ORIGINAL ARTICLE

Blockade of deubiquitinase YOD1 degrades oncogenic PML/RARα and eradicates acute promyelocytic leukemia cells

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Abstract In most acute promyelocytic leukemia (APL) cells, promyelocytic leukemia (PML) fuses to retinoic acid receptor α (RARα) due to chromosomal translocation, thus generating PML/RARα oncoprotein, which is a relatively stable oncoprotein for degradation in APL. Elucidating the mechanism regulating the stability of PML/RARα may help to degrade PML/RARα and eradicate APL cells. Here, we describe a deubiquitinase (DUB)-involved regulatory mechanism for the maintenance of PML/RARα stability and develop a novel pharmacological approach to degrading PML/RARα by inhibiting DUB. We utilized a DUB siRNA library to identify the ovarian tumor protease (OTU) family member

Abbreviations: APL, acute promyelocytic leukemia; ATO, arsenic trioxide; ATRA, all-trans retinoic acid; cAMP, cyclic adenosine monophosphate; EARD, endoplasmic reticulum-associated degradation; FLT3/ITD, internal tandem duplication within FLT3; G5, ubiquitin isopeptidase inhibitor I; HOTAIRM1, HOXA transcript antisense RNA myeloid-specific 1; JAMM, Jab1/Pab1/MPN domain-containing protease; LATS, large tumor suppressor kinase; MDM2, murine double minute 2; MINDY, motif-interacting with ubiquitin-containing novel DUB family; MJD, machado-Joseph domain-containing protease; OUT, ovarian tumor; PLZF, promyelocytic leukemia zinc finger; PML, promyelocytic leukemia; RARα, retinoic acid receptor α; RNF4, ring finger protein 4; S100A3, S100 calcium binding protein A3; TAZ, transcriptional co-activator with PDZ-binding motif; TGFβ, transforming growth factor β; TRIB3, tribbles pseudokinase 3; UCH, ubiquitin carboxyl terminal hydrolase; UCHL1, ubiquitin c-terminal hydrolase L1; USP, ubiquitin specific protease; YAP, yes-associated protein.

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YOD1: A novel therapeutic target for APL

1. Introduction

Acute promyelocytic leukemia (APL) is characterized by the t(15;17)(q24;q21) chromosomal translocation, which fuses promyelocytic leukemia (PML) to retinoic acid receptor α (RARα) and generates PML/RARα oncprotein. PML/RARα is a clinically acknowledged therapeutic target to cure APL. First, PML/RARα is considered to be critical for APL pathogenesis. The expression of PML/RARα is sufficient to initiate APL in mice, and the proliferation of APL-initiating cells depends on the expression of PML/RARα. Second, PML/RARα is a useful diagnostic biomarker for APL, and its detection is performed because PML/RARα-driven APL tends to be sensitive to all-trans retinoic acid (ATRA) and arsenic trioxide (ATO), which are both targeted drugs to initiate PML/RARα proteolysis. Thus, triggering the degradation of oncogenic PML/RARα has been considered as a successful strategy to treat APL.

After incorporation of ATRA and ATO into the APL management paradigms, the prognosis has dramatically improved for APL patients. Nonetheless, significant challenges still remain. For instance, serious adverse events such as hepatotoxicity, gastrointestinal symptoms, water-sodium retention, and nervous system damage frequently occur in the clinical setting. Furthermore, some patients develop resistance during therapy, and relapse and refractory responses are still observed in patients under treatment. Studies incorporating genome sequencing analyses have demonstrated that genetic mutations in the PML moiety (e.g., L218P and A216V) or RARα moiety (e.g., ΔF286 and R276Q) of PML/RARα disrupt its binding to ATRA or ATO, thus hindering the subsequent degradation process, which ultimately leads to resistance to ATRA and ATO treatment. Therefore, novel strategies to trigger PML/RARα degradation would be valuable for treating APL, particularly drug-resistant APL patients.

PML/RARα is a relatively stable oncprotein with a 24-h half-life for degradation in APL cells. The mechanisms currently recognized for regulating the stability of PML/RARα were discovered through treatment with ATRA and ATO. Specifically, ATO destabilizes PML/RARα through the ubiquitination proteasome pathway, by conjugating SUMO to the PML portion and recruiting the ubiquitin ligase ring finger protein 4 (RNF4). ATRA binds to the RARα portion and triggers the ligand-dependent degradation of PML/RARα. Autophagic degradation of PML/RARα is also activated upon exposure to ATRA and ATO. Cyclic adenosine monophosphate (cAMP) stabilizes N-Myc in neuroblastoma, and USP28 is required for MYC stability. Some DUBs have also been described to manifest oncogenic activities, such as ubiquitin c-terminal hydrolase L1 (UCHL1), which is activated upon exposure to ATRA or ATO, thus hindering the subsequent degradation process, which leads to resistance to ATRA and ATO treatment. Therefore, novel strategies to trigger PML/RARα degradation would be valuable for treating APL, particularly drug-resistant APL patients.

2. Materials and methods

2.1. Cells and culture

Human embryonic kidney HEK-293T and 293FT cell lines were supplied by Invitrogen (Grand Island, NY, USA). Monkey kidney COS-7, human H1299 and the human myeloid leukemia (non-APL) U937 and HL60 cell lines were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The NB4 human myeloid leukemia cell line was kindly gifted from Dr. Lingtao Wu (University of Southern California, California).
CA, USA), the NB4R1 cell line was a kind gift from Dr. He Huang (Zhejiang University, Hangzhou, China) and the NB4R2 cell line was kindly provided by Dr. Jian Zhang (Shanghai Jiao Tong University, Shanghai, China). Primary APL blasts (Leu-1–12) extracted from the bone marrow of patients (Children’s Hospital of Zhejiang University and First Affiliated Hospital of Zhejiang University, Hangzhou, China) were isolated using Ficoll-Paque PREMIUM (Cytiva). Written informed consents from patients and approval from the Institutional Research Ethics Committee of the hospital were obtained before the use of these clinical materials for research purposes.

Primary patient blasts (Leu-1–12) and normal human HSCs were cultured in IMDM medium supplemented with recombinant human SCF (50 ng/mL; R&D Systems), recombinant human IL-3 (10 ng/mL; R&D Systems), recombinant human IL-6 (5 ng/mL; R&D Systems), hydrocortisone (10 μmol/L; Sigma–Aldrich, St. Louis, MO, USA), l-glutamine (2 mmol/L), 20% fetal bovine serum (Gibco BRL) and 1% penicillin/streptomycin. Human myeloid leukemia (NB4, NB4R1, NB4R2 and U937 cells) and 1% penicillin/streptomycin.

All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO2 and passaged for a maximum of 2 months. All cell lines were routinely tested for mycoplasma using a MycoPlaque PREMIUM (Cytiva). Written informed consents from patients and approval from the Institutional Research Ethics Committee of the hospital were obtained before the use of these clinical materials for research purposes.

Antibodies against YOD1 (25370-1-AP) were purchased from Proteintech. Antibodies against PML (db9346), HA (db2603), β-actin (db10001) and GAPDH (db106) were purchased from Diagbio. The anti-Flag antibody (HOA012FL01) was purchased from HuaBio.

HEK-293T cells were cotransfected with PML/RARα-Flag, Renilla and DUB siRNAs (Dharmacon) per well in the presence of jetPrime (Polynplux). After 36 h, the cell lysate was analyzed with a Dual Luciferase Reporter Assay System (Promega). Relative firefly expression was determined as the ratio of Firefly-to-Renilla luciferase activity. The fold change of the relative firefly expression was normalized to that of the scramble control.

Virus production, titration and transduction were performed as described previously.

Real-time PCR
Real-time PCR was used to detect the mRNA levels of PML/RARA and YOD1. GAPDH was used as an internal standard. The primers used are shown in Supporting Information Table S2.

Inmunoprecipitation
When detecting the interaction between PML/RARα and YOD1, cells were lysed in RIPA lysis buffer (50 mmol/L Tris-base, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate, pH = 7.4), containing leupeptin, PMSF, and Na3VO4 for 30 min on ice, then centrifuged at 16,363 × g for 30 min at 4°C. To observe the polyubiquitination of PML/RARα, cells were lysed in 4% SDS buffer (4% SDS, 150 mmol/L NaCl and 50 mmol/L triethylamine, pH = 8.0). Then, the cell lysate was incubated with a 30 μL suspension of beads at 4°C overnight.

Finally, the beads were washed five times with wash buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 1% NP40, pH = 8.0) before boiling. The boiled samples were then separated by SDS-PAGE and subjected to immunoblotting analysis with the indicated antibodies.

In vitro ubiquitination assay
COS7 cells were cotransfected with pCDNA3.0-PML/RARα-HA (L, S or mutant) and PRK5-His-Ub plasmid. After 36 h, cells were treated with MG132 (10 μmol/L) for 8 h before harvest, followed by cell lysis in 4% SDS buffer (4% SDS, 150 mmol/L NaCl, and 50 mmol/L triethylamine, pH = 8.0). Then, ubiquitinated PML/RARα-HA (L, S or mutant) was pulled down by immunoprecipitation with HA beads and subsequently incubated with recombinant wild-type GST-YOD1 or mutant GST-YOD1(C160S) at 37°C for 6 h. When detecting the effect of DUB inhibitors, recombinant wild-type GST-YOD1 or mutant GST-YOD1(C160S) was preincubated with different DUB inhibitors separately at 37°C for 1 h. The ubiquitination of PML/RARα was detected by Western blotting with a His antibody.
2.8. Cellular proliferation analysis

The cell count and viability were determined with manual counting by the trypan blue (Sigma) exclusion method in Burker chambers. Nonviable and dead cells were identified as those that had taken up trypan blue dye.

2.9. Soft agar formation assay

The wells of a 6-well plate were coated with 2 mL of 0.5% soft agar (Sigma), and the agar mixture was allowed to solidify at room temperature. Then, the cells were mixed in 0.3% soft agar to plate the upper layer. After the agar solidified, the cultures were placed into a humidified cell culture incubator set to 37 °C. The cells were incubated several days until colonies formed. Then, nitrotetrazolium blue chloride was added to each well to stain the cells. The cell number used for colony formation assay is 1000 (NB4 cells) or 2000 (NB4R1 and NB4R2 cells) cells per well. The stained clones were photographed using a camera, and were automatically counted using ImageJ.

Relative clonal formation rate (%) = (The number of clones in shYOD1 group/ The number of clones in the shCtrl group) × 100. (1)

2.10. Cell apoptosis analysis

The apoptosis quantification was detected by PI/Annexin V-staining, and Annexin V-stained (PI−/Annexin V+ and PI+/ Annexin V−) cells were analyzed to measure the cell apoptosis rate.

2.11. Cell differentiation analysis

To assess CD11b expression, a fluorochrome conjugate of monoclonal antibody was used to detect CD11b expression following the protocol from the manufacturer (BD Biosciences).

2.12. A cell-based YOD1 inhibitor screening assay

H1299 cells stably expressing PML/RARα were seeded into 24-well plates, and the next day, the cells were exposed to the library of 31 reported DUB inhibitors at a concentration of 5 μmol/L for 12 h. Then, the protein levels of PML/RARα were assessed by Western blotting. The relative protein levels of PML/ RARα were determined as the ratio of PML/RARα to GAPDH, and the ratio of PML/RARα/GAPDH was normalized to level of the solvent control.

2.13. Recombinant YOD1 purification

Plasmids containing GST-YOD1 or GST-YOD1 (C160S) were transformed into Escherichia coli BL21 (DE3) cells. After induction with isopropyl β-D-thiogalactoside (IPTG), the bacteria in buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.76 mmol/L KH2PO4 and 10 mmol/L Na2HPO4·12H2O, pH = 7.4) were lysed by a High-pressure Bacteria Breaker (Union Biotech). The GST-fusion protein was purified using a GST affinity column (GE healthcare). The eluted protein was dialyzed overnight in 3 L of dialysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5 mmol/L EDTA and 10% glycerol, pH = 8.0) at 4 °C.

2.14. Animal studies

To generate an orthotopic xenograft model, NB4 or NB4R1 cells were transduced with shCtrl or shYOD1 (#1 and #2) lentivirus. The mice were sublethally pretreated with cyclophosphamide. Then, NB4 cells (1 × 106 cells) or NB4R1 cells (2 × 106 cells) were injected into 6- to 7-week-old NOD/SCID mice (SLRC Laboratory Animal Inc.) via the tail vein. Some mice were killed on the indicated days (30 days for those implanted with NB4 cells) after transplantation. First, the bone marrow cells were stained by Wright−Giemsa to determine the tumor burden in the mice. Next, the leukemic cells were confirmed by analyzing the proportion of human CD45-positive and mouse CD45-negative (hCD45+ mCD45−) cells in the bone marrow as described previously28. It is reported that successful leukemia engraftment was defined by the presence of > 1% human CD45+ murine CD45− cells in the recipient BM or PB29−31. For other mice, the survival times were recorded.

To perform the xenograft experiment, 2 × 106 NB4R2 cells were injected subcutaneously into 4- to 5-week-old female nude mice (SLRC Laboratory Animal Inc.). When the tumors reached a volume from 200 to 400 mm3, the mice were allocated by tumor volume into four groups. The mice were treated with G5 (20 or 40 mg/kg; i.v.) every other day or with ATRA (20 mg/kg; i.g.) every other day for 13 days. The tumor volume was recorded until the animals were sacrificed.

The Animal Research Committee at Zhejiang University approved all animal studies and animal care was provided in accordance with the institutional guidelines.

2.15. Statistical analysis

For all parameters measured, the values for all samples under different experimental conditions were averaged, and the standard deviation (SD) or Standard error (SE) was calculated. The statistical significance of differences between groups was determined by unpaired two-tailed Student’s t-test analysis, one-way analysis of variance (ANOVA) with Tukey’s tests or Log-rank test. The results are considered significant when P < 0.05 (n.s.), P < 0.05; *P < 0.05; **P < 0.01; and ***P < 0.001.

3. Results

3.1. YOD1 is identified as a key DUB to modulate the stability of the PML/RARα protein

To identify the potential DUBs modulating the stability of PML/ RARα, we utilized a cell-based dual-luciferase reporter screening assay combined with a siRNA library which targeted 98 recognized human DUBs. As shown in Fig. 1A, PML/RARα-Firefly was used to evaluate the PML/RARα protein levels, and Renilla served as the internal control. After cells were transfected with the DUB siRNA library for 36 h, the firefly and Renilla luciferase activity levels were measured and used to calculate the relative Firefly activity. The results show that the siRNAs targeting three DUBs (USP28, USP37 and YOD1) significantly attenuated the protein level of PML/RARα, by approximately 50% (Fig. 1B). To further validate the regulatory effect of these three DUB candidates on the protein abundance of PML/RARα, we constructed two independent shRNAs targeting each DUB. The results demonstrate that the protein level of PML/RARα was obviously...
reduced by shYOD1, while it was not reduced by shUSP28 and moderately decreased by shUSP37 (Fig. 1C). Meanwhile, YOD1 suppression also decreased the level of the short isoform of PML/RARα (PML/RARα(S)), which is another major isoform of PML/RARα, differing from PML/RARα(L) that mainly used in this article (Supporting Information Fig. S1A). These results collectively indicate that YOD1 silencing can downregulate the protein level of PML/RARα, inspiring us to further study the role of YOD1 in regulating the stability of PML/RARα.

Next, we evaluated the regulatory effect of YOD1 in the cellular context of APL. As expected, YOD1 knockdown significantly decreased the level of endogenous PML/RARα in NB4 cells (Fig. 1D). More importantly, the shYOD1-induced decline in PML/RARα protein level was completely abolished by the proteasome inhibitor MG132 (Fig. 1E), whereas PML/RARα mRNA level was not affected by shYOD1 (Fig. S1B), indicating that YOD1 regulates the protein level of PML/RARα in a proteasome-dependent manner. Consistent with this finding, overexpression of YOD1 obviously increased the protein levels of both PML/RARα(L) and PML/RARα(S) (Fig. 1F). These results strongly indicate that YOD1 functions as a critical regulator for the ubiquitination proteasome pathway-dependent degradation of PML/RARα.

3.2. YOD1 silencing decreases the protein levels of drug-resistant PML/RARα mutants

Since the regulatory effect of YOD1 on the protein stability of PML/RARα has been validated, we wonder whether YOD1 can also manipulate the stability of drug-resistant PML/RARα mutants. First, two ATRA-resistant APL cell strains (NB4R1 and NB4R2 cells) harboring mutated PML/RARα (L) were obtained32. Western blot analysis confirmed that ATRA significantly degraded wild-type PML/RARα in NB4 cells, whereas the protein levels of mutated PML/RARα (ΔF286) in NB4R1 cells were partially decreased and those of mutated PML/RARα (Q411X) in NB4R2 cells were negligibly affected by

Figure 1  YOD1 is identified as a key deubiquitinase to modulate the stability of the PML/RARα protein. (A) Schematic diagram of the dual-luciferase reporter system utilized to screen deubiquitinases (DUBs) modulating the stability of PML/RARα. (B) Relative firefly activity of PML/RARα-pCDNA3.0-PML/RARα-firefly-overexpressing HEK-293T cells were transfected with siRNAs targeting 98 DUBs. The inhibition ratios of the top 3 most effective DUB siRNAs that significantly reduced the relative Firefly were indicated. (C) Effect of USP28, USP37 and YOD1 knockdown on the protein level of PML/RARα. PML/RARα-overexpressing HEK-293T cells were infected with shUSP28, shUSP37 or shYOD1 lentivirus, and the protein levels of PML/RARα and YOD1 were measured by Western blotting. (D) Effect of shYOD1 on the protein level of endogenous PML/RARα. NB4 cells were infected with lentivirus-shYOD1 (#1 and #2) for 72 h, and then, the protein expression levels of PML/RARα and YOD1 were measured by Western blotting. (E) Effect of MG132 on the shYOD1-induced PML/RARα decline. NB4 cells infected with shYOD1 (#1 and #2) were treated with or without MG132 (40 nmol/L) for 72 h, and then subjected to Western blotting. (F) Effect of YOD1 on the protein levels of exogenous long form (L) and short form (S) of PML/RARα as determined by Western blotting. HEK-293T cells were cotransfected with PML/RARα-HA (L or S) and YOD1-Flag plasmids. (C)–(F) Data are performed at least three individual experiments and one representative image is shown.
ATRA (Fig. 2A, Left). In contrast to the effect of ATRA, shYOD1 not only caused a notable reduction in the wild-type PML/RARα level in NB4 cells but also almost completely eliminated drug-resistant PML/RARα mutants in NB4R1 and NB4R2 cells (Fig. 2A, Right). These results provide the possibility that silencing YOD1 can degrade drug-resistant PML/RARα mutants to overcome ATRA resistance.

To further evaluate the modulatory effect of shYOD1 on various drug-resistant PML/RARα mutants, we introduced more ATRA- and ATO-resistant forms of PML/RARα with distinct clinical missense mutations detected in APL patients. As shown in Fig. 2B, in contrast to wild-type PML/RARα, the two ATRA-resistant PML/RARα mutants (ΔF286 and R276Q) showed little responsiveness to ATRA-induced degradation. By contrast, when exposed to shYOD1, both wild-type PML/RARα and ATRA-resistant PML/RARα mutants (ΔF286 and R276Q) were significantly decreased. Meanwhile, although ATO-resistant forms of PML/RARα (A216V and L218P) failed to degrade upon ATO treatment, their protein abundance were also effectively diminished by shYOD1 (Fig. 2C). These results further confirm the
modulatory effect of shYOD1 on various drug-resistant PML/RARα mutants.

Furthermore, experiments were performed to determine the effect of YOD1 overexpression on drug-resistant PML/RARα mutants. Similar accumulation of PML/RARα protein levels induced by YOD1 overexpression was also observed on all four drug-resistant PML/RARα mutants, including the ATRA-resistant mutants (ΔF286 and R276Q) and the ATO-resistant mutants.
Figure 4  Depletion of YOD1 leads to effective APL eradication in vitro and in vivo. (A) The silencing efficiency of different shRNAs (#1 and #2) against YOD1 in NB4 cells. The protein levels of YOD1 were measured by Western blotting. (B) Proliferation of NB4 cells infected with lentivirus-shYOD1 (#1 and #2), as determined by trypan blue exclusion test for the indicated times. (C) Colony formation assay of NB4 cells transduced with lentivirus-shYOD1 (#1 and #2). 1000 cells were seeded per well and the colony numbers were counted after several days. Relative clonal formation rate is the ratio of the number of clones in each group to the number of clones in the shCtrl group. (D) Fluorescence-activated cell sorting (FACS) quantification of apoptosis with PI+/Annexin V− or PI−/Annexin V+ NB4 cells. NB4 cells were infected with shYOD1 for 5 days. (E) The silencing efficiency of different shRNAs (#1 and #2) against YOD1 in NB4R1 and NB4R2 cells. (F) Cell proliferation of NB4R1 and NB4R2 cells infected with lentivirus-shYOD1s, as determined by trypan blue exclusion test for the indicated times. (G) Colony formation assay of NB4R1 and NB4R2 cells transduced with lentivirus-shYOD1s. 2000 cells were seeded per well and the colony numbers were counted after several days. Relative clonal formation rate is calculated as 4C. (H) FACS quantification of apoptosis with PI+/Annexin V+ or PI−/Annexin V+ NB4R1 and NB4R2 cells. Cells were infected with shYOD1s for 5 days. (A)–(H) Data are presented as mean ± SD (n = 3); *P < 0.05, **P < 0.01, and ***P < 0.001 vs. shCtrl or indicated. The significance analysis was conducted by one-way ANOVA analysis. (I)–(K) The effect of YOD1 on the NB4 tumor burden of NOD/SCID mice. NOD/SCID mice were transplanted with NB4-shCtrl or NB4-shYOD1 (#1 and #2) cells. (I) and (J) The mice were sacrificed 30 days after cell transplantation to detect the APL burden in bone marrow. Cell morphological analysis of bone marrow cells obtained and subjected to Wright–Giemsa staining (I). Blue arrow: APL blasts. Scale bar: 50 μm. The population of human CD45-positive and mouse CD45-negative (hCD45+/mCD45−) leukemia cells in the bone marrow of NOD/SCID mice were determined (J). Data are presented as mean ± SD (n = 3); ***P < 0.001 vs. shCtrl. The significance analysis was conducted by one-way ANOVA analysis. (K) The survival times of the NOD/SCID mice were recorded (n = 7); ***P < 0.001 vs. indicated. The significance analysis was conducted by Log-rank test. (L)–(N) The effect of YOD1 on the NB4R1 tumor burden of NOD/SCID mice. NOD/SCID mice were transplanted with NB4R1-shCtrl or NB4R1-shYOD1 (#1 and #2) cells. (M) Cell morphological analysis of bone marrow cells. (M) The population of hCD45+ mCD45− leukemia cells in the bone marrow. Data are presented as mean ± SD (n = 3). (N) The survival times of the NOD/SCID mice were recorded (n = 6); ***P < 0.001 vs. indicated. The significance analysis was conducted by Log-rank test.
(L218P and A216V) (Fig. 2D and E). Taken together, these results strongly indicate that YOD1 regulates the stability of both the wild-type and mutant forms of PML/RARα.

3.3. YOD1 deubiquitinates wild-type PML/RARα and its drug-resistant mutants

Given that YOD1 serves as a critical regulator of the stability of wild-type PML/RARα and its drug-resistant mutants, we next determined the mechanisms underlying this modulatory effect. First, we performed a co-immunoprecipitation assay to examine the physical interaction between YOD1 and PML/RARα. Results reveal that exogenously expressed YOD1-Flag and PML/RARα-HA (both the L and S isoforms) were robustly coprecipitated (Fig. 3A). In a reciprocal immunoprecipitation experiment, over-expressed HA-tagged PML/RARα could also be detected in the immunocomplex of YOD1-Flag (Fig. 3B). Similarly, different drug-resistant PML/RARα mutants were also in contact within YOD1, as confirmed by immunoprecipitation (Fig. 3C). Thus, these results indicate that YOD1 physically interacts with both the wild-type and mutant forms of PML/RARα.

DUBs enhance the stability of proteins by directly deconjugating the ubiquitin chain from substrates to enable their escape from proteasomal degradation. Thus, we first performed a deubiquitination assay in cells, and the results show that overexpression of YOD1 significantly reduced the ubiquitination level of wild-type PML/RARα, including both L and S isoforms (Fig. 3D). In line with these results, YOD1 also notably downregulated the ubiquitination levels of drug-resistant PML/RARα mutants (ΔF286, R276Q, L218P and A216V) (Fig. 3E), strongly supporting that YOD1 may deubiquitinate of PML/RARα. Therefore, an in vitro deubiquitination assay was utilized to validate the deubiquitinating effect of YOD1 on PML/RARα. We pulled down ubiquitinated PML/RARα proteins from cells and incubated them with recombinant wild-type YOD1 or its catalytically inactive mutant YOD1 (C160S) in a cell-free system. Results reveal that purified YOD1 effectively deubiquitinated PML/RARα in vitro, while YOD1 (C160S) failed to deubiquitinate PML/RARα (Fig. 3F), confirming the importance of YOD1 catalytic activity to PML/RARα deubiquitination. Similar results were also observed in in vitro deubiquitination experiment with all four drug-resistant PML/RARα mutants (ΔF286, R276Q, L218P and A216V) (Fig. 3G). Collectively, these results clearly demonstrate that YOD1 interacts with PML/RARα and functions as the deubiquitinase of PML/RARα to regulate its protein stabilization. Moreover, the evident deubiquitinating effect of YOD1 on drug-resistant mutants suggests that current drug-resistant mutational hotspots within PML/RARα does not interfere with its deubiquitination by YOD1.

3.4. Depletion of YOD1 leads to effective APL eradication in vitro and in vivo

Considering the observations that inhibiting YOD1 promotes proteasomal degradation of PML/RARα, we further assessed whether targeting YOD1 could arrest APL progression. First, two shRNAs targeting YOD1 were introduced into NB4 cells (Fig. 4A). Because PML/RARα promotes the proliferation of APL cells, we first determined the antiproliferative effect of shYOD1 on APL cells. Notably, YOD1 knockdown potently inhibited the proliferation (Fig. 4B) and colony formation ability (Fig. 4C) of NB4 cells. Meanwhile, PML/RARα is also known to protect APL cells from apoptosis and acts as a transcriptional repressor of RARα-target genes to block myeloid differentiation. Therefore, we detected the effects of shYOD1 on APL cell apoptosis and cell differentiation. We found that YOD1 depletion induced significant apoptosis of NB4 cells (Fig. 4D). Surprisingly, YOD1 depletion did not induce obvious apoptosis in non-APL AML cell lines (U937 and HL60) (Supporting Information Fig. S2A). Additionally, shYOD1 also significantly promoted CD11b expression in NB4 cells, which is a typical differentiation marker, but the CD11b expression of U937 and HL60 cell lines did not increase upon YOD1 knock down (Fig. 5B). These results indicate that silencing YOD1 in APL cells can suppress cell proliferation, induce cell apoptosis and cell differentiation in a PML/RARα-dependent manner.

Next, to confirm that YOD1 could be an effective therapeutic target in AML, we evaluated the effect of YOD1 inhibition on normal human hematopoietic stem cells (HSCs). The results demonstrate that YOD1 depletion showed little cytotoxicity in HSCs (Fig. S2C), which demonstrating the low potential toxic and side effects of targeting YOD1 and further supporting the hypothesis that targeting YOD1 may be a promising treatment of APL patients.

Then we further assessed the influence of YOD1 knockdown in drug-resistant APL cells. Genetic depletion of YOD1 was performed with shYOD1 (#1 and #2) in two drug-resistant APL cell strains (NB4R1 and NB4R2 cells) (Fig. 4E). Surprisingly, when transduced with shYOD1 lentivirus, most drug-resistant NB4R1 and NB4R2 cells lost the ability to proliferate and only multiplied twice in 5 days, whose proliferation rates were much lower than that transduced with shCtrl lentivirus (Fig. 4F). Similarity, the colony formation abilities of NB4R1 and NB4R2 cells were significantly suppressed by shYOD1 (Fig. 4G). Moreover, the numbers of apoptotic NB4R1 and NB4R2 cells were also remarkably increased by shYOD1 treatment (Fig. 4H). Taken together, these data validate that depletion of YOD1 exhibits strong suppressive effect on both APL and drug-resistant APL cells.

Furthermore, to test whether the suppressive activity of shYOD1 in APL cells could be reproduced in vivo, we utilized the APL xenograft mouse model. First, NB4 cells transduced with lentivirus containing shCtrl and shYOD1 (#1 and #2) were intravenously transplanted into NOD/SCID mice. When the shCtrl leukemic mice were moribund, we sacrificed three mice in each group to detect the APL burden in bone marrow. The morphological analysis of bone marrow cells showed that leukemic blasts were apparent in the shCtrl group, while they were rare in the shYOD1 groups (Fig. 4I). Moreover, we detected the population of hCD45^-mCD45^- cells in the bone marrow, which directly reflected the overall leukemic burden. Strikingly, the proportion of hCD45^-mCD45^- cells in the shCtrl group significantly increased (>1%), while these cells remained undetected (<1%) in the shYOD1 groups (Fig. 4J). Thus, both morphological analysis and proportion of hCD45^-mCD45^- demonstrate that YOD1 knockdown reduces the human APL burden in leukemic mice. Furthermore, all the shCtrl leukemic mice died rapidly within 1 month, while the overall survival time was dramatically prolonged in the shYOD1 (#1 and #2) groups (Fig. 4K). More importantly, similar therapeutic effects of shYOD1 were also observed in NB4R1 xenograft mice, as assessed by reduced APL burden (Fig. 4L and M) and prolonged overall survival time (Fig. 4N). Collectively, these results demonstrate that shYOD1 represses the invasion of leukemic blasts in both APL and drug-resistant APL xenograft models, further supporting that YOD1 is a promising therapeutic target for APL, particularly for drug-resistant APL treatment.
3.5. G5 inhibits the deubiquitination activity of YOD1 and triggers PML/RARα degradation

Since our data indicate that targeting YOD1 induces enzyme-dependent degradation of PML/RARα, we were inspired to apply a YOD1 inhibitor to eliminate PML/RARα for APL treatment. However, no small-molecule inhibitor of YOD1 has been reported to data. In order to give priority to the degradation effect of possible inhibitors on PML/RARα, we screened small molecules based on the level of PML/RARα protein. As shown in Fig. 5A and Supporting Information Fig. S3, we added a library of 31 reported DUB inhibitors to cells that stably expressed PML/RARα and then measured their effect on the protein level of PML/RARα. Three inhibitors (G5, EOAI3402143 and spautin-1) were found to...
Figure 6  
G5 shows a strong inhibitory effect on APL to overcome resistance by targeting YOD1. (A) Proliferation of NB4 cells treated with G5 (0, 50, 100, and 200 nmol/L) and ATRA (1 μmol/L), as measured by trypan blue exclusion test for the indicated times. (B) Colony formation assay of NB4 cells treated with G5 (100 nmol/L) and ATRA (100 nmol/L). 1000 cells were seeded per well and the colony numbers were counted after several days. Relative clonal formation rate is the ratio of the number of clones in each group to the number of clones in the control group. (C) FACS quantification of apoptosis with PI⁻/Annexin V⁺ or PI⁺/Annexin V⁺ NB4 cells. The cells were treated with G5 (0, 50, 100, and 200 nmol/L) for 48 h. (D)–(E) Proliferation of NB4R1 and NB4R2 cells. The cells were treated with G5 (0, 50, 100, and 200 nmol/L) and ATRA (1 μmol/L), and a trypan blue exclusion test was performed for the indicated times. (F)–(G) Colony formation assay of NB4R1 and NB4R2 cells treated with G5 (100 nmol/L) and ATRA (100 nmol/L). 2000 cells were seeded per well and the colony numbers were counted after several days. Relative clonal formation rate is the ratio of the number of clones in each group to the number of clones in the control group. (H) FACS quantification of apoptosis with PI⁻/Annexin V⁺ or PI⁺/Annexin V⁺ NB4R1 and NB4R2 cells. The cells were treated with G5 (0, 50, 100, and 200 nmol/L) for 48 h. (A)–(H) Data are presented as mean ± SD (n = 3); n.s., P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001 vs. Control or indicated. The significance analysis was conducted by one-way ANOVA analysis. (I) The effect of G5 on tumor growth in an NB4R2 xenograft nude mouse model. NB4 xenografts were established by subcutaneous injection of cells into nude mice (n = 6). G5 (20 or 40 mg/kg) was administered to the mice by intravenous injection into the tail vein every other day. ATRA was administered by intragastric injection every other day. (I) Tumor growth of the NB4R2 xenografts. Tumor volume growth curves are based on mean ± SE. (J) Tumor weight of the NB4R2 xenografts on Day 13. Data are presented as mean ± SD. (K) Effects of G5 on tumor size and tumor weight at pre-dose and post-dose. RTV, relative tumor volume; T/C (%) = RTV_Treatment/RTV_Control × 100. Criteria for therapeutic activity: T/C (%), optimal growth inhibition ≤ 50 = effective. Data are represented as mean ± SD. (K)–(L) n.s., P > 0.05, ***P < 0.001 vs. Control. The significance analysis was conducted by one-way ANOVA. (L)–(M) FACS quantification of apoptosis with PI⁻/Annexin V⁺ or PI⁺/Annexin V⁺ primary APL cells. Primary APL cells derived from the peripheral blood of patients at diagnosis were treated with G5 (200 nmol/L) and ATO (2 μmol/L) for 72 h. Data are presented as mean ± SD (n = 12); ***P < 0.001 vs. indicated. The significance analysis was conducted by two-tailed unpaired Student’s t-test.
dramatically reduce the PML/RARα protein level. Among them, G5 exhibited the greatest effect of lowering PML/RARα protein level.

Next, to determine whether the inhibitors-induced PML/RARα decline is dependent on YOD1, we conducted the in vitro deubiquitination assay. As displayed in Fig. 5B, recombinant YOD1 was pretreated with DUB inhibitors or DMSO and then incubated with ubiquitinated PML/RARα. Finally, the ubiquitination level of PML/RARα was measured. Results show that G5 significantly destroyed the YOD1-induced decline of PML/RARα ubiquitination level, suggesting that the deubiquitination activity of YOD1 was almost completely blocked by G5 (Fig. 5C). By contrast, EOAI3402143 and spautin-1 did not affect the deubiquitination activity of YOD1 on the PML/RARα, indicating that they may affect the stability of PML/RARα through other mechanisms. Moreover, the inhibitory effect of G5 on the deubiquitination activity of YOD1 was concentration-dependent (Fig. 5D), further indicating G5 as a potent inhibitor of YOD1.

Finally, we then evaluated whether G5 could trigger the degradation of endogenous wild-type PML/RARα and its drug-resistant mutants. Results show that G5 potently reduced PML/RARα protein in NB4 cells, while EOAI3402143 slightly affected PML/RARα protein levels and spautin-1 exhibited no effect at the same concentrations (Fig. 5E). More encouragingly, G5 also triggered the strong degradation of mutated PML/RARα in NB4R1 and NB4R2 cells (Fig. 5F). Together, these data demonstrate that G5 promises to be an effective YOD1 inhibitor to induce PML/RARα degradation by inhibiting the deubiquitination activity of YOD1.

3.6. G5 shows a strong inhibitory effect on APL to overcome resistance by targeting YOD1

Since G5 was shown to drive PML/RARα degradation by inhibiting YOD1, we next assessed the capacity of G5 to suppress APL and relieve drug resistance. Treatment with G5 at 50–200 nmol/L effectively impaired the proliferation of NB4 cells, with most cells losing the ability to multiply, while ATRA at 1 μmol/L showed only an approximate 50% suppressive effect on NB4 cells (Fig. 6A). Moreover, G5 destroyed most of the clonal formation ability of NB4 cells with higher efficiency than ATRA (Fig. 6B). In addition, G5 potently induced apoptosis of NB4 cells in a concentration-dependent manner (Fig. 6C). Then, we attempted to identify the inhibitory effect of G5 on drug-resistant APL cells. Compared with the little suppressive ability of ATRA, G5 dramatically decreased the proliferation capacity of both the NB4R1 and NB4R2 cell lines (Fig. 6D and E). Similar results were obtained in the colony formation assay. G5 decreased the colony number of drug-resistant APL cells (NB4R1 and NB4R2), while ATRA failed to reduce their colony number (Fig. 6F and G). In addition, significant apoptosis of these drug-resistant APL cells were obtained in the colony formation assay. G5 decreased the colony number of drug-resistant APL cells (NB4R1 and NB4R2), indicating that they may affect the stability of PML/RARα through other mechanisms. Moreover, the inhibitory effect of G5 on the deubiquitination activity of YOD1 was concentration-dependent (Fig. 5D), further indicating G5 as a potent inhibitor of YOD1.

Studies have proposed that YOD1 is involved in several biological activities in mammalian cells, including endoplasmic reticulum-associated degradation (EARD)47, the clearance of damaged lysosomes48 and antigen cross-presentation49. Moreover, YOD1 is closely associated with cancers. YOD1 is highly expressed in human liver cancer tissue and promotes cell migration and colony formation. Mechanistically, YOD1 regulates the hippo signaling pathway by regulating the ubiquitination level of

4. Discussion

Currently, ATRA/ATO with chemotherapy is the standard of care for APL, which still remaining hurdles including serious adverse events and drug resistance6,8. For ATRA/ATO-sensitive APL patients, once side effects occurred too severely to continue treatment. Our study validates YOD1 as a potential therapeutic target for APL, thus new compounds (such as YOD1 inhibitors) may well be the choice for such patients. More importantly, there are currently no drugs available for ATRA/ATO-resistant APL patients. Our results show that depletion of YOD1 exhibits strong suppressive effect on drug-resistant APL cells in vitro and in vivo, indicating that YOD1 is a promising therapeutic target for drug-resistant APL treatment.

Most fusion proteins, including PML/RARα, are recognized as major drivers of human tumorigenesis37,38. Due to their powerful roles in tumorigenesis and development, oncofusion proteins are ideal therapeutic targets1,39. But there still remain many challenges in targeting oncofusion proteins. On the one hand, tyrosine kinase oncofusions are considered druggable by kinase inhibitors, but prolonged treatment always leads to mutation-mediated acquired drug-resistance40. On the other hand, transcription factor oncofusions are difficult to target directly41. Notably, DUB dysregulation has been found to contribute to abnormal accumulation of various cancer-promoting proteins, such as MYC26, KRAS42, yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ)43, and murine double minute 2 (MDM2)44. Thus, we hypothesized that degrading oncofusion proteins by targeting DUB could be a promising therapeutic strategy for oncofusion-driven tumors. Studies have confirmed the role of DUBs in regulating the stability of some oncofusion proteins, including promyelocytic leukemia zinc finger/retinoic acid receptor α (PLZF/RARα)-USP3757 and internal tandem duplication within FLT3 (FLT3/ITD)-USP1046. Our study indicates that YOD1, the specific DUB of PML/RARα, is a key factor to maintain PML/RARα stability. Inhibiting YOD1 triggers significant clearance of the PML/RARα oncoprotein, thus ultimately exerts a therapeutic effect on APL. This discovery sets a great example for the realization of oncofusion protein degradation by targeting DUBs as a practicable therapeutic strategy in oncofusion-driven tumors.

Studies have proposed that YOD1 is involved in several biological activities in mammalian cells, including endoplasmic reticulum-associated degradation (EARD)47, the clearance of damaged lysosomes48 and antigen cross-presentation49. Moreover, YOD1 is closely associated with cancers. YOD1 is highly expressed in human liver cancer tissue and promotes cell migration and colony formation. Mechanistically, YOD1 regulates the hippo signaling pathway by regulating the ubiquitination level of
the ubiquitin ligase ITCH targeting large tumor suppressor kinase (LATS)\textsuperscript{50,51}. These studies demonstrate the oncogenic function of YOD1 and indicate that YOD1 is a potential target for hepatic carcinoma. However, the functions of YOD1 in other cancers require further studies. Our current study identifies PML/RAR\textalpha as a new substrate of YOD1 and indicates YOD1 as an appropriate target for APL treatment. Importantly, we have proved both the specificity and safety of targeting YOD1 by evaluating the effects on non-APL cell lines and normal human HSCs, which showed little cytotoxicity due to lack of PML/RAR\textalpha (Fig. S2). Additionally, Dai et al.\textsuperscript{52} constructed Yod1 gene knockout (KO) mice and found that the Yod1-KO mice bore pups normally without embryonic lethality, and no significant pathological phenotype was observed in the Yod1-KO mice. These studies may suggest the relative safety of inhibiting YOD1. Taken together, our results indicate that YOD1 can be a potential drug target for treating APL.

Most DUBs contain catalytically active cysteine residues, making them highly possible to be intervened by small compounds. DUBs are classified into six families, including ubiquitin carboxyl terminal hydrolase (UCH) family, USP family, OTU family, Machado–Joseph domain-containing protease (MJD) family, Jab1/Par1/MPN domain-containing protease (JAMM) family and motif-interacting with ubiquitin-containing novel DUB family (MINDY). However, the degree of research on DUB inhibitors varies considerably in different DUB families. The current studies on small-molecule inhibitors of DUBs mainly focus on UCH family and USP family, rather than OTU family\textsuperscript{53}. Notably, OTU family is Ub linkage-specific\textsuperscript{54}, which indicates the relatively small probability of mutual compensation among different OTUs, even though they show structural similarity. Therefore, OTU inhibitors, such as YOD1 inhibitors, may exhibit higher efficiency than other DUB family inhibitors. In this work, we generated a screening model based on the stability of PML/RAR\textalpha and speculated that G5 would be the first YOD1 inhibitor and the first OTU inhibitor.

A previous study revealed that G5 targeted the ubiquitin-proteasome system by inhibiting ubiquitin isopeptidases\textsuperscript{55}. Later, G5 was identified to target the deubiquitinating activity of BRCC3 and subsequently inhibited NLRP3 inflammasome activation\textsuperscript{54,55}. G5 has also been reported to induce the apoptosis of IMR90-E1A human fibroblasts at a concentration of 1.25 μmol/L and apoptosis-resistant double-deficient BAX/BAK (DKO) mouse embryo fibroblasts at a concentration of 5 μmol/L\textsuperscript{56}. However, there is no current research that directly investigates the possible role of G5 in tumor cells. Our results reveal the obvious suppressive effect of G5 on leukemia cells for the first time. Notably, the concentration of G5 (200 nmol/L) to induce APL cell apoptosis was found to be much lower than that needed to induce normal cell apoptosis. More importantly, because of the obvious inhibitory effects of G5 on drug-resistant APL, our work indicates that G5 may be effective in overcoming APL drug resistance.

5. Conclusions

In summary, our study provides new insights into the function of YOD1 in APL progression by deubiquitinating and stabilizing the PML/RAR\textalpha oncoprotein. Then we demonstrate that suppression of YOD1 by shRNA or its newfound inhibitor G5 triggers the degradation of PML/RAR\textalpha to inhibit drug-resistant APL both in vitro and in vivo. Taken together, our study not only reveal the DUB-related regulatory mechanism on ubiquitination degradation of PML/RAR\textalpha and validate YOD1 as a potential therapeutic target for APL, but also suggest G5 as a YOD1 inhibitor to be the promising candidate to cure APL, particularly drug-resistant APL patients.

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Author contributions

Meidan Ying and Xuejing Shao conceived the study and analyzed data; Meidan Ying, Xuejing Shao, Yingqian Chen and Wenxin Du wrote the manuscript. Xuejing Shao and Yingqian Chen performed dual-luciferase reporter system screening and cell-based YOD1 inhibitor screening assay; Xuejing Shao, Yingqian Chen, Shaowei Bing and Wenxin Du performed the immunoprecipitation and Western blotting procedure; Yingqian Chen and Wei Wang performed the in vitro ubiquitination assay; Yingqian Chen, Wei Wang, Xingya Zhang and Minyi Cai performed the cellular proliferation analysis, soft agar formation assay and cell apoptosis assay; Xuejing Shao, Yingqian Chen and Wei Wang performed the animal studies; Ji Cao, Xiaojun Xu, Qiaojun He and Bo Yang conceived the experiments and helped organize the paper.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2021.10.020.

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