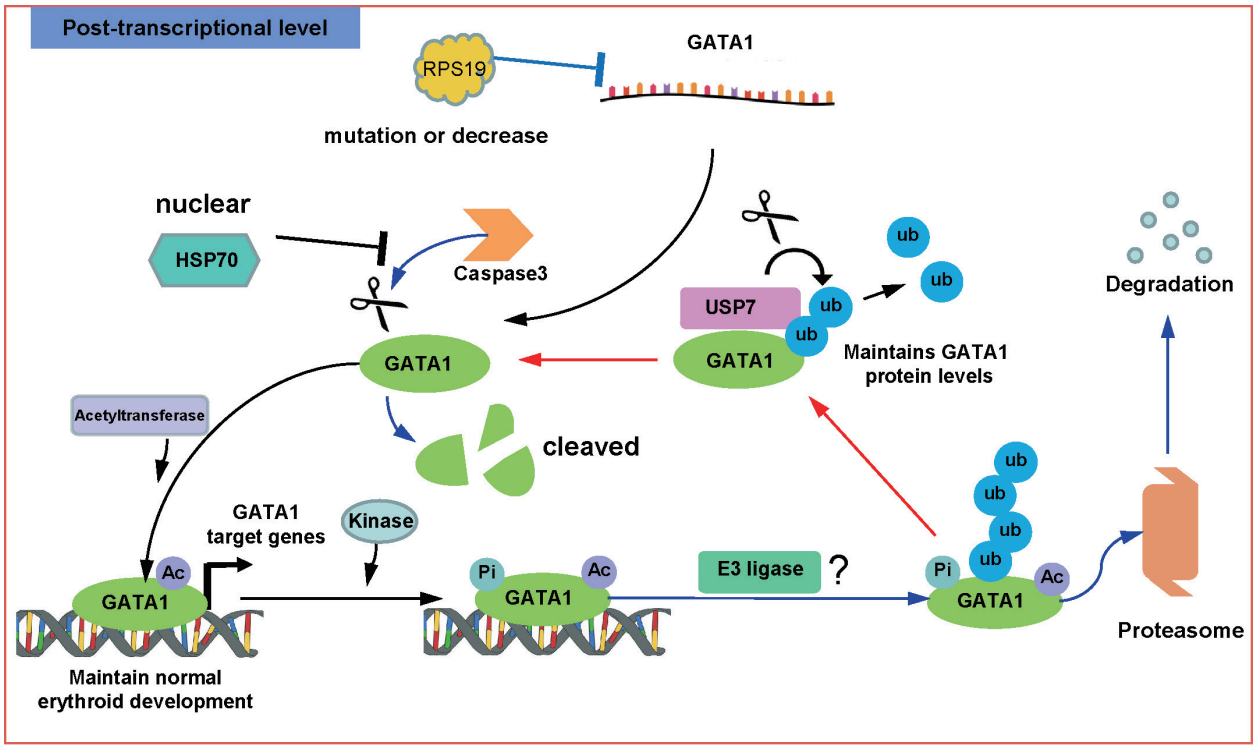

Figure 6. A schematic model of post-transcriptional regulation of GATA1. Post-transcriptional regulation of GATA1 includes the translational and post-translational levels. The translational level of GATA1 is mainly controlled by RPS19. Decrease or mutation of RPS19 results in reduced translation of GATA1. At the post-translational level, the nuclear HSP70 protects GATA1 from caspase 3 cleavage. In addition, acetylation and phosphorylation of GATA1 cooperate as the signal for ubiquitylation of GATA1 to degradation. USP7 interacts directly with GATA1 and maintains stability of GATA1 by removing the poly-ubiquitylation.

is known about the mechanisms by which the proteome is remodeled. Previous studies demonstrated that the balance between ubiquitination and deubiquitination plays important roles in homeostasis of cellular protein pools.[45] In the present study, we documented, for the first time, the role of a deubiquitylase, USP7, in erythroid differentiation. We further documented that the mechanism is stabilization of the erythropoiesis master regulator GATA1.

USP7 is a member of a deubiquitinating enzyme family that contains more than 90 genes.[46] USP7 expression is ubiquitous in different cell types: mice with knocked out USP7, which are homozygous for a null allele, show embryonic growth arrest and die between embryonic day 6.5 and 7.5.[47] Furthermore, conditional knockout mice showed that USP7 is required for development of the central nervous system and functional regulatory T cells.[48,49] We expect that deletion of USP7 in erythroid cells *in vivo* will led to altered erythropoiesis. We are in the process of generating such conditional knockout mice to define the function of USP7 *in vivo*. Besides USP7, many other deubiquitinating enzymes are also expressed in erythroid cells, although at lower levels than USP7. It will be interesting in future studies to identify the functional roles of other deubiquitylases during erythroid differentiation. Since each deubiquitinating enzyme has different substrate specificity,[47] it is likely that members of the deubiquitinating enzyme family may regulate different aspects of erythropoiesis via different mechanisms.

As the key transcriptional factor for erythropoiesis, GATA1 protein expression is tightly regulated at several levels. These include translational control by ribosome levels,[26,41] stabilization by HSP70 from caspase 3 cleavage,[27,38-40] and degradation by acetylation and phosphorylation-associated ubiquitination.[24] Here we show that knockdown of USP7 by shRNA or inhibition of USP7 activity by USP7-specific inhibitors led to dramatic decreases in GATA1 protein levels. Interestingly, USP7 knockdown or inhibition had no effects on the protein levels of RPS19 or HSP70, strongly suggesting that USP7 affects the stability of GATA1 in a direct manner. This notion is supported by our findings that USP7 binds GATA1 directly and stabilizes GATA1 by de-ubiquitination. Specifically, USP7 catalyzes the removal of K48-linked poly-ubiquitin which is a proteasome degradation signal for proteins. Based on our findings and that of others, we propose a schematic model for post-transcriptional regulation of GATA1 (Figure 6). GATA1 functions in the context of multi-protein complexes that include interacting proteins such as FOG1.[50-53] Although USP7 knockdown or inhibition did not affect the level of FOG1 or NuRD complex (*Online Supplementary Figure S8*), we cannot exclude the possibility that these important GATA1 cofactors or modifications can modulate GATA1-USP7 interactions during erythroid development. Further study is therefore warranted to investigate whether USP7 binds other cofactors such as FOG1 and/or different modifications of GATA1.

Altered expression of GATA1 has been reported in myelodysplastic syndromes[39,54] and β-thalassemia.[40] However, the mechanisms of the altered GATA1 expression remains to be fully defined. It has been reported that USP7 is associated with several human diseases.[55-57] Given the close relationship between USP7 and GATA1, demon-

strated in our present study, it will be interesting in future studies to examine whether the altered expression of GATA1 may be associated with changes in USP7 expression in certain blood disorders.

In summary, we have uncovered a previously unrecognized role for a deubiquitylase, USP7, in human terminal erythroid differentiation and have identified USP7 as a deubiquitylase of GATA1. Our findings provide new and novel insights into mechanisms of regulating human erythropoiesis.

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