

# UBC9 silencing-mediated PPAR $\alpha$ desumoylation induces inhibition of cell proliferation by ferroptosis in acute myeloid leukemia

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## Keywords

acute myeloid leukemia, UBC9, PPAR $\alpha$ , ferroptosis, sumoylation

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## Abstract

### Introduction

Inhibited acute myeloid leukemia (AML) proliferation is accompanied by downregulated peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ), which however can be stabilized via sumoylation. This study investigated how PPAR $\alpha$  sumoylation impacts on AML cell growth.

### Material and methods

Human AML HL-60 and tohoku hospital pediatrics-1 (THP-1) cells were treated with the PPAR $\alpha$  inhibitor, GW6471 (10  $\mu$ M), for 24 and 48 h. THP-1 cells were exposed to the PPAR $\alpha$  agonist, pirinixic acid (10  $\mu$ M), after the expression of the small ubiquitin-like modifier proteins (SUMO)-conjugating enzyme UBC9 was manipulated. The interaction between PPAR $\alpha$  and SUMO1 was detected by immunoprecipitation assay. HL-60 and THP-1 cell viability, apoptosis and ferroptosis were measured via cell counting kit-8 assay, flow cytometry, BODIPY-C11 staining and/or colorimetric assay. UBC9, glutathione peroxidase 4 (GPX4), recombinant solute carrier family 7, member 11 (SLC7A11) and PPAR $\alpha$  expressions were analyzed by qRT-PCR or Western blot.

### Results

GW6471 treatment for 24 and 48 h suppressed viability, promoted apoptosis and lipid peroxidation, increased the level of Fe<sup>2+</sup>, and decreased the expressions of GPX4, SLC7A11 and PPAR $\alpha$  in HL-60/THP-1 cells. PPAR $\alpha$  antibody induced enrichment of PPAR $\alpha$  and SUMO1 in THP-1 cells, which was attenuated after UBC9 silencing. UBC9 silencing resulted in viability decrease, apoptosis and lipid peroxidation promotion, Fe<sup>2+</sup> upregulation, and GPX4, SLC7A11 and PPAR $\alpha$  downregulation in THP-1 cells, which were all counteracted by pirinixic acid.

### Conclusions

UBC9 silencing-induced PPAR $\alpha$  desumoylation induces suppression of AML cell growth by ferroptosis.

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**UBC9 silencing-mediated PPAR $\alpha$  desumoylation induces inhibition of cell proliferation by ferroptosis in acute myeloid leukemia**

**Running title:** Effects of PPAR $\alpha$  on AML

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**Methods:** Human AML HL-60 and tohoku hospital pediatrics-1 (THP-1) cells were treated with the PPAR $\alpha$  inhibitor, GW6471 (10  $\mu$ M), for 24 and 48 h. THP-1 cells were exposed to the PPAR $\alpha$  agonist, pirinixic acid (10  $\mu$ M), after the expression of the small ubiquitin-like modifier proteins (SUMO)-conjugating enzyme UBC9 was manipulated. The interaction between PPAR $\alpha$  and SUMO1 was detected by immunoprecipitation assay. HL-60 and THP-1 cell viability, apoptosis and ferroptosis were measured via cell counting kit-8 assay, flow cytometry, BODIPY-C11 staining and/or colorimetric assay. UBC9, glutathione peroxidase 4 (GPX4), recombinant solute carrier family 7, member 11(SLC7A11) and PPAR $\alpha$  expressions were analyzed by qRT-PCR or Western blot.

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**Conclusion:** UBC9 silencing-induced PPAR $\alpha$  desumoylation induces suppression of AML cell growth by ferroptosis.

**Keywords:** acute myeloid leukemia; PPAR $\alpha$ ; sumoylation; UBC9; ferroptosis

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## Introduction

Acute myeloid leukemia (AML) develops from the malignant transformation of the stem cell precursors of the myeloid lineage (1), constituting 15% to 20% of leukemia in the United States, with 21,450 cases diagnosed in 2019 (2). The progression of AML is driven by genetic variations that trigger neoplastic changes and further ensue clonal proliferation (1). Excessive proliferation and subsequent accumulation of immature myeloid cells in the bone marrow and peripheral blood interfere with the generation of normal blood cells, resulting in abnormal hematopoiesis (4). AML is a biologically hematologic disease group, characterized by malignant clonal proliferation of immature myeloid cells in the bone marrow, leading to disruption of normal hematopoiesis and bone marrow failure (5). Currently, in the context of advance in therapies for AML, the survival curve of patients still remains stagnant, due to disease relapse as a consequence of unhindered cell proliferation following hematopoietic stem cell transplantation (6). Therefore, novel approaches to potently suppress expansion of AML cells are in dire need of exploration.

Ferroptosis is a newly identified regulated cell death featured by the accumulation of iron-dependent lipid peroxides to fatal levels (7). Morphologically, biologically and genetically separate from apoptosis, ferroptosis is deemed as regulated necrosis (8). Glutathione peroxidase 4 (GPX4) is a key regulator of ferroptosis and plays a crucial role in converting lipid hydroperoxides to non-toxic lipid (9). Additionally, inhibition of recombinant solute carrier family 7, member 11 (SLC7A11), the light chain of system xc<sup>-</sup>, attenuates GSH level and GPX4 activity, resulting in the accumulation of lethal lipid peroxides and the induction of ferroptosis (10). Inducing ferroptosis enhances anti-leukemic activity (11) and serves as a mechanism underlying chemotherapy targeting AML (12).

Ferroptosis and cell fate are impacted by genetic cues (13). Recent improvement in the understanding of the genetic variations in AML progression contributes to developing some promising novel therapies for better outcomes (14). Adipose triacylglyceride lipase (ATGL) silencing-induced inhibition of AML cell proliferation is concomitant with downregulation of peroxisome proliferator-activated receptor

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alpha (PPAR $\alpha$ ) (15). PPAR $\alpha$  is a ligand-activated transcription factor, belonging to the NR1C nuclear receptor subfamily that has many members implicated in large-scale remodeling of lipid homeostasis (16), which affects the sensitivity of cells to ferroptosis (17). Besides, PPAR $\alpha$  agonists alleviate Erastin-induced ferroptotic death in liver cancer cells (18). Nevertheless, whether PPAR $\alpha$  inhibition blocks AML cell growth through inducing ferroptosis has yet to be verified.

Sumoylation is a reversible post-translational modification modulating protein stability, nuclear-cytosolic transport, and transcriptional regulation (19). Sumoylation is mediated by small ubiquitin-like modifier proteins (SUMOs), like SUMO1, which is covalently attached to and detached from other proteins in cells to alter their function (20). PPAR $\alpha$  is susceptible of sumoylation modification, and desumoylating PPAR $\alpha$  can promote its ubiquitination-mediated degradation (21). Through a String-predicted PPAR $\alpha$ -protein interaction map, UBE2I (ubiquitin carrier protein 9, UBC9), the only E2 SUMO-conjugating enzyme, was identified as a protein able to interact with PPAR $\alpha$ . A previous study has documented that knockdown of UBC9 inhibits AML cell proliferation (22).

Accordingly, we made a hypothesis that blocking sumoylation modification of PPAR $\alpha$  poses PPAR $\alpha$  degradation, which inhibits cell proliferation in AML by facilitating ferroptosis. The present study set out to test this hypothesis through establishing an *in vivo* AML model under the influence of the PPAR $\alpha$  inhibitor or the PPAR $\alpha$  agonist plus UBC9 silencing.

## **Materials and methods**

### **Cell culture**

Human AML HL-60 cell lines and THP-1 cell lines were purchased from American Type Culture Collection (ATCC; CCL-240 and TIB-202, Manassas, VA, USA). HL-60 cells were cultured in ATCC-formulated Iscove's Modified Dulbecco's Medium (30-2005, ATCC, USA), supplemented with 20% fetal bovine serum (FBS; HY-P2352, MedChemExpress, Monmouth Junction, NJ, USA). A complete medium comprising ATCC-formulated RPMI-1640 medium (30-2001, ATCC, USA), 10% FBS and 0.05 mM 2-mercaptoethanol (21985023, ThermoFisher, Waltham, MA, USA)

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was used to maintain THP-1 cells. Cell culture was performed with 5% CO<sub>2</sub> at 37°C.

### **Cell transfection**

THP-1 cells were transfected with small interfering RNA targeting UBC9 (siUBC9; SR305005, OriGene, Rockville, MD, USA) or its negative control (siNC; PS100001, OriGene, USA) utilizing Lipofectamine 3000 transfection reagent (L3000015, ThermoFisher, USA). In short,  $1 \times 10^4$  THP-1 cells were seeded in each well of 96-well plates and cultured to form an 80% confluent monolayer. The above plasmids and Lipofectamine 3000 transfection reagent were diluted and incubated together for 15 min at 37°C to form gene-lipid complexes. The complexes were then incubated for 48 h with the monolayer, and the transfection efficiency was checked by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

### **QRT-PCR**

Total RNA from transfected/nontransfected THP-1 cells were obtained using Trizol reagents (15596026, ThermoFisher, USA) with a diluted concentration of 100 µg/mL, followed by reverse transcription employing a reverse transcription kit (K1622, Yaanda Biotechnology, Beijing, China) to generate cDNA. QPCR was conducted to achieve cDNA amplification in a PCR System (7500 Fast Real-Time, ThermoFisher, USA) with the help of TB Green Premix Ex Taq (RR420Q, Takara, Tokyo, Japan). The reaction conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 58°C for 60 sec. Relative mRNA levels were examined with the  $2^{-\Delta\Delta Ct}$  method (23) and normalized against the level of GAPDH. The primers used included: UBC9, forward: 5'-AAAAATCCCGATGGCACGATG-3', reverse: 5'-CTTCCCACGGAGTCCCTTTC-3'; GAPDH, forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'.

### **Immunoprecipitation (IP) assay**

Pierce Co-Immunoprecipitation kit (26149, ThermoFisher, USA) was utilized to examine the interaction between PPAR $\alpha$  and SUMO1. Simply put, siUBC9-transfected THP-1 cells were lysed using IP Lysis/Wash Buffer. After being precleared by Agarose Resin, the obtained lysates were centrifuged (2000 $\times$ g) at 4°C

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for 20 min to harvest supernatant. Agarose Resin-coupled PPAR $\alpha$  antibody (ab227074, 1:100, Abcam, Cambridge, UK) or normal Rabbit IgG (ab171870, Abcam, UK) was used to precipitate target proteins in the supernatant. After an overnight incubation at 4°C, the immunocomplexes were eluted using elution buffer (21009, ThermoFisher, USA) for 5 min and finally detected by Western blot to analyze the number of PPAR $\alpha$  and SUMO1 proteins.

### **Cell treatment**

HL-60 and THP-1 cells were treated with 10  $\mu$ M PPAR $\alpha$  inhibitor, GW6471, (sc-300779, Santa Cruz Biotechnology, Santa Cruz, CA, USA) pre-dissolved by dimethyl sulphoxide (sc-358801, Santa Cruz Biotechnology, USA) in their medium at 37°C for 24 and 48 h (18). The PPAR $\alpha$  agonist, pirinixic acid, (S8029, 10  $\mu$ M, Selleckchem, Houston, TX, USA) pre-dissolved in ethanol was applied to incubate transfected/nontransfected THP-1 cells at 37°C for 16 h (18). Untreated cells acted as the control group.

### **Viability assay**

HL-60 and transfected/nontransfected THP-1 cells ( $2 \times 10^3$ ) were seeded in each well of 96-well plates, and subjected to the treatment mentioned above. Each well was then filled with cell counting kit (CCK)-8 reagent (10  $\mu$ L, CA1210, Solarbio, Beijing, China) and incubated for 2 h at 37°C. The absorbance at 450 nm was read by a microplate reader (EMax Plus, Molecular Devices, Sunnyvale, CA, USA).

### **Apoptosis assay**

HL-60 and transfected/nontransfected THP-1 cells were treated as mentioned above, and their apoptosis rate was determined employing Annexin V-FITC Early Apoptosis Detection kits (#6592, Cell Signaling Technology, Danvers, MA, USA). Briefly, the treated cells ( $1 \times 10^6$ ) were harvested via centrifugation at  $2000 \times g$  for 10 min. After being washed with pre-cooled phosphate buffered saline (PBS; #9808, Cell Signaling Technology, USA), the cells were resuspended in Annexin-V binding buffer. The cell suspension (96  $\mu$ L) was incubated with 1  $\mu$ L Annexin V-FITC and 12.5  $\mu$ L propidium iodide solution for 10 min in the dark. Lastly, the stained cells were again suspended with Annexin-V binding buffer, and analyzed by a flow cytometer

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(Cytoflex, Beckman Coulter, Brea, CA, USA).

### **BODIPY-C11 staining**

Following the above-mentioned treatment, HL-60 and transfected/nontransfected THP-1 cells were stained with 5  $\mu$ M BODIPY-C11 (D3861, ThermoFisher, USA) in 1 mL of their media for 20 min at 37°C. After that, excessive stain was removed via PBS wash, and the cells were filtered using a 0.4  $\mu$ m nylon filter. The activity of lipid peroxidation was reflected by the level of lipid reactive oxygen species (ROS), and its fluorescence was detected by the flow cytometer.

### **Fe<sup>2+</sup> level assessment**

Intracellular levels of Fe<sup>2+</sup> were measured using iron kits (ab83366, Abcam, USA). HL-60 and transfected/nontransfected THP-1 cells ( $2 \times 10^5$ /well) were plated in 6-well plates, treated as above mentioned, and reacted with assay buffer for 30 min at 37°C. Then, after incubation with 1  $\mu$ mol Fe<sup>2+</sup> probes for 60 min at 37°C, the cells were subjected to absorbance detection by the microplate reader at 593 nm.

### **Western blot**

HL-60 and transfected/nontransfected THP-1 cells following the above-mentioned treatment were lysed using RIPA Lysis Buffer (20-188, Sigma-Aldrich, St. Louis, MO, USA) to isolate total protein. Then, the protein concentration was determined using the bicinchoninic acid kit (A53227, ThermoFisher, USA). After being denatured by boiling at 95°C for 5 min, 30  $\mu$ g isolated proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (1615100, BIO-RAD, Hercules, CA, USA), electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane (1620256, BIO-RAD, USA), and blocked in 5% skim milk for 1 h at room temperature. Primary antibodies for PPAR $\alpha$  (ab227074, 52 kDa, 1:500, Abcam, UK), SUMO1 (ab32058, 12 kDa, 1:1000, Abcam, UK), SLC7A11 (ab175186, 55 kDa, 1:1000, Abcam, UK), GPX4 (ab125066, 17 kDa, 1:1000, Abcam, UK) and GAPDH (ab8245, 37 kDa, 1:500, Abcam, UK) were utilized to probe the membrane overnight at 4°C. The membrane was then washed thrice with TWEEN-20 PBS (28352, ThermoFisher, USA), followed by incubation with Goat anti-Rabbit IgG secondary antibodies (ab97051, Abcam, UK)

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for 2 h at room temperature. On an imaging system (LAS-3000, Fujifilm, Tokyo, Japan), proteins were developed with Clarity™ Western ECL Substrate (1705060, BIO-RAD, USA). The grayscale value of each protein band was determined using ImageJ software (3.0 version, National Institutes of Health, Bethesda, MA, USA).

### **Statistical analysis**

All statistical values were presented as mean  $\pm$  standard deviation (SD) from experiments repeated thrice. Statistical analysis was performed with GraphPad prism (version 8.0, GraphPad Software Inc., San Diego, CA, USA). Data among multiple experimental groups were compared via one-way analysis of variance (ANOVA), followed by Tukey post hoc test. Statistical significance was concluded at  $P < 0.05$ .

### **Results**

#### **GW6471 treatment for 24 and 48 h decreased viability and promoted apoptosis and ferroptosis of HL-60/THP-1 cells.**

Treatment with GW6471, the PPAR $\alpha$  inhibitor, for 24 and 48 h led to decreased viability (Fig. 1A-B,  $P < 0.05$ ), markedly promoted apoptosis (Fig. 1C-E,  $P < 0.001$ ), and enhanced activity of lipid peroxidation in HL-60 and THP-1 cells (Fig. 1F-H,  $P < 0.001$ ). Besides, HL-60 and THP-1 cells treated with GW6471 for 24 and 48 h exhibited an increased level of Fe<sup>2+</sup> (Fig. 1I-J,  $P < 0.01$ ).

#### **GW6471 treatment for 24 and 48 h dwindled the expressions of ferroptosis inhibition-related proteins and PPAR $\alpha$ in HL-60/THP-1 cells.**

Moreover, the expressions of ferroptosis inhibition-related proteins, GPX4 and SLC7A11, were decreased in HL-60 and THP-1 cells after 24 and 48 h of GW6471 treatment (Fig. 2A-E,  $P < 0.05$ ). Undoubtedly, at 24 and 48 h post GW6471 treatment, HL-60 and THP-1 cells presented an evident downregulation of PPAR $\alpha$  expression (Fig. 2F-H,  $P < 0.001$ )

#### **UBC9 silencing attenuated the PPAR $\alpha$ antibody-induced enrichment of PPAR $\alpha$ and SUMO1 in THP-1 cells.**

Since THP-1 cells showed stronger lipid peroxidation activity than HL-60 cells after GW6471 treatment, THP-1 cells were used in the subsequent experiments. By transfection with siUBC9, UBC9, the only E2 SUMO-conjugating enzyme, was

silenced in THP-1 cells (Fig. 3A,  $P < 0.001$ ). IP assay was conducted to verify the UBC9-mediated sumoylation of PPAR $\alpha$ . As shown in Fig. 3B, PPAR $\alpha$  antibodies precipitated both PPAR $\alpha$  protein and SUMO1 protein in siNC-transfected THP-1 cells, but precipitated a fewer number of these two proteins in siUBC9-transfected THP-1 cells (Fig. 3B).

**UBC9 silencing-induced viability decrease and promotion of apoptosis and ferroptosis of THP-1 cells were counteracted by pirinixic acid.**

UBC9 silencing decreased the viability of THP-1 cells (Fig. 4A,  $P < 0.01$ ), which however was resisted by the PPAR $\alpha$  agonist, pirinixic acid (Fig. 4A,  $P < 0.001$ ). Also, THP-1 cells transfected with siUBC9 showed an increased rate of apoptosis (Fig. 4B-C,  $P < 0.001$ ), and this siUBC9-induced apoptosis promotion was mitigated by pirinixic acid (Fig. 4B-C,  $P < 0.001$ ). Moreover, UBC9 silencing augmented the activity of lipid peroxidation and the level of Fe<sup>2+</sup> in THP-1 cells (Fig. 4D-F,  $P < 0.001$ ), which were reversed by pirinixic acid (Fig. 4D-F,  $P < 0.001$ ).

**UBC9 silencing-induced GPX4, SLC7A11 and PPAR $\alpha$  downregulation was counteracted by pirinixic acid.**

The expressions of GPX4 and SLC7A11 as well as PPAR $\alpha$  were diminished in THP-1 cells due to UBC9 silencing (Fig. 5A-E,  $P < 0.001$ ), while such downregulation of GPX4, SLC7A11 and PPAR $\alpha$  in THP-1 cells was neutralized partly by pirinixic acid (Fig. 5A-E,  $P < 0.05$ ).

## **Discussion**

Despite the recent approval of novel promising therapies for AML, treatment of AML still remains a challenge due to the frequent disease relapse (6, 24). Relapse arises from unhindered cell proliferation, which is often associated with cytogenetic abnormalities in AML (25). Targeting these abnormalities can contain the progression of AML (26). Moreover, genes involved in ferroptosis signaling pathways have been reported to impact on AML patients' prognosis (27). The present study uncovered that destabilizing PPAR $\alpha$  can promote ferroptosis to inhibit AML cell proliferation.

PPAR $\alpha$ , the first identified member of the NR1C nuclear receptor subfamily, is predominantly expressed in the liver (28). PPAR $\alpha$  can be activated by peroxisome

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proliferators, which elicit peroxisome proliferation and liver cancer in mice (29). PPAR $\alpha$  contributes to mono(2-ethylhexyl) phthalate-caused acceleration of ovarian cancer development (30). Conversely, intestinal PPAR $\alpha$  has been recorded to protect mice against colon carcinogenesis (31). Previously, Luo et al. reported that the ubiquitin-dependent proteasomal degradation of PPAR $\alpha$  by Tribbles homolog 3 reduces apoptosis and autophagy of AML cells, implying that PPAR $\alpha$  plays a tumor-suppressive role in AML (32). Notably, contrary to Luo's finding, our study presented that PPAR $\alpha$  inhibition caused by GW6471 treatment decreased AML/THP-1 cell viability and enhanced cell apoptosis, suggesting that PPAR $\alpha$  was oncogenic in AML. Our results are supported by Li's study where PPAR $\alpha$  expression is downregulated when ATGL silencing induces inhibition of AML cell proliferation (15).

In response to ligand stimulation, PPAR $\alpha$  functions as a transcriptional program that greatly augments fatty acid uptake and breakdown through fat acid oxidation (33). PPAR $\alpha$  can act as a transcriptional activator inducing Multidrug Resistance Protein2 (Mdr2) expression to mediate hepatobiliary cholesterol transport in mice (34). By enhancing PPAR $\alpha$  activity, pharmacological fibrates are able to lower triglyceride (35). In addition, PPAR $\alpha$  activation by fasting promotes hepatic fatty acid oxidation and gluconeogenesis (29). Ferroptosis is a form of programmed cell death, the sensitivity of which can be increased with accumulation of oxidized cellular membrane phospholipids (36). Since PPAR $\alpha$  enhances fatty acid oxidation to reduce lipid accumulation (37), PPAR $\alpha$  is expected to antagonize ferroptosis, which is corroborated by previous findings that PPAR $\alpha$  activation mitigates Erastin-induced liver cancer cell ferroptosis (18). Ferroptosis occurs when fenton reaction induces iron-dependent lethal lipid peroxidation, and Fe<sup>2+</sup> is needed for conversion into Fe<sup>3+</sup> to run the reaction (38). GPX4 is an enzyme preventing the toxicity of lipid peroxides to maintain the homeostasis of membrane lipid bilayers (39). To initiate ferroptosis, GPX4 inactivation is caused by RSL3, leading to the accumulation of lipid peroxides (7). SLC7A11 is a key component of system x<sub>c</sub><sup>-</sup>, through which extracellular cystine is transported into cells by exchange of intracellular glutamate and then converted into

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cysteine, and cysteine is required for the generation of glutathione, whose depletion also gives rise to GPX4 inactivation (40). Also, repression of SLC7A11 impairs cysteine import to deplete glutathione, thereby inactivating GPX4 to trigger ferroptosis (41). Ferroptosis is more immunogenic than apoptosis in causing cell death (8), and stimulating ferroptotic pathway may contribute to immunotherapy against cancers including AML (42, 43). Promoting ferroptosis by all-trans retinoic acid derivative has been proven to facilitate AML cell differentiation and thereby prevent AML progression (44). Our study provided a new finding that GW6471-caused PPAR $\alpha$  inhibition boosted ferroptosis of AML/THP-1 cells by elevating lipid peroxidation and Fe<sup>2+</sup> levels and inhibiting the production of GPX4 and SLC7A11, hinting that repressing PPAR $\alpha$  activity can retard AML cell growth.

Sumoylation, a type of reversible post-translational modification, can modify genes to alter their functions in AML, thus affecting AML progression (22). Desumoylation leads to ubiquitination-mediated degradation of PPAR $\alpha$  (21). UBC9 is a SUMO-conjugating enzyme that transfers activated small SUMOs like SUMO1 to various protein substrates (45), prior to the linking of SUMO to the protein by an E3 ligase (20). Our IP assay confirmed that UBC9 silencing destabilized PPAR $\alpha$  by preventing SUMO1 from ligating PPAR $\alpha$  to desumoylating PPAR $\alpha$  in THP-1 cells. Knockdown of UBC9 has been reported to inhibit AML cell proliferation (22), which is similar to our results that UBC9 silencing, like PPAR $\alpha$  inhibition, resulted in decrease in cell viability and levels of GPX4, SLC7A11 and PPAR $\alpha$ , and promotion of apoptosis and ferroptosis in THP-1 cells. Furthermore, increasing PPAR $\alpha$  activity was found to reverse all the above effects of UBC9 silencing. Our results taken together indicated that UBC9 silencing desumoylated PPAR $\alpha$  to induce PPAR $\alpha$  inhibition, which inhibited AML cell growth by facilitating ferroptosis. Our study highlighted the potential of the ferroptosis pathway as a therapeutic target for AML, not only improving the understanding of ferroptosis, but also providing potential new therapeutic strategies for the clinical treatment of AML. Nonetheless, there were some limitations and shortcomings. Given only investigation at the cellular level, further validation of the results in animal studies is required.

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## Conclusion

To conclude, the present study demonstrates that UBC9 silencing-induced desumoylation results in PPAR $\alpha$  inhibition, whereby ferroptosis is facilitated to suppress AML cell growth. Our findings provide a rationale for AML treatment with PPAR $\alpha$  inhibitor.

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## Conflict-of-Interest

The authors declare no conflicts of interest.

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**FIGURE LEGENDS****Figure 1. GW6471 treatment for 24 and 48 h decreased viability and promoted apoptosis and ferroptosis of HL-60/THP-1 cells. (A/B/C/D/E/F/G/H).**

HL-60/THP-1 cells were treated with the PPAR $\alpha$  inhibitor, GW6471 (10  $\mu$ M), for 24 and 48 h. (A/B). Cell viability was measured by cell counting kit-8 assay. (C/D/E). Cell apoptosis was determined by flow cytometry with Annexin V-FITC/PI staining. (F/G/H). The activity of lipid peroxidation of cells was determined by flow cytometry with BODIPY-C11 staining. (I/J). The level of Fe<sup>2+</sup> in cells was assessed by colorimetric assay. The values of the two ends of horizontal lines were compared by \*; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$  (Ctr, Control)

**Figure 2. GW6471 treatment for 24 and 48 h diminished the expressions of ferroptosis inhibition-related proteins and PPAR $\alpha$  in HL-60/THP-1 cells. (A/B/C/D/E/F/G/H).**

The expressions of GPX4, SLC7A11 and PPAR $\alpha$  in HL-60/THP-1 cells treated with the PPAR $\alpha$  inhibitor, GW6471 (10  $\mu$ M), for 24 and 48 h, were analyzed by Western blot, with GAPDH used as the normalizer. The values of the two ends of horizontal lines were compared by \*; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$  (GPX4, glutathione peroxidase 4; PPAR $\alpha$ , peroxisome proliferator-activated receptor alpha)

**Figure 3. UBC9 silencing attenuated the PPAR $\alpha$  antibody-induced enrichment of PPAR $\alpha$  and SUMO1 in THP-1 cells. (A).**

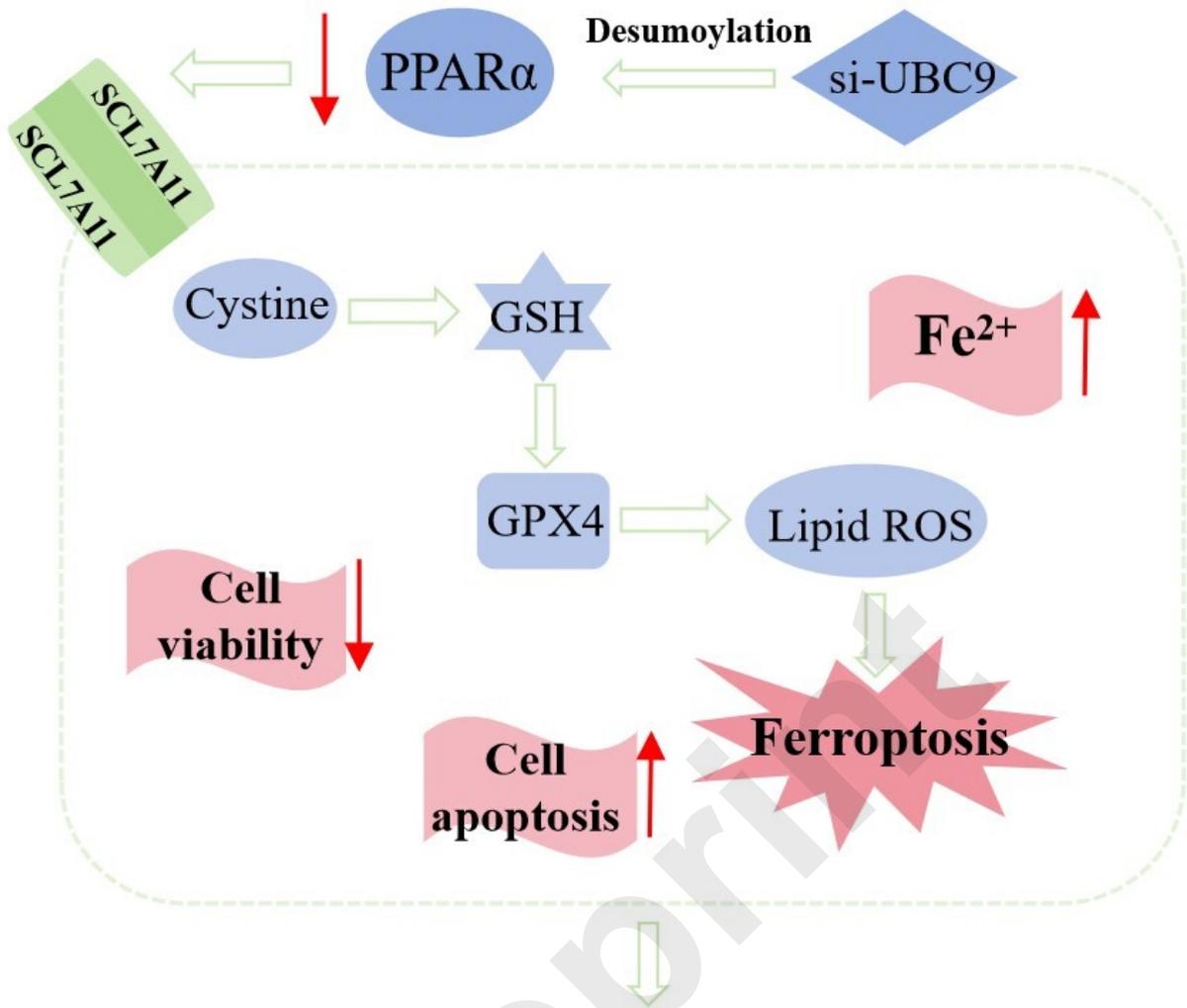
The expression of UBC9 in siUBC9/siNC-transfected THP-1 cells was analyzed by qRT-PCR, with GAPDH used as the normalizer. (B). The interaction between PPAR $\alpha$  and SUMO1 was determined by immunoprecipitation assay in siUBC9/siNC-transfected THP-1 cells. The values of the two ends of horizontal lines were compared by \*; \*\*\*  $P < 0.001$  (UBC9, ubiquitin carrier protein 9; siUBC9, small interfering RNA targeting UBC9; siNC, small interfering RNA targeting negative control; PPAR $\alpha$ , peroxisome proliferator-activated receptor alpha; SUMO1, small ubiquitin-related modifier 1; qRT-PCR, quantitative reverse transcription polymerase chain reaction; Ctr, Control)

**Figure 4. UBC9 silencing-induced viability decrease and promotion of apoptosis and ferroptosis of THP-1 cells were counteracted by pirinixic acid. (A/B/C/D/E/F).**

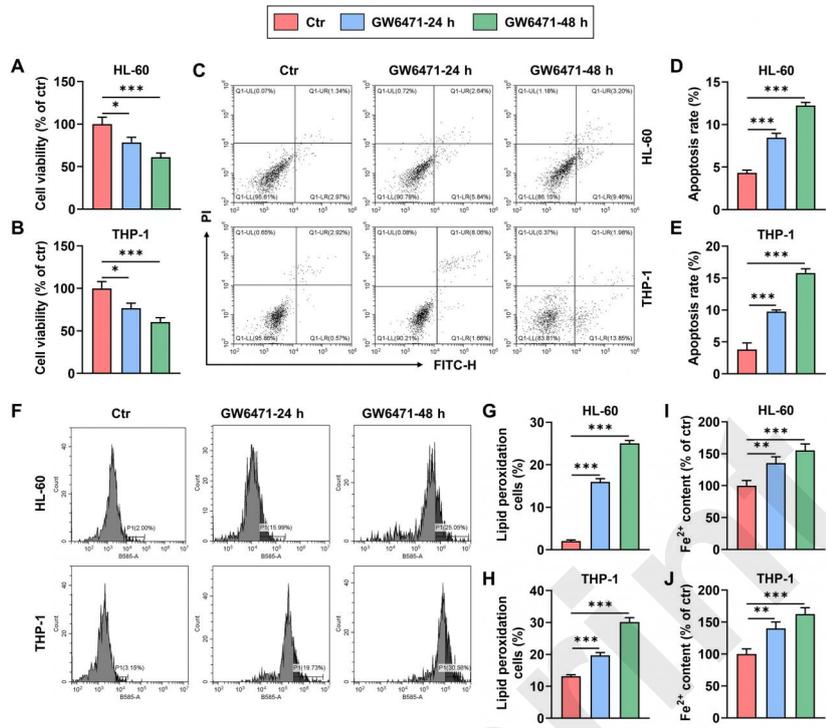
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THP-1 cells were transfected with siUBC9/siNC followed by incubation with/without the PPAR $\alpha$  agonist, pirinixic acid (10  $\mu$ M), for 16 h. (A). Cell viability was measured by cell counting kit-8 assay. (B/C). Cell apoptosis was determined by flow cytometry with Annexin V-FITC/PI staining. (D/E). The activity of lipid peroxidation of cells was evaluated by flow cytometry with Annexin V-FITC/PI staining and BODIPY-C11 staining. (F). The level of Fe<sup>2+</sup> in cells was assessed by colorimetric assay. The values of the two ends of horizontal lines were compared by \*; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$  (UBC9, ubiquitin carrier protein 9; siUBC9, small interfering RNA targeting UBC9; siNC, small interfering RNA targeting negative control; qRT-PCR, quantitative reverse transcription polymerase chain reaction; Ctr, Control)

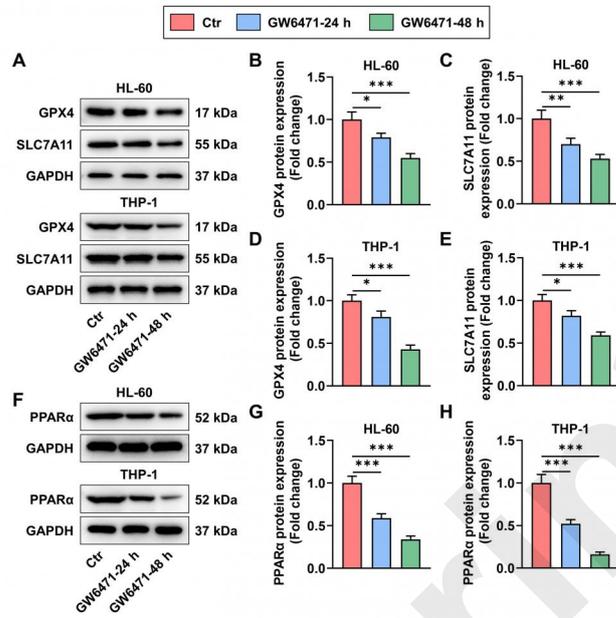
**Figure 5. UBC9 silencing-induced GPX4, SLC7A11 and PPAR $\alpha$  downregulation was counteracted by pirinixic acid.** (A/B/C/D/E). The expressions of GPX4, SLC7A11 and PPAR $\alpha$  in THP-1 cells transfected with siUBC9/siNC followed by incubation with/without the PPAR $\alpha$  agonist, pirinixic acid (10  $\mu$ M), for 16 h, were analyzed by Western blot, with GAPDH used as the normalizer. The values of the two ends of horizontal lines were compared by \*; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$  (UBC9, ubiquitin carrier protein 9; siUBC9, small interfering RNA targeting UBC9; siNC, small interfering RNA targeting negative control; GPX4, glutathione peroxidase 4; PPAR $\alpha$ , peroxisome proliferator-activated receptor alpha; Ctr, Control)



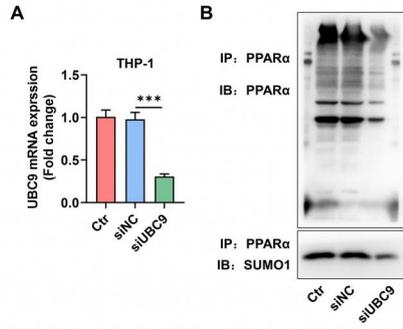
**Acute Myeloid Leukemia**



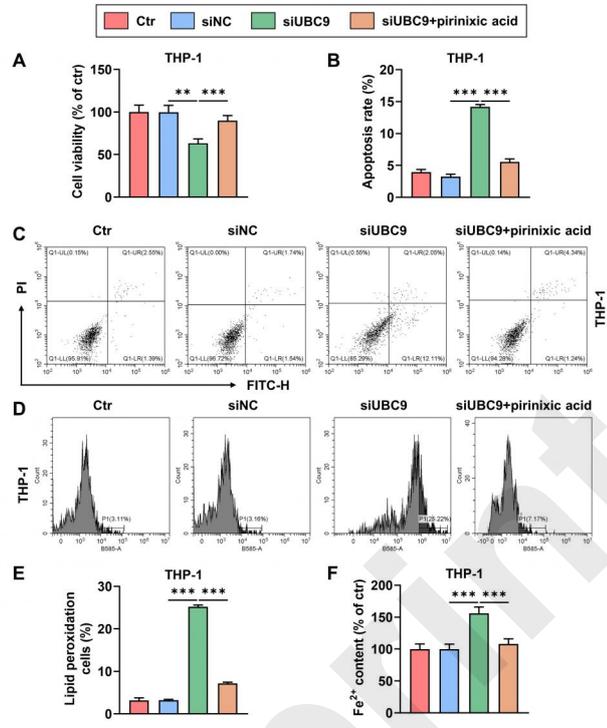
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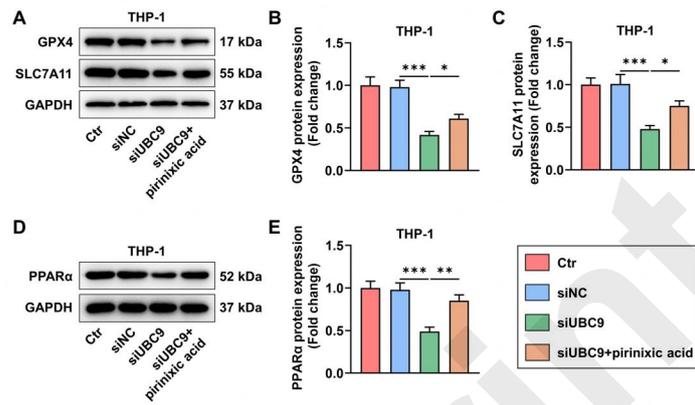
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C D E F G H I J K L M N



G H I J K L M N



F G H I J K L M N