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Mol Cancer Res. Author manuscript; available in PMC 2024 July 02.

Published in final edited form as:

Mol Cancer Res. 2024 January 02; 22(1): 94–103. doi:10.1158/1541-7786.MCR-23-0115.

PRL2 phosphatase promotes oncogenic KIT signaling in leukemia cells through modulating CBL phosphorylation

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Author Contributions

HC, YB, MK, ZYZ, and YL designed the research. HC, YB, MK, SX, SB, WC, SC, JM, FNM, CY, YY, KS, OS, JS, LR, YJ, HL, PJ, ZYZ and YL performed experiments and data analysis. JMC, HSB, LSL, JKA, EAE, MS, WT, HB, DTH, and LCP provided reagents to the study. HC, YB, ZYZ, and YL interpreted data, wrote, and edited the manuscript.

Competing of interests Statement

The authors declared no competing interests.

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Abstract

Receptor tyrosine kinase KIT is frequently activated in acute myeloid leukemia (AML). While high *PRL2* (*PTP4A2*) expression is correlated with activation of SCF/KIT signaling in AML, the underlying mechanisms are not fully understood. We discovered that inhibition of PRL2 significantly reduces the burden of oncogenic KIT-driven leukemia and extends leukemic mice survival. PRL2 enhances oncogenic KIT signaling in leukemia cells, promoting their proliferation and survival. We found that PRL2 dephosphorylates CBL at tyrosine 371 and inhibits its activity toward KIT, leading to decreased KIT ubiquitination and enhanced AKT and ERK signaling in leukemia cells.

Introduction

Aberrant activation of receptor tyrosine kinase signaling, such as oncogenic FLT3 and KIT signaling, promotes proliferation and survival of acute myeloid leukemia (AML) cells (1–4). Activating mutations in *KIT* are confined to either the extracellular (exon 8 mutations) or the PTK2 domain (D816 mutations). Both classes of *KIT* mutations have been identified predominantly in core binding factor (CBF) leukemia (5–6). There are several small molecule inhibitors, including dasatinib, midostaurin, and avapritinib, can target *KIT* mutations. However, these drugs have not been FDA approved for treating leukemia patients with *KIT* mutations (5–6).

E3 ubiquitin ligase CBL is responsible for the ubiquitination of both FLT3 and KIT in normal hematopoietic and leukemia cells (7). CBL is a tumor suppressor in hematological malignancies and Cbl deficiency drives myeloproliferative neoplasms (MPN) development in mice (8–9). Both somatic and germline *CBL* mutations are frequently found in myeloid malignancies (10–14). In response to cytokine stimulation, CBL is phosphorylated and activated (7). However, how CBL phosphorylation is regulated in leukemia cells is fully understood.

The **p**hosphatase of **r**egenerating **l**iver (PRL) family of phosphatases, also known as protein tyrosine phosphatase 4A (PTP4A), are a group of proteins that have been shown to have oncogenic potentials in human cancers, including hematological malignancies (15–16). PRL2, encoded by *PTP4A2* gene, is highly expressed in some human AML subtypes (17). PRL2 enhances KIT signaling in normal hematopoietic stem and progenitor cells (HSPCs) as well as leukemia cells (17–19); however, the underlying mechanism is not fully understood.

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Material and Methods

Mice

B6.SJL and NSG mice were purchased from the Jackson Laboratories. We maintained *Prl2*$^{+/+}$ and *Prl2*$^{-/-}$ mice in animal facilities of Indiana University School of Medicine and Northwestern University Feinberg School of Medicine. All experimental procedures were approved by Indiana University School of Medicine and Northwestern University Feinberg School of Medicine Institutional Animal Care and Use Committee (IACUC).

AML cell lines

We obtained human AML cell lines from ATCC and authenticated all cell lines by SRT profiling. We determined that all cell lines were mycoplasma negative.

Primary AML cells

Collection and processing of primary AML samples were performed as described before (20). We conducted studies involving human patient samples in accordance with the Declaration of Helsinki. Chongqing University Three Gorges Hospital and Northwestern University institutional review board approved these studies.

Proliferation assays

For proliferation assay using primary AML patient samples, 1×10^5 cells were treated with or without gradient concentration of PRLi in 96 well plate. After 24 hours, cell proliferation assays were performed as described previously (20). Experiments were performed on SpectraMax iD3.

Cell cycle analysis

We treated primary AML cells with DMSO or PRLi for 24 hours and then performed cell cycle analysis as described previously (20). Experiments were performed on DxFLEX flow cytometers (Beckman) and analyzed by using the FlowJo_v10 software.

Apoptosis assays

We treated primary AML cells with DMSO or PRLi for 24 hours and then performed apoptosis assays as described previously (20).

Transplantation assays

To determine the role of PRL2 in KitD814V induced leukemia, we transplanted 1×10^6 KitD814V transduced *Prl2*$^{+/+}$ and *Prl2*$^{-/-}$ Lin$^-$ cells into lethally irradiated recipient mice (B6.SJL) via tail vein injection.

To determine the role of PRL2 in leukemia development, 3×10^6 lentivirus transduced Kasumi-1cells (GFP$^+$) were injected into sublethally irradiated (2.5 Gy) NSG mice via tail vein. shCtrl or shPRL2 was used to study PRL2 deficiency. shLuciferase was used to perform *in vivo* imaging.

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PRLi treatment *in vivo*

PRLi were dissolved in DMSO at 25mg/ml stocking concentration and saved in −80°C freezer. PRLi stock solution or DMSO was diluted in PBS before administration. Mice were treated with PRLi (25mg/kg, i.p.) or DMSO daily for 21 days.

In vivo imaging system

Bioimaging of leukemia burden in NSG mice transplanted with human leukemia cells was performed as described previously (20). The data was analyzed by the Aura software.

Flow cytometry

Flow cytometry analysis of human leukemia cells in peripheral blood, bone marrow and spleen of NSG mice was performed as described previously (20).

Production of Retrovirus and Lentivirus

Production and transduction of human AML cells with retroviruses or lentiviruses was performed as described previously (17–20).

Colony formation unit assays

The colony formation of human leukemia cells was performed as described previously (20).

Generation of murine mast cells

Generation of murine mast cells from low-density BM mononuclear cells was described previously (21).

Immunoblotting analysis

Immunoblot was performed as described previously (17–20). pAKT-S473 (9271), AKT (4691S), pERK-Y202/204 (9101), ERK (9102), β-Actin (3700), Ubiquitin (3936), PLCγ (5690), SHP2 (3397), CBL-human(8447s), Kit (3074S), CBL-Y731 (3554), were obtained from Cell signaling. CBL-mouse(C40320) was obtained from Transduction. PRL2 (05–1583) and pTyr (05–321) were obtained from Sigma Aldrich. pCBL-Y371 antibody has been described previously (22).

Ubiquitination assays

Kit positive mast cells from *Prl2*$^{+/+}$ and *Prl2*$^{-/-}$ mice were stimulated with SCF for indicated time points and then Kit ubiquitination assays were performed as described previously (23). We obtained Anti-c-KIT (#3074) and anti-ubiquitin (P4D1, #3936) antibodies from Cell Signaling. We obtained Anti-ubiquitin (FK2, #ST1200), anti-ubiquitin (Lys48-specific, clone Apu2, #05–1307) and anti-ubiquitin (Lys63-specific, clone Apu3, #05–1308) antibodies from Millipore. We obtained Anti-Actin (#sc-47,778) and anti-GAPDH (#sc-365062) antibodies from Santa Cruz Biotechnology.

GST pull down assays

GST pull down assays using purified GST, GST-PRL2 and GST-PRL2-CSDA proteins in Kasumi-1 cells were performed as described previously (20).

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Immunoprecipitation (IP) assays

Immunoprecipitation (IP) assays were performed as described previously (20).

Statistical Analysis

Statistical analysis was performed as described previously (17–20).

TCGA sequencing data

Transcription of *PRL2* (*PTP4A2*) and all data on clinical, cytogenetic characteristics, and survival were derived from TCGA. DEGs were used to do Gene-set enrichment analysis by GSEA v4.2.2.

Data Availability

All data from this study are available upon request from corresponding authors.

Results

KIT signaling gene signatures are significantly enriched in AML with high *PRL2* expression

To examine the potential role of PTP4A2 in human leukemia, we analyzed the published TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) dataset. We found that signaling by KIT in disease (Fig. 1A), KIT pathway (Fig. 1B), signaling by SCF/KIT (Fig. 1C), regulation of KIT signaling (Fig. 1D), and KIT receptor signaling pathway (Fig. 1E) gene signatures were enriched in *PRL2* (*PTP4A2*) high group compared to *PRL2* (*PTP4A2*) low group in AML. These findings suggest that PRL2 may be a key regulator of KIT signaling in AML.

PRL2 is important for oncogenic KIT-driven leukemia

Ectopic KitD814V expression induces the development of myeloproliferative neoplasms (MPN) in mice (24) and we found that loss of PRL2 significantly delays the development of KitD814V-driven MPN in mice (Fig. 2A), demonstrating that PRL2 is important for KitD814V-driven MPN *in vivo*.

Kasumi-1 and SKNO-1 are human AML cell lines with *KIT* mutations (17). To examine the impact of PRL2 deficiency on human leukemia cell proliferation, we have developed two shRNAs targeting different regions of human *PRL2* and showed that these shRNAs can efficiently decrease PRL2 proteins in human leukemia cells (17). We focused our studies on one of the PRL2 shRNA and found that PRL2 deficiency decreases the colony formation and proliferation of both Kasumi-1 and SKNO-1 cells (Fig. 2B–G). Furthermore, knockdown of PRL2 increases the apoptosis of SKNO-1 cells (Fig. 2H–I).

To determine the role of PRL2 in leukemia development, 3×10^6 Kasumi-1 cells expressing a control or PRL2 shRNA were transplanted into sublethally-irradiated NSG mice. We found that PRL2 deficiency significantly extended the survival of recipient mice transplanted with Kasumi-1 cells (Fig. 2J). Notably, we observed decreased the number of human cells ($hCD45^+$) in peripheral blood (PB), bone marrow (BM), and spleen of recipient mice repopulated with Kasumi-1 cells expressing PRL2 shRNA (Fig. 2K).

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Furthermore, knockdown of PRL2 significantly decreased splenomegaly seen in recipient mice transplanted with Kasumi-1 cells (Fig. 2L).

PRLi treatment extends the survival of immunodeficient mice repopulated with *KIT* mutated AML cells

To further substantiate the PRL2 knockdown results, we utilized compound 43, a small molecule PRL inhibitor (PRLi) that blocks PRL trimerization, which is essential for PRL function (17, 25). We found that PRLi treatment reduces the colony formation of both Kasumi-1 and SKNO-1 cells (Fig. 3A–B). To determine the efficacy of PRLi on human leukemia cells *in vivo*, we transplanted luciferase-labeled Kasumi-1 cells into sublethally-irradiated NSG mice via tail vein injection. One week after the transplantation, NSG mice were with PRLi (25 mg/kg, I.P.) or vehicle (10% DMSO) daily for three weeks. Leukemia burden in NSG mice was monitored via bioluminescence imaging. Imaging of luminescence showed that PRLi treatment dramatically decreases leukemia burden compared with the control group (Fig. 3C). The radiance of the NSG mice was significantly reduced after exposure to PRLi (Fig. 3D). Furthermore, NSG mice transplanted with human leukemia cells showed extended survival following PRLi treatment (Fig. 3E). PRLi also considerably decreased the engraftment of human leukemia cells in NSG mice (Fig. 3F) and reduced spleen weight of NSG mice (Fig. 3G).

To determine whether primary human AML cells with *KIT* mutations are sensitive to PRLi treatment, we treated primary *KIT* mutated AML cells with PRLi or DMSO and performed proliferation, cell cycle and apoptosis analysis. PRLi decreases the viability of *KIT* mutated AML cells *in vitro* (Fig. 3H). In addition, PRLi treatment of primary AML cells with *KIT* mutation led to G0/G1 cell cycle arrest and decreased the number of cells in S and G2M phases of the cell cycle (Fig. 3I). Furthermore, PRLi treatment increases the apoptosis of primary human AML cells with *KIT* mutation (Fig. 3J). Thus, we demonstrated that primary human AML cells with *KIT* mutations are sensitive to PRLi treatment.

PRL2 enhances KIT signaling in hematopoietic and leukemia cells

We first examined the impact of SCF stimulation on Kit phosphorylation in $Prl2^{+/+}$ and $Prl2^{-/-}$ hematopoietic stem and progenitor cells (HSPCs). We observed decreased phosphorylation of Kit, ERK1/2, and AKT in *Prl2* null HSPCs following SCF stimulation (Fig. 4A). We then utilized BM-derived mast cells (MCs) to examine the impact of PRL2 on Kit signaling (Fig. 4B) (21). We found that $Prl2^{-/-}$ MCs showed enhanced Kit internalization compared to $Prl2^{+/+}$ cells following SCF stimulation (Fig. 4C). Kit expression on the surface of *Prl2* null MCs were decreased during SCF-induced differentiation (Fig. 4D). In addition, expression of a shRNA targeting PRL2 or a catalytically inactive PRL2-CSDA mutant resulted in decreased KIT expression on the surface of human AML cell line MO7e (Fig. 4E).

PRL2 regulates KIT stability and ubiquitination in hematopoietic cells

To measure the half-life of Kit protein, we treated serum starved MCs with cycloheximide and determined the levels of Kit by immunoblot analysis. Loss of *Prl2* increased the half-life of Kit in MCs (Fig. 5A). Furthermore, loss of *Prl2* enhanced Kit ubiquitination

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following SCF stimulation in MC cells (Fig. 5B). It has been suggested that Cbl-mediated c-Kit ubiquitination-internalization-degradation is through mono-ubiquitination-lysosome pathway (23, 26–27). To determine if PRL2 deletion can also enhance Cbl mediated c-Kit monoubiquitination, we compared the commercially available antibodies P4D1 and FK2 against Ub to differentiate monoubiquitin, which can be recognized by P4D1, or polyubiquitin which can be recognized by both P4D1 and FK2 (26). We confirmed that c-Kit undergo mono-ubiquitination upon SCF stimulation, which was further increased by PRL2 deletion (Fig. 5C). Consistent with this observation, we were unable to detect any K48- or K63- polyubiquitination signal in c-Kit IP (Fig. 5C). However, the detailed sites for mono-ubiquitination need further evaluation.

In response to cytokine stimulation, CBL is phosphorylated and activated, leading to ubiquitination and degradation of KIT (22–23, 28). However, how CBL phosphorylation is regulated in leukemia cells remains elusive. Notably, GSEA analysis revealed that high PRL2 expression is correlated with CBL signaling in human leukemia patients (Fig. 5D). We found that PRL2-CSDA mutant showed increased interaction with KIT and CBL compared to WT PRL2 in GST pulldown assays (Fig. 5E). We found that PRL2 interacts with both CBL and KIT in Kasumi-1 and SKNO-1 cells (Fig. 5F–G). We also found that ectopic PRL2 expression increases the levels of KIT in HEK293 cells (Fig. 5H) and we confirmed the PRL2 and CBL interaction in HEK293 cells expressing KITD814V (Fig. 5I).

PRL2 dephosphorylate CBL at tyrosine 371 in *KIT* mutated AML cells

CBL becomes activated upon Tyrosine 371 phosphorylation (28–29). Indeed, we found that knockdown of PRL2 increases CBL phosphorylation at tyrosine 371, whereas the levels of CBL phosphorylation at tyrosine 731 was not affected by PRL2 inhibition in both Kasumi-1 and SKNO-1 cells (Fig. 6A–B). Further, PRL2-CSDA also increased CBL tyrosine 371phosphorylation in Kasumi-1 and SKNO-1 cells (Fig. 6C–D). Thus, we demonstrate the PRL2 associates with CBL and dephosphorylates CBL at tyrosine 371 in leukemia cells with *KIT* mutations.

Discussion

Protein tyrosine phosphatases (PTPs) control tyrosine phosphorylation mediated cellular signaling (30–31). Mutations in *KIT* lead to aberrant KIT activation in leukemia (3) and we demonstrated that PRL2 enhances oncogenic KIT signaling and promotes leukemia cell proliferation and survival. Furthermore, we found that KIT is a CBL substrate in leukemia cells and loss of PRL2 increases CBL phosphorylation at tyrosine 371. Finally, we showed that PRL2 is important for oncogenic KIT-driven leukemia development and PRLi treatment extends the survival of leukemic mice transplanted with *KIT* mutated human AML cells.

Studies found that CBL activity is stimulated by phosphorylation of a Tyr residue in a LHR (26–28). Structural and biochemical studies showed that phosphorylation of Tyr 371 activates CBL by eliminating autoinhibition and enabling direct participation of phosphotyrosine in the activation of E2~ubiquitin complex for catalysis (26). This activation is required for receptor tyrosine kinase ubiquitination (26–28). We found that PRL2 associates with and dephosphorylates CBL at Tyr 371 in human leukemia cells with *KIT*

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mutations. Furthermore, genetic and pharmacological inhibition of PRL2 activity increases CBL Tyr 371 phosphorylation in human leukemia cells with *KIT* mutations. FLT3 is another oncogenic receptor tyrosine kinase aberrantly activated in leukemia (32–33) and we reported that PRL2 interacts with CBL and dephosphorylates CBL on Tyr 371 in human leukemia cells with *FLT3* mutations (20). Thus, PRL2 enhances both oncogenic FLT3 and KIT signaling through modulating CBL phosphorylation in leukemia cells.

In summary, our studies have uncovered a novel mechanism that fine-tunes oncogenic KIT signaling in leukemia cells and identified PRL2 as a novel therapeutic target in myeloid neoplasms with *KIT* mutations.

Acknowledgements

YL was supported by fundings from NIH (R01HL150624, R56DK119524, and R56AG052501), DoD (W81XWH-18-1-0265 and W81XWH-19-1-0575), the Leukemia &Lymphoma Society (Translational Research Program award 6581-20) and the St. Baldrick's Foundation (Scholar Award). YB, JM, FNM, and ZYZ were supported by fundings from NIH (R01CA069202) and the Robert C. and Charlotte Anderson Chair Endowment. SB was supported by an F31 Award (F31HL160120) from NIH. HC was supported by Natural Science Foundation of Chongqing, China (cstc2020jcyj-msxmX0969). The authors would like to thank the NUseq Core, Quantitative Data Science Core, Pathology Core, Flow Cytometry Core, and Imaging core at Northwestern University Feinberg School of Medicine and Robert H. Lurie Comprehensive Cancer Center.

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Implications:

Our studies uncover a novel mechanism that fine-tunes oncogenic KIT signaling in leukemia cells and will likely identify PRL2 as a novel therapeutic target in AML with *KIT* mutations.

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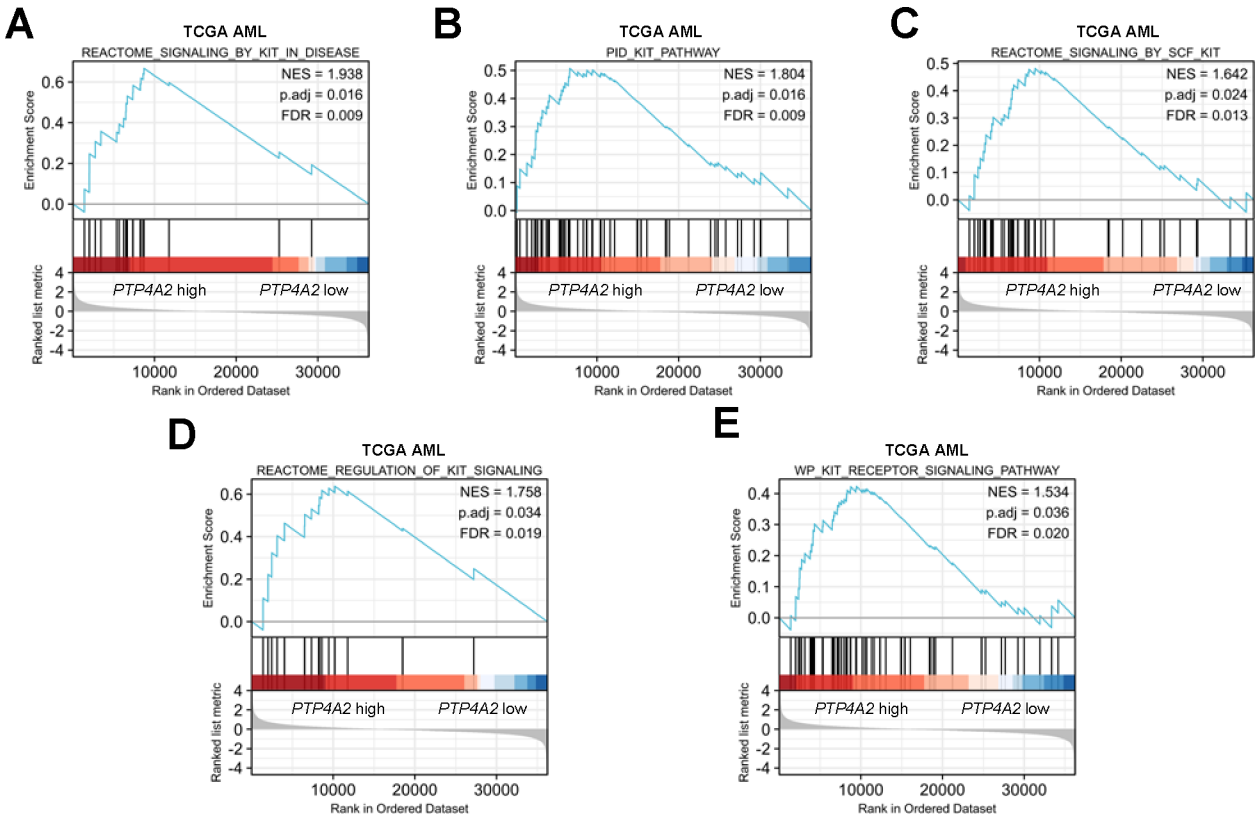

Figure 1.

High PRL2 expression is associated with enhanced KIT signaling in AML. (A) Signaling by KIT in disease, (B) KIT pathway, (C) signaling by SCF-KIT, (D) regulation of KIT signaling, and (E) KIT receptor signaling pathway were enriched in AML patients with *PRL2* high expression compared to AML patients with *PRL2* low expression.

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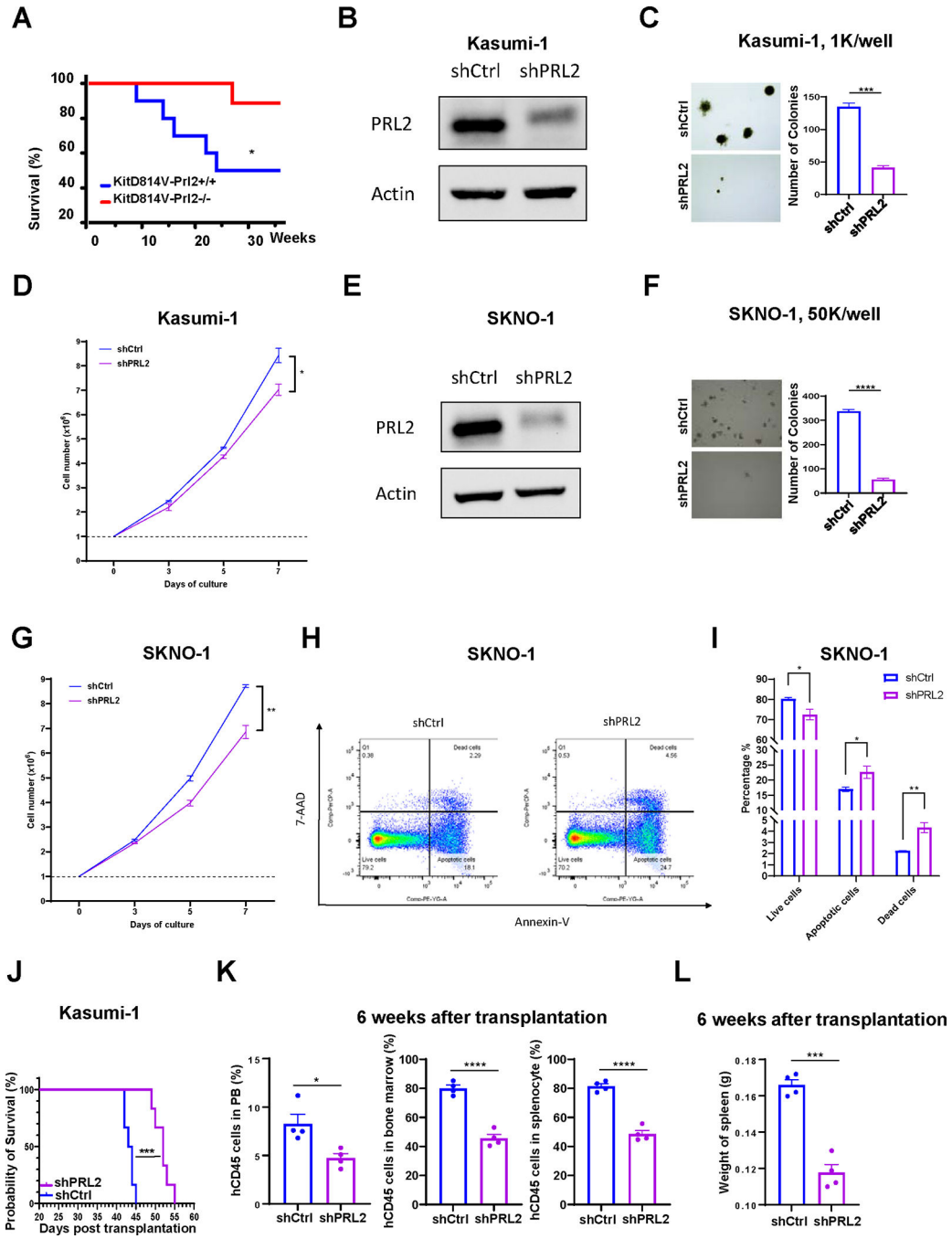

Figure 2.

Genetic inhibition of PRL2 represses oncogenic KIT driven AML *in vivo*. A, *Prl2*$^{+/+}$ and *Prl2*$^{-/-}$ hematopoietic progenitor cells expressing KitD814V were transplanted into lethally irradiated recipient mice and the survival of the mice were monitored; n= 9 mice per group. B, the levels of PRL2 protein were decreased by a shRNA targeting *PRL2* in Kasumi-1 cells. C, Colony formation of Kasumi-1 cells expressing a control or PRL2 shRNA; n=3 independent experiments. Representative images of colonies are shown. D, Proliferation of Kasumi-1 cells expressing a control or PRL2 shRNA; n=3

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independent experiments. E, PRL2 shRNA decreases the levels of PRL2 in SKNO-1 cells. F, Colony formation of SKNO-1 cells expressing a control or PRL2 shRNA; n=3 independent experiments. Representative images of colonies are shown. G, Proliferation of SKNO-1 cells expressing a control or PRL2 shRNA; n=3 independent experiments. H-I Knockdown of PRL2 significantly increases the apoptosis of SKNO-1 cells; n=3 independent experiments performed in duplicate. J, 3×10^6 Kasumi-1 cells expressing shCtrl or shPRL2 were transplanted into sublethally-irradiated NSG mice and animal survival was monitored; n=6 mice per group. K, Percentage of Kasumi-1 cells expressing a control shRNA or shPRL2 (GFP^+) in peripheral blood (PB), bone marrow (BM), and spleen of NSG mice were determined by flow cytometry analysis; n=4 mice per group. L, the spleen weight of NSG mice were measured after trasnplantation; n=4 mice per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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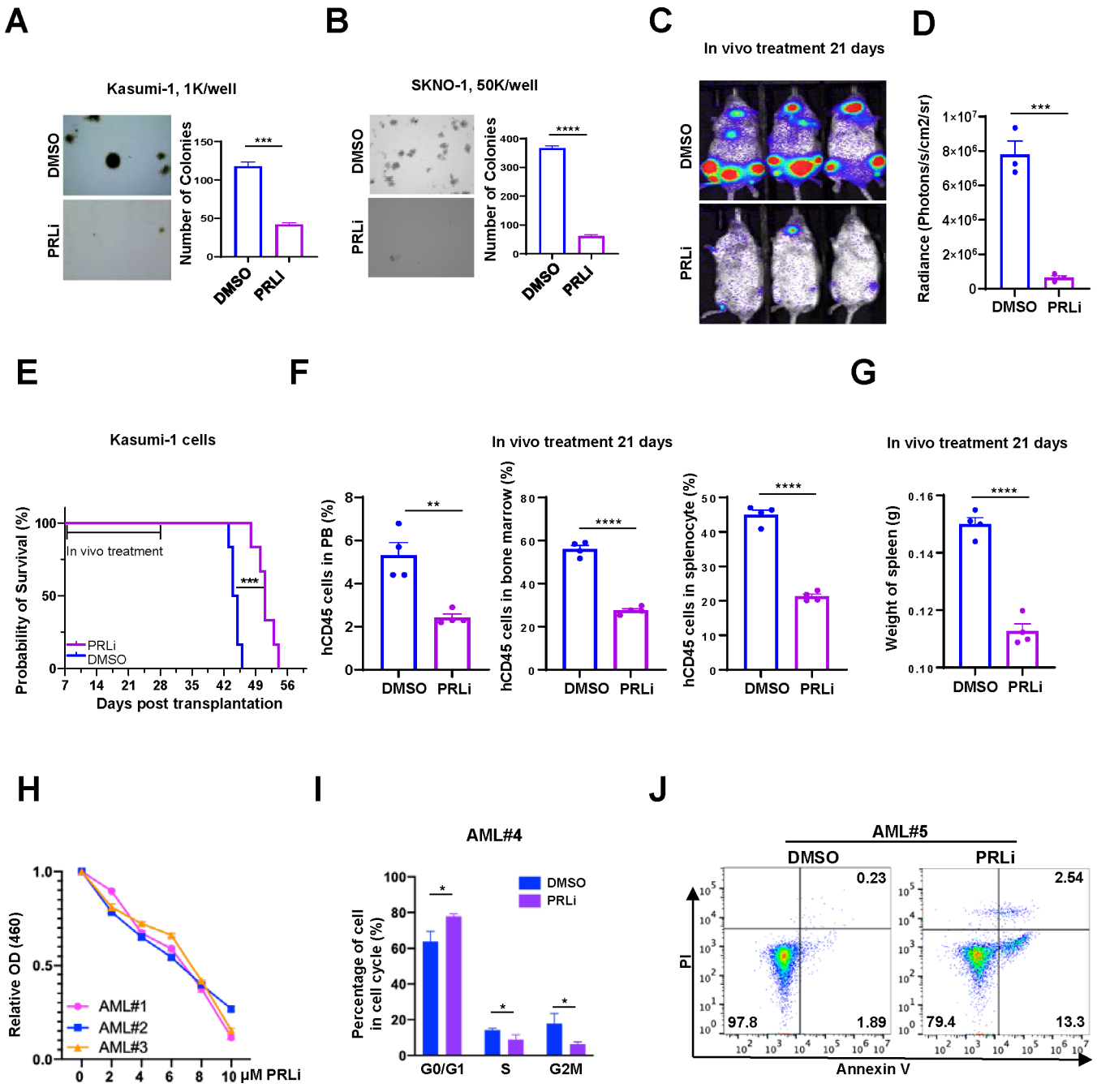

Figure 3.

PRLi treatment delays the development of oncogenic KIT driven AML *in vivo*.

A, Colony formation of Kasumi-1 cells following PRLi or DMSO treatment; n=3 independent experiments. Representative images of colonies are displayed. B, Colony formation of SKNO-1 cells following PRLi or DMSO treatment; n=3 independent experiments. Representative images of colonies are displayed. C, 3×10^6 Kasumi-1 cells expressing luciferase were transplanted into sublethally irradiated NSG mice via tail vein injection. One week after the transplantation, NSG mice were treated with DMSO or PRLi (25mg/kg, I.P.) daily for three weeks. We utilized In Vivo Image System (IVIS) to

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monitor leukemia burden in NSG mice once a week for three weeks (n=3 mice per group). D, Quantitative results from bioimaging shown in Figure 3C. E, Survival of NSG mice following DMSO or PRLi treatment; n=6 mice per group. F, Flow cytometry analysis of human $CD45^+$ cells in NSG mice following three weeks of DMSO or PRLi treatment; n=4 mice per group. G, the spleen weights of NSG mice transplanted with Kasumi-1 cells following three weeks of DMSO or PRLi treatment; n=4 mice per group. H, PRLi treatment decreased the viability of primary human AML cells with *KIT* mutation (AML#1–3, *KIT-D816V*). I, Cell cycle analysis of primary AML cells with *KIT* mutation (AML#4, *KIT-D816V*) at 24 hours following DMSO or PRLi (10 μM) treatment. J, Apoptosis analysis of primary AML cells with *KIT* mutation (AML#5, *KIT-D816V*) at 24 hours following DMSO or PRLi (10 μM) treatment. **p<0.01, ***p<0.001, ****p<0.0001.

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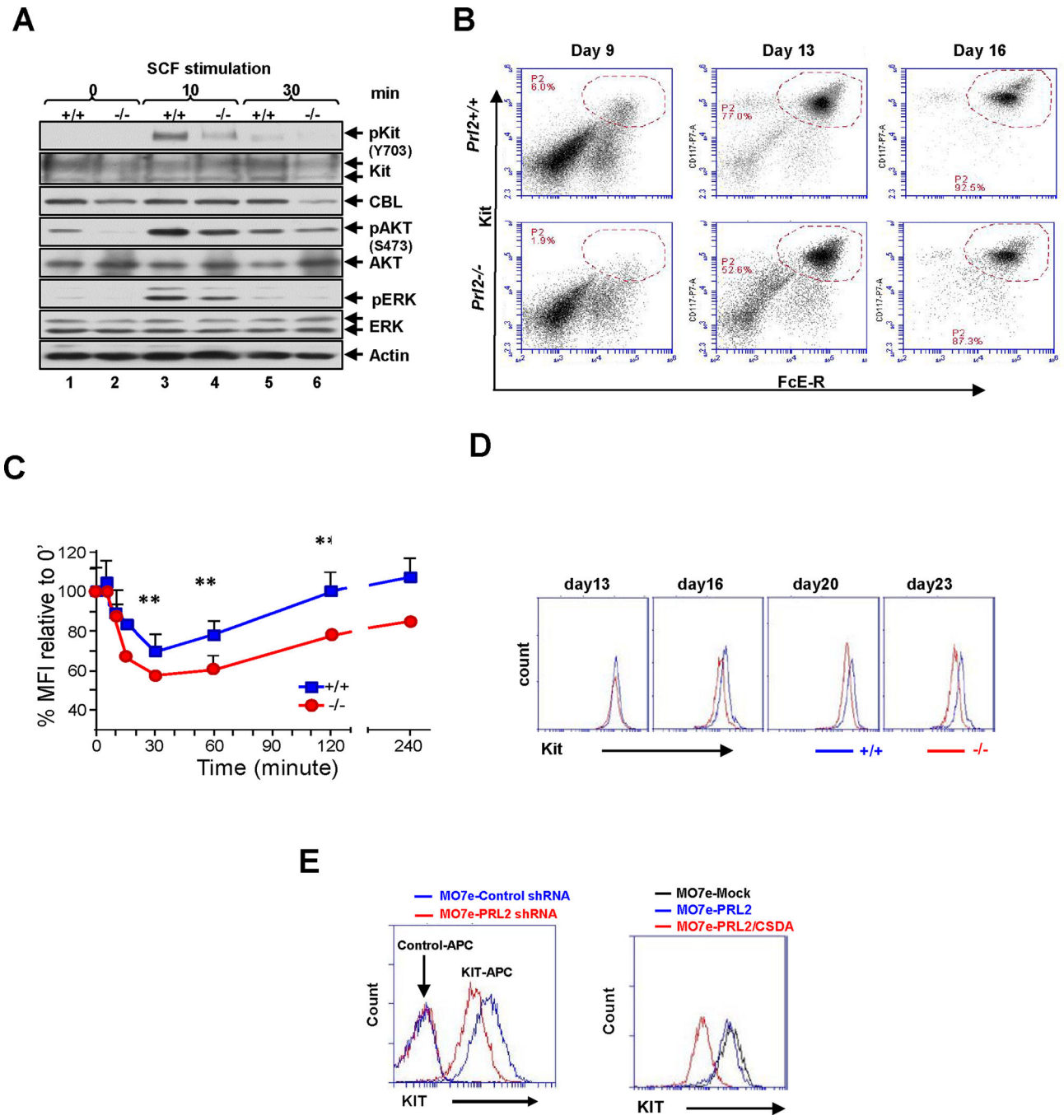

Figure 4.

PRL2 regulates KIT internalization in hematopoietic and leukemia cells. A, PRL2 enhances KIT phosphorylation in HSPCs following SCF stimulation. B, Mast cells (MCs) were induced from BM cells *in vitro*. C, Expression of Kit on MC surface following SCF stimulation; n=3. D, Loss of PRL2 increases Kit internalization in MCs. E, PRL2 deficiency increases KIT internalization in MO7e cells (Left). Expression of PRL2-CSDA, but not PRL2, increases KIT internalization in MO7e cells (Right). **p<0.01.

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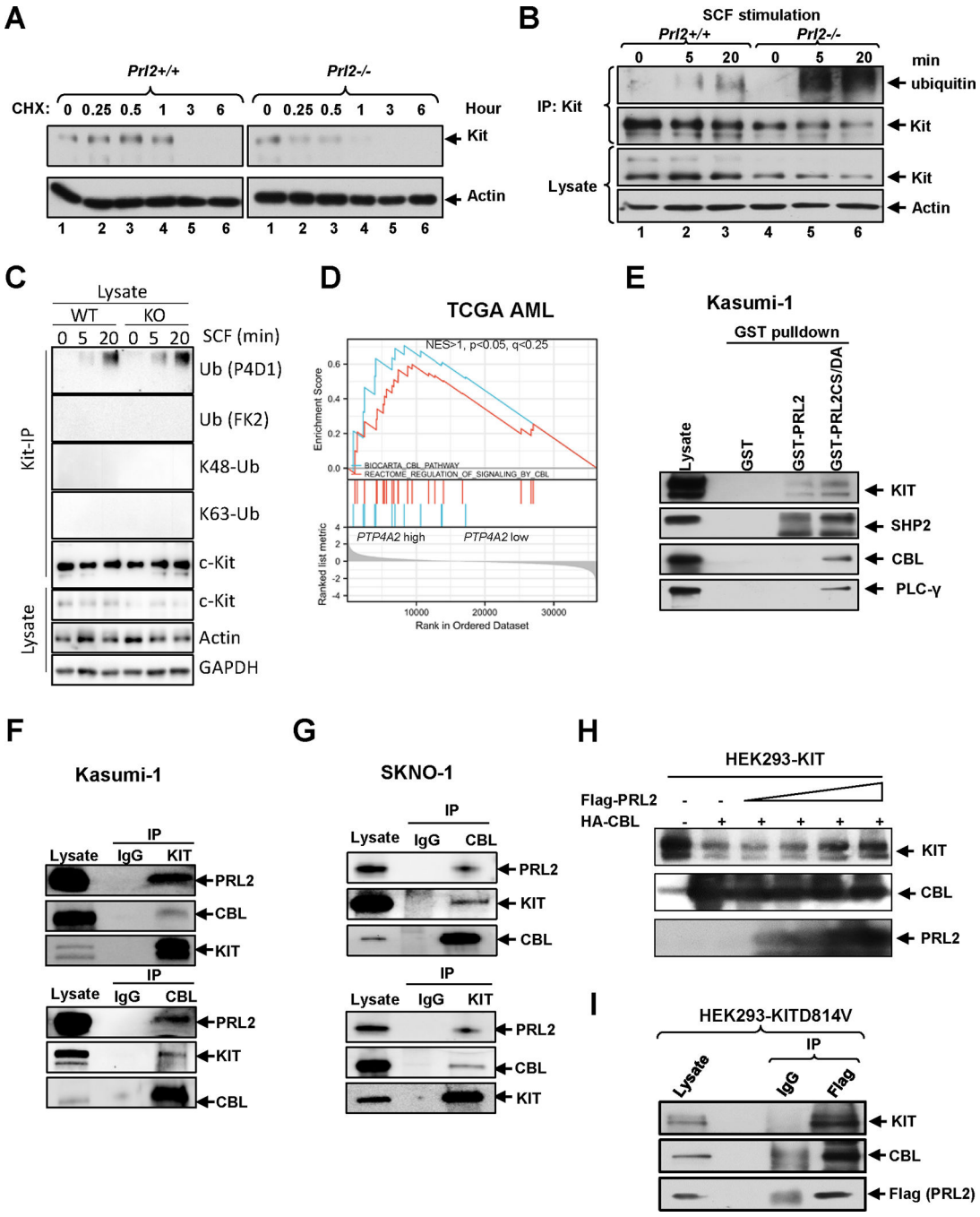

Figure 5.

PRL2 regulates Kit turnover in hematopoietic cells. A, Loss of Prl2 decreases Kit half-life in MCs. B, Kit ubiquitination in *Prl2*$^{+/+}$ and *PRL2*$^{-/-}$ MCs stimulated with SCF. C, Kit is monoubiquitinated in MCs. D, CBL pathway and regulation of signaling by CBL were enriched in AML patients with high *PRL2* expression compared to AML patients with low *PRL2* expression. E, PRL2-CSDA show enhanced association with CBL and KIT compared to PRL2 in Kasumi-1 cells as determined by GST pull-down assays. G, Co-immunoprecipitation assays showed that PRL2 interacts with KIT and CBL in SKNO-1

cells. H, Overexpression of PRL2 increases the level of KIT in HEK293 cells. I, PRL2 interacts with CBL in HEK293 cells expressing KITD814V.

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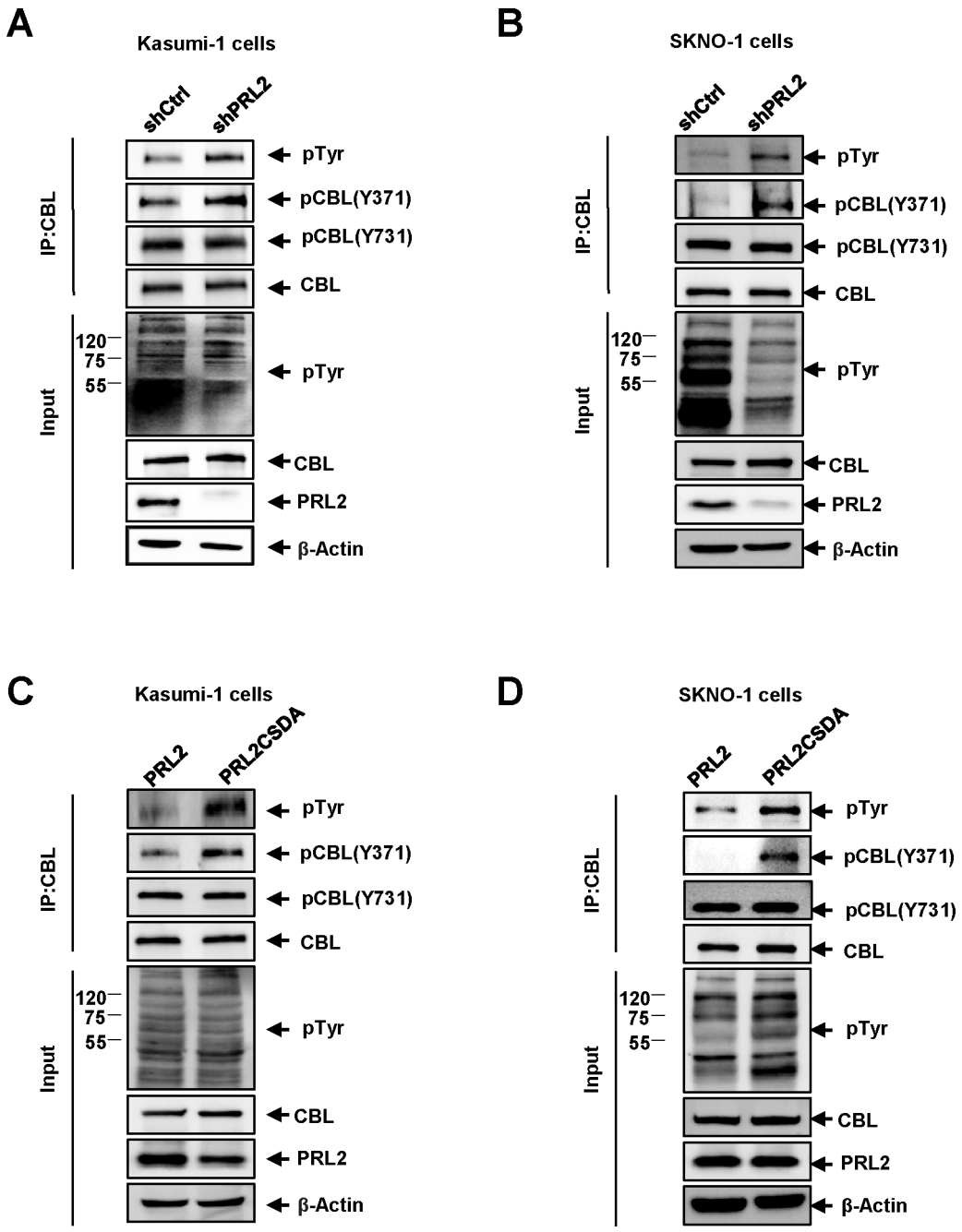

Figure 6.

PRL2 dephosphorylates CBL at tyrosine 371 in leukemia cells. A, Knockdown of PRL2 increases CBL phosphorylation at Y371 in Kasumi-1 cells. B, Knockdown of PRL2 increases CBL phosphorylation at Y371 in SKNO-1 cells. C, Ectopic expression of PRL2-CSDA increases CBL phosphorylation at Y371 in Kasumi-1 cells. D, Ectopic expression of PRL2-CSDA increases CBL phosphorylation at Y371 in SKNO-1 cells.