SUPPLEMENTARY APPENDIX

Deubiquitylase USP7 regulates human terminal erythroid differentiation by stabilizing GATA1

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Supplementary Methods

Reagents and antibodies. Antibodies used in this study include: USP7 (A300-033A) from Bethyl Laboratories Inc. (TX, USA); HSP70 (ab2787), KLF1 (ab175372) from Abcam (MA, USA); GATA1 N1 (sc-266) (for WB), LSD1 (sc-271720), SOX6 (sc-393314), IKAROS (sc-398265), HBG (sc-21756), RPS19 (sc-100836), RCC1(sc-374325) and GAPDH (FL-335) from Santa Cruz Biotechnology (TX, USA); HA-tag (C29F4) and GATA1 (D52H6) from Cell Signaling Technology (MA, USA) (for IP, IF and WB),FLAG-tag (M185-3L) from Medical & Biological Laboratories (Nagoya, Japan).

Western blot analysis. For western blot analysis, cells were lysed in cold RIPA buffer (Beyotime, China) in the presence of 1×protease inhibitor cocktail and 1×PhosStop (Roche, Isere, France). Protein concentration was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, MA, USA). Equal amounts of proteins were loaded on to polyacrylamide gels. Cytoplasmic and nuclear proteins were isolated using a nuclear/cytoplasm protein extraction kit (Thermo FisherScientific, USA), according to the manufacturer's instructions.

Lentivirus Infection: Lentiviruses were packaged according to the manufacturer's protocol (Invitrogen, MA, USA), and viral titers were measured using HEK293T cells. A total of 30 million lentiviral particles were incubated with polybrene and added to 0.5 million $CD34^+$ cells on day 3 or 4. Puromycin (1 μg/ml) was used for selection of transduced cells.

RNA isolation and quantitative real-time PCR. Total RNA was isolated from cultured human erythroid cells using TRIzol (Invitrogen) according to the standard protocol. 1 μg total RNA was reverse-transcribed using the HiScript II One Step RT-PCR Kit (Vazyme Biotech Co., Ltd., Nanjing, China). Quantitative PCR was performed using a qPCR system (Eppendorf, Hamburg, Germany). All mRNA expression levels were normalized to GAPDH and calculated using the $2^{-\triangle\triangle CT}$ method.

Immunofluorescence. For immunofluorescence, cells were fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100 (PBS) and then blocked with bovine serum albumin.

Slides were incubated with the indicated primary antibodies overnight, followed by incubation with Cy3-conjugated secondary antibody for 1h at 25°C. The nuclei were stained with DAPI (Sigma), and images were visualized using a confocal microscope (Zeiss LSM 510, Germany).

Immunoprecipitation. For immunoprecipitation of ectopically expressed Flag-tagged proteins, cells were lysed in IP buffer (P0013, Beyotime, China) and freshly supplemented protease inhibitor (Roche, France). Cell extracts were incubated with the monoclonal M2/Flag-agarose beads (Selleck, USA) at 4°C overnight or 25°C for 2 h. After three washes with lysis buffer, bound proteins were eluted by boiling or with Flag peptide (Sigma, USA) in IP buffer for 2 h at 4°C. For endogenous immunoprecipitation assays, cells were lysed in cold IP buffer (P0013, Beyotime, China) supplemented with protease-inhibitor cocktail (Roche, France), 5-10% of the cell extract was saved as the input, and the rest was incubated with primary antibody at 4°C overnight and then with protein A/G agarose beads (Santa Cruz, USA) for 2 h at 4°C. After three washes with the IP buffer, bound proteins were eluted by boiling with 2×SDS loading buffer.

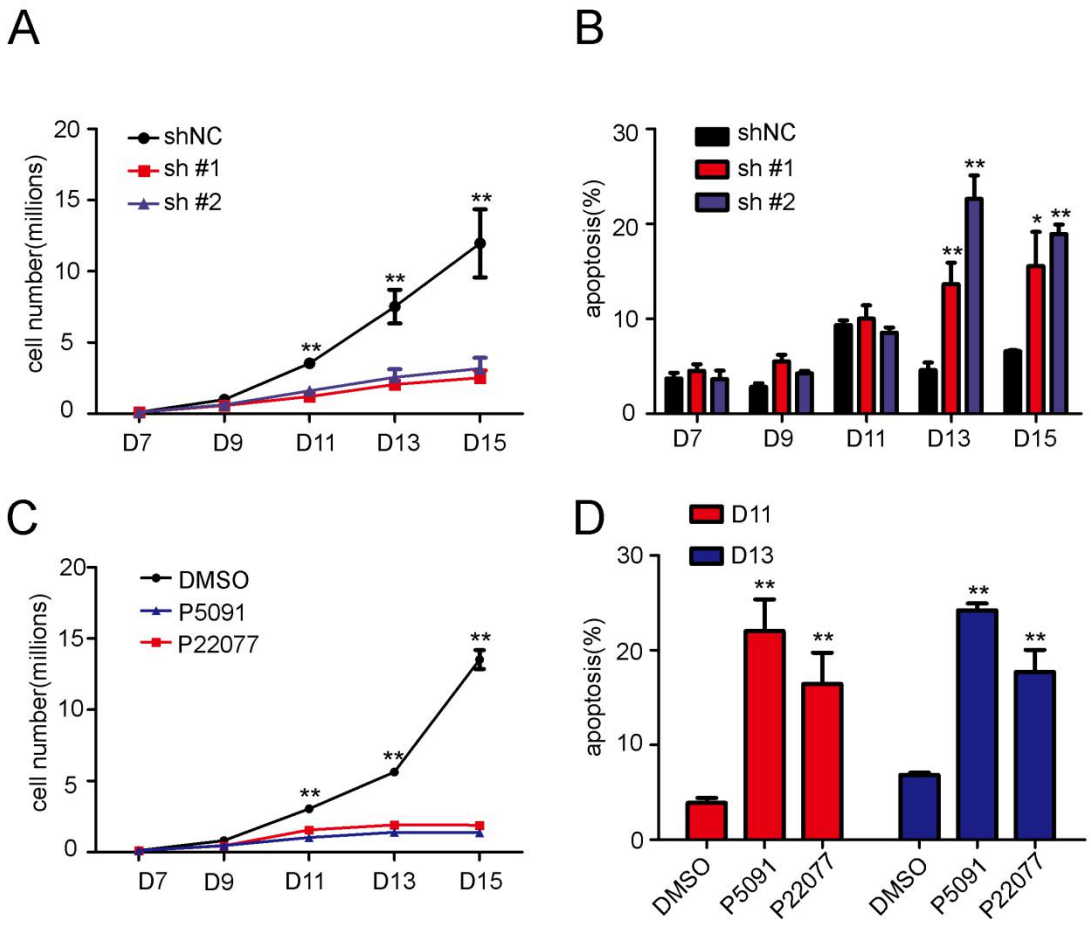
Glutathione-S-transferase (GST) pull-down. The protein products from the pCMV-Flag-USP7 construct were purified under high stringent conditions (10 MmTris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, and 1% NP-40) and bound proteins were eluted with Flag peptide (Sigma, USA). Bacterial-expressed GST or GST-GATA1 bound to glutathione-Sepharose 4B beads (GE Healthcare, IL, USA) was incubated with purified Flag-USP7 for 2 h at 4°C. Following the washing ofthe beads with IP buffer, the bound proteins were eluted by boiling with 2×SDS loading buffer, followed by western blot.

***In vivo* ubiquitylation and deubiquitylation assay.** Cells were subsequently lysed in lysis buffer (2% SDS, 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, and 1% NP-40) at 95°C for 10 min followed by addition of nine volumes of dilution buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, and 1% NP-40). Sonicated samples were incubated with anti-Flag antibody and immunoprecipitation was performed as describe above. For the deubiquitination assay with USP7 inhibitor, the USP7 inhibitors or DMSO were added 8 h

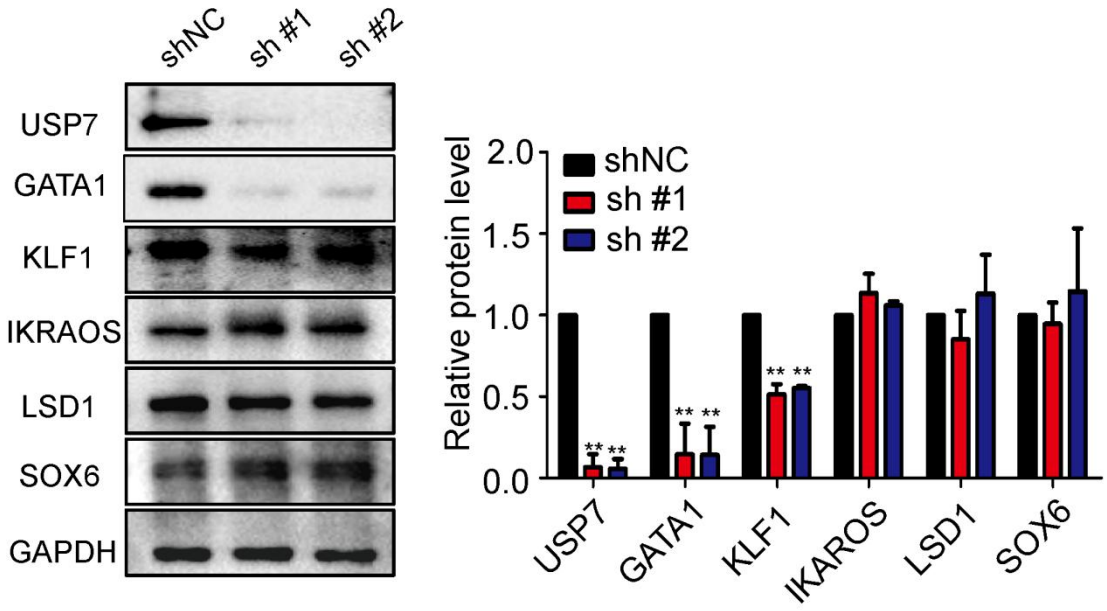
before harvesting of the samples. The proteins were released from the beads by boiling in 2×SDS loading buffer and analyzed by immunoblotting with anti-HA or anit-ubiquitin antibody.

***In vitro* deubiquitylation assay.** The ubiquitin linked GATA1 protein products were purified under highly stringent conditions (10 Mm Tris-HCL, pH 8.0, 1 M NaCl, 1 mM EDTA, and 1% NP-40). The reaction was carried out at 37°C for 2 h in 100 μl reaction buffer (20 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$, 50 mM NaCl, 1 mM 2-mercaptoethanol, 10% glycerol) containing: 30 μl surry beads of HA-UB-GATA1 and 20 nM USP7 (UBPbio, USA). The beads were washed thrice with IP buffer. The proteins bound to beads were released by boiling in 50 μl of 2×SDS loading buffer for 10 min, followed by western blot analysis.

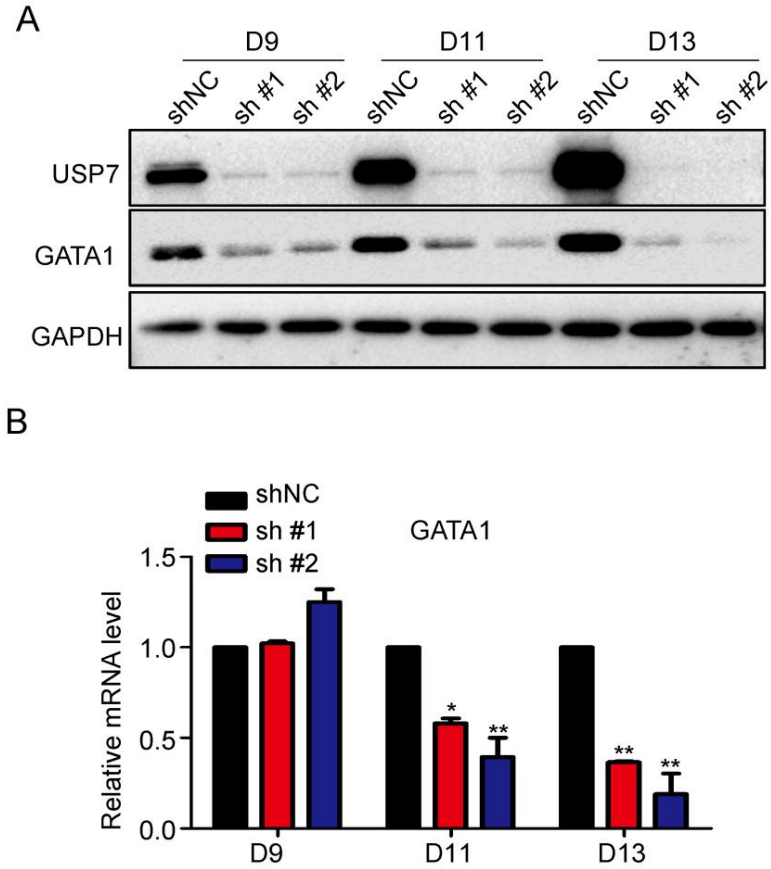
Supplementary figures and figure legends



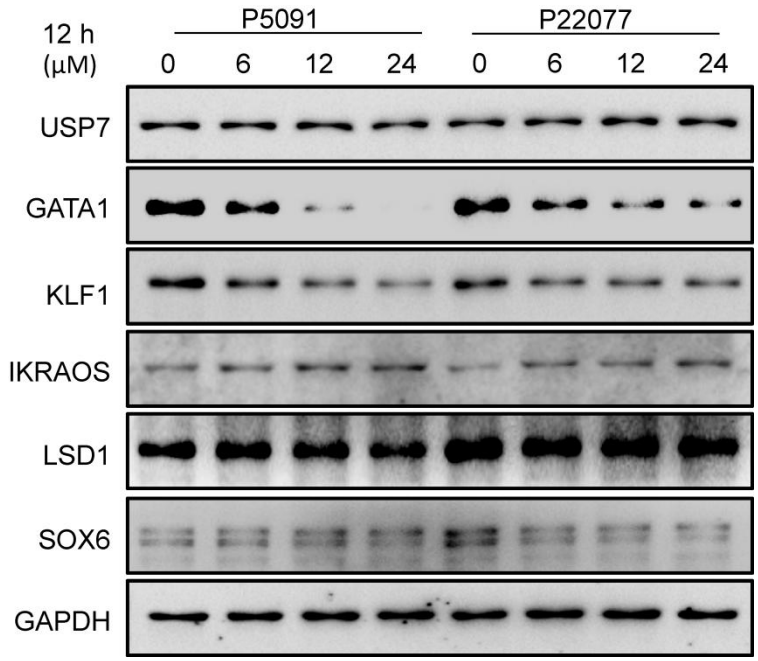
Sup Fig 1. USP7 knockdown or inhibition inhibit proliferation of erythroblast. (A) Erythroid cell growth curves determined by manual cell counting of erythroblasts infected with USP7 shRNAs. Results are shown as mean±SD (**, p<0.01). **(B)** Bar graph presentation of apoptosis of erythroblasts after infected USP7 shRNAs. Results are shown as mean±SD (**, p<0.01). **(C)** Erythroid cell growth curves determined by manual cell counting of erythroblasts treated with either DMSO or the USP7 inhibitors P5091 (5 μM) and P22077 (7.5 μM). Results are shown as mean±SD (**, p<0.01). **(D)** Bar graph presentation of apoptosis of erythroblasts treated with USP7 inhibitors. Results are shown as mean±SD (**, p<0.01).



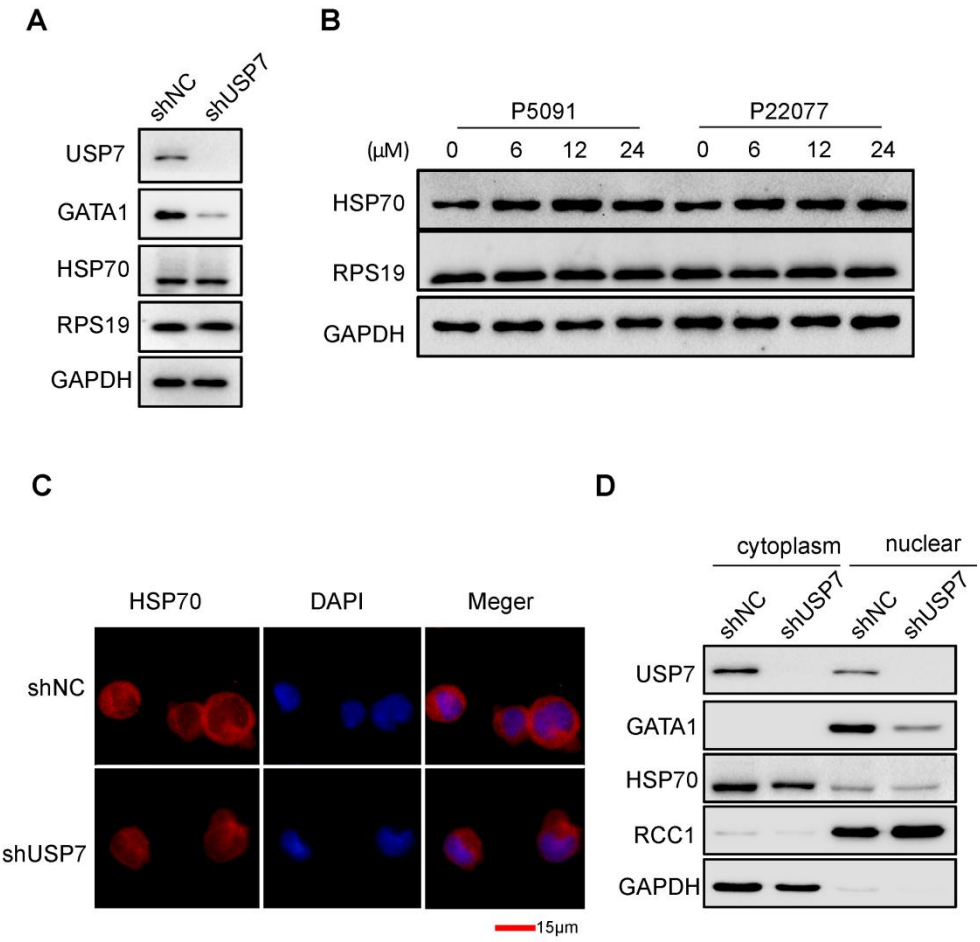
Sup Fig 2. The expression of several transcription factors following UPS7 knockdown. Whole-cell extracts obtained on day 9 from erythroblasts infected with the control or USP7 shRNA were subjected to western blot to detect the levels of the indicated proteins. GAPDH was used as a loading control. The right panel shows the quantitative analysis of protein expression data from three independent experiments. Data are presented as the means±SD of three independent experiments (**, $p<0.01$).



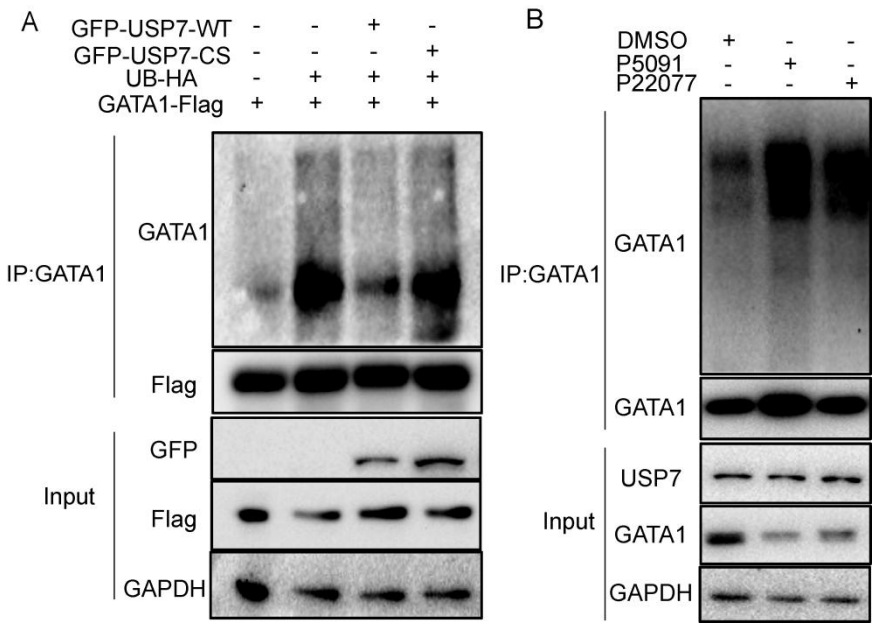
Sup Fig3.The protein and mRNA levels of GATA1 following USP7 knockdown. (A) Representative western blot of GATA1 and USP7 level in erythroblasts infected with the control or USP7 shRNA at indicated time points. **(B)** The bar graph showing the mRNA level of GATA1 at indicated time points. Data are presented as the means±SD of three independent experiments (*, $p<0.05$, **, $p<0.01$).



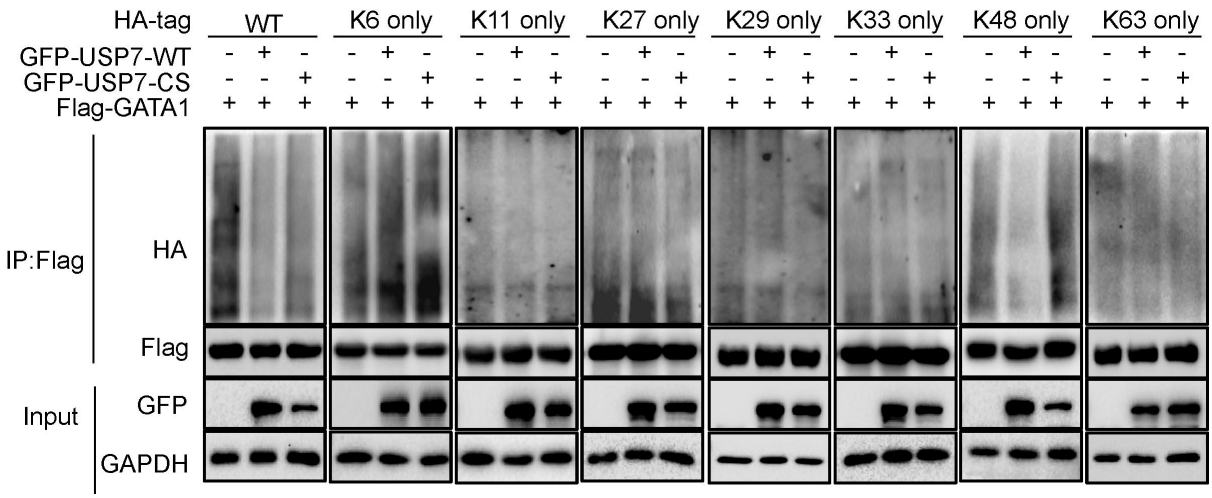
Sup Fig 4. The expression of several transcription factors following USP7 inhibition. Representative western blots analysis of erythroblasts that were treated with different doses of USP7 inhibitors (P5091 or P22077) 12 h, protein was extracted for immunoblot with USP7, GATA1, KLF1, LSD1, SOX6, IKROS and GAPDH on day 9.



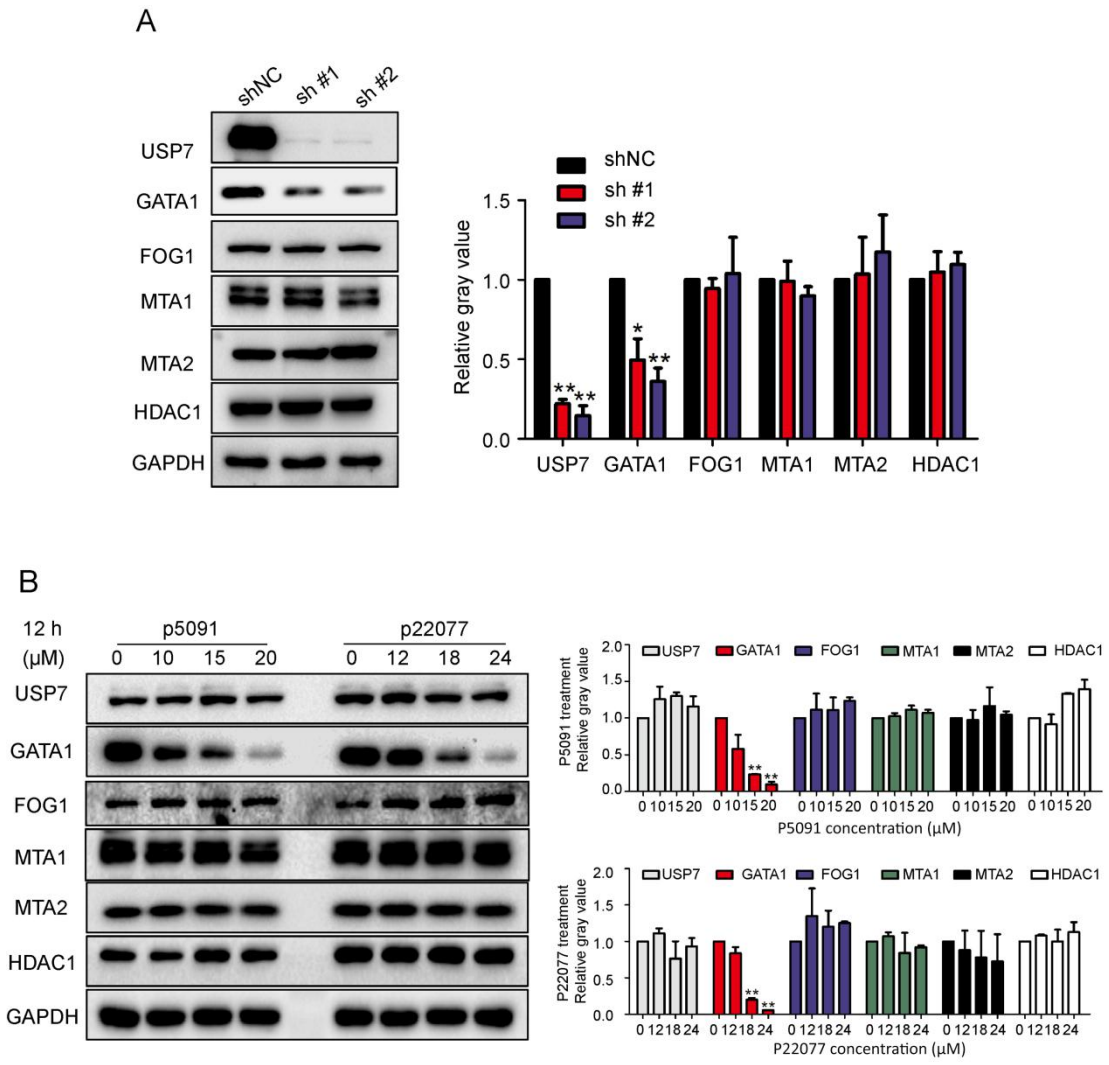
Sup Fig 5. The effects of USP7 knockdown or inhibition on HSP70 or PRS19. (A) Representative western blots of HSP70 and RPS19 levels in erythroblasts infected with the control or USP7 shRNA lentviruses for 48 h on day 9. **(B)** Representative western blots analysis of erythroblasts that were treated with different doses of USP7 inhibitors (P5091 or P22077). Protein was extracted for immunoblot withHSP70 and RPS19. **(C)** Representative image of immunofluorescence staining for HSP70 (Cy3-RED) in USP7 knockdown cells on day9; DAPI staining shows the nucleus. **(D)** Representative western blot analysis of erythroblasts that were infected with control or USP7 shRNA lentivirus, after the nuclear and cytoplasmic fraction protein was extracted for immunoblot with HSP70, USP7 and GATA1 on day9. GAPDH and RCC1were used as the loading control for cytoplasm and nuclear fraction, respectively.



Sup Fig 6. USP7 affects GATA1 ubiquitination. (A) Representative western blot analysis of ubiquitin by anti-GATA1 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. (B) Erythroblasts at day 9 treated with USP7 inhibitors (P5091 15 μM and P22077 15 μM) for 12 h. GATA1 was immunoprecipitated with anti-GATA1 antibody and immunoblotted with anti-GATA1.



Sup Fig 7. USP7 removes the K48-chain polyubiquitylation of GATA1. Representative western blots showing the ubiquitin chain type in HEK293T cells ectopically expressing USP7-WT, USP7-CS or vector combine with Flag-GATA1, and HA-tag ubiquitin WT or the mutants containing only one lysine (K6, K11, K27, K29, K33, K48 or K63). After transfection for 40 h, cells were treated with MG132 for 8 h before collection.



Sup Fig 8. The effects of USP7 knockdown or inhibition on the expression of FOG1 and NuRD complex. (A) Representative western blots showing USP7 knockdown by infected with the control or USP7 shRNA lentivirus, were subjected to western blot to detect the levels of the indicated proteins on day 9 from erythroblasts. GAPDH was used as a loading control. Data are presented as the means±SD of three independent experiments (*, $p<0.05$, **, $p<0.01$). (B) Representative western blots analysis of erythroblasts that were treated with different doses of USP7 inhibitors (P5091 or P22077) for 12 h, subjected to western blot to detect the levels of the indicated proteins. GAPDH was used as a loading control. Data are presented as the means±SD of three independent experiments (**, $p<0.01$).