PYCR1 regulates TRAIL-resistance in non-small cell lung cancer cells by regulating the redistribution of death receptors

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Abstract. Although recombinant human TNF-related apoptosis-inducing ligand (TRAIL) protein exhibits anti-tumor activity in a number of lung and liver cancer cells and tumor-bearing animals, TRAIL resistance has substantially restricted its clinical application. Pyrroline-5-carboxylate reductase 1 (PYCR1) is a key enzyme in the regulation of proline synthesis. PYCR1 is highly expressed in various types of malignant tumor, in which it has been implicated in 5-fluorouracil resistance. However, the possible relationship between PYCR1 and TRAIL resistance remains unclear. In the present study, both reverse transcription-quantitative PCR and western blotting were performed. The results indicated that H1299 cells had higher PYCR1 expression levels and were less sensitive to TRAIL compared with the TRAIL-sensitive cell line, H460. PYCR1 knockdown in H1299 cells increased TRAIL sensitivity, increased the localization of death receptors (DRs) on the cell surface and activated Caspase-3/8. By contrast, overexpression of PYCR1 in H1299 cells decreased TRAIL sensitivity, reduced the distribution of DRs on the cell surface and suppressed the activation of Caspase-3/8. Taken together, these results suggested that PYCR1 promoted TRAIL resistance in the non-small cell lung cancer cell line, H1299, by preventing redistribution of DRs to the plasma membrane. This in turn inhibited TRAIL-mediated cell apoptosis by reducing the activation of Caspase-3/8.

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Introduction

According to statistics regarding the global burden of cancer released by the International Agency for Research on Cancer, there were ~1.8 million cases of mortality resulting from lung cancer worldwide in 2020, far surpassing those from other cancer types and ranking first in terms of the cancer mortality rate (1). As previously reported, metabolic reprogramming is an important characteristic of cancer, with both the Warburg effect and essential amino acid (such as proline) metabolism alteration having been previously implicated in this reprogramming (2). Proline has been documented to exert protective effects against stress-induced cell death and apoptosis in mammalian cells in culture to meet their rapid proliferative needs (3,4). The housekeeping enzyme that catalyzes the last step of proline biosynthesis, pyrroline-5-carboxylate reductase 1 (PYCR1), utilizes NAD(P)H as a cofactor to catalyze the transformation of 5-pyrrolinecarboxylic acid to proline (5). In addition, PYCR1 was previously found to be one of the most commonly upregulated metabolic enzymes in malignant tumors such as in lung, liver and prostate cancer, where was also shown to be closely associated with the occurrence and development of disease (6).

The *PYCR1* gene is located on chromosome 17q2.3 and encodes a 33.4-kDa enzyme that consists of 319 amino acids (7). Phang *et al* (8) previously found that the PYCR1 protein is mainly localized to the mitochondria and forms a decameric structure of two main structural domains, namely the N- and C-terminal domains. The N-terminal domain folds to bind NAD(P)H, whilst the C-terminal domain contains an α-helix to bind other substrates, such as Pyrroline 5-carboxylate (P5C) or L-Thiazolidine-4-carboxylate

(T4C,thiaproline) (9,10). When first discovered, *PYCR1* gene mutations were found to be closely associated with the loss of skin elasticity, premature aging and antioxidant stress activation and mitochondrial regulation (11-13). Bogner *et al* (6) previously analyzed the RNA-sequencing data of 9,736 tumor and 8,587 normal tissues from 28 different cancer types from the gene expression profiling database, GEPIA2. Expression of the *PYCR1* gene was found to be increased in 79% (22/28) of the cancer types tested, with a >4X increase observed in 16 types of cancers compared with that in the corresponding normal tissues. It has also previously been reported that high expression levels of the *PYCR1* gene in most solid tumors may serve an oncogenic role by promoting tumor cell proliferation whilst inhibiting apoptosis (6). By contrast, downregulation of the *PYCR1* gene with small interfering RNA (siRNA) has been documented to inhibit cell proliferation and invasion whilst promoting apoptosis in breast cancer (14).

Recombinant human (rh) TNF-related apoptosis inducing ligand protein (TRAIL) has been reported to exert specific antitumor properties in human melanoma WM793 and lung cancer cells H460 whilst remaining non-toxic to normal cells (15,16). However, a number of tumor cell types (such as colorectal cancer cells and non-small cell lung cancer cells) can evade TRAIL-mediated killing either directly (in a process known as primary resistance) or by developing acquired resistance to TRAIL (in a process known as secondary resistance) following treatment (16,17). The long-term clinical efficacy of TRAIL is therefore restricted by such drug resistance.

PYCR1 has also been implicated in drug resistance. Meng *et al* (9) previously found, via structural and biochemical analyses, that the catalytic sites of human PYCR1 are located in a circular groove structured by the N-terminal domain, which contains the conserved dinucleotide-binding Rossmann motif (sequence, GXGXXA/G; part of the Rossmann fold), and dimerization of the C-terminal domain. The fine changes of PYCR1 result in an opening and closing of the binding groove to facilitate the cofactor to enter and leave the active site. Glu221 as a conformational switch for cofactor selectivity for thiaproline dehydrogenase activity.Furthermore, the thermal stability and fine changes of the PYCR1 enzyme was associated with the oxidation of the antitumor drug, thiaproline. In another study, She *et al* (18) found that silencing expression of the *PYCR1* gene significantly increased the sensitivity of lung adenocarcinoma to cisplatin. Yan *et al* (19) also previously revealed that the sensitivity of HCT116 and DLD1 colon cancer cells to 5-fluorouracil was significantly increased following knockdown of PYCR1 expression. Ding *et al* (14) found that PYCR1 is highly expressed in doxorubicin-resistant breast cancer cells, while downregulation of PYCR1 expression using siRNA transfection significantly enhanced the cytotoxicity of doxorubicin. However, the relevance and molecular mechanism underlying the function of the *PYCR1* gene, in addition to its potential role in TRAIL resistance, remains unclear.

In the present study, the association between the *PYCR1* gene and TRAIL resistance was explored.

Materials and methods

Reagents and plasmids. rhTRAIL was purchased from Sino Biological, Inc. (cat. no. 10409-HNAE). A negative control siRNA with no homology to other genes ('NC' hereafter) was used in RNA interference (Suzhou GenePharma Co., Ltd.). siRNA1 or siRNA2 against PYCR1 gene ('siRNA1' or 'siRNA2' hereafter) were used to knock down PYCR1 expression in H1299 cells(Suzhou GenePharma Co., Ltd.). The pGCMV/MCS/Neo vector ('pEX-3' hereafter) was used as the control vector (Suzhou GenePharma Co., Ltd.). The full-length *PYCR1* gene was inserted between the *Sal*I/*Bam*HI cloning sites of pEX-3 to construct the PYCR1-overexpression vector ('pEX-3-PYCR1' hereafter) (Suzhou GenePharma Co., Ltd.).

Cells. The human bronchial epithelial cell line, HBE, and the human non-small cell lung cancer (NSCLC) cell lines, H460 (TRAIL-sensitive) and H1299 (TRAIL-resistant cell line) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and preserved by Hubei Key Laboratory of Tumor Microenvironment and Immunotherapy at China Three Gorges University (Yichang, China). The cells were cultured under 5% CO_2 at 37˚C in a humidified incubator in RPMI-1640 medium (cat. no. PM150110; Procell Life Science & Technology Co., Ltd.) containing 10% (v/v) fetal bovine serum (cat. no. 164210; Procell Life Science & Technology Co., Ltd.) and Penicillin-Streptomycin Solution (cat. no. PB180120; Procell Life Science & Technology Co., Ltd.).

RNA interference, plasmids transfection and generation of stable cell lines. First, *PYCR1* expression was knocked down by siRNA transfection. siRNAs were used to knock down *PYCR1* expression in H1299 cells. The sequences of the negative control (NC) and siRNA against *PYCR1* (siRNA1 and siRNA2) are listed in Table SI. Cells were first seeded into 6-well plates and transfected once the density reached 60-70%. Following the manufacturer's protocol, TurboFect transfection reagent (cat. no. R0531; Thermo Fisher Scientific, Inc.) was used for transient transfection. A total of $1x10^5$ adherent cells were cultured with 2 ml RPMI-1640 medium containing 10% (v/v) fetal bovine serum for 16 h at 37˚C before transfection. The transfection mixture was prepared immediately before transfection. Specifically, 2 μg DNA was diluted in 200 μl serum-free RPMI-1640 medium. For this, the transfection reagent was gently mixed before being immediately added (4 μl) to the diluted DNA mixture by pipetting. This mixture was then incubated at room temperature for 15-20 min. Subsequently, 200 μl of the transfection reagent + DNA mixture was added to each well. The growth medium was not removed from the cells prior to adding the transfection reagent + DNA mixture. The plate was then gently shaken immediately after the addition of the transfection mixture to evenly distribute the complexes. The cells were incubated under 5% CO_2 at 37˚C for 48 h, before transfection efficacy was assessed by RT-qPCR or western blotting. Then cells were divided into four groups and named NC, siRNA1, NC+TRAIL and siRNA1+TRAIL. In siRNA1+TRAIL group, H1299 cells were transfected by siRNA1 and then treated with 50 ng/ml TRAIL at 37˚C for 48 h. The expression of PYCR1, DR4 and DR5 gene were detected by RT-qPCR or western blotting.

To determine explain the association between *PYCR1* and TRAIL resistance, transgenic cell line with stable overexpression of *PYCR1* was constructed. Following transfection

as aforementioned, the cells were transfected with pEX-3 or pEX-3-PYCR1 vector and then selectively screened using G418 sulfate solution (cat. no. G4024; Wuhan Servicebio Technology Co., Ltd.). A G418 screening gradient (100-1,000 μg/ml) was added to untransfected H1299 cells at 37˚C, with medium + G418 changes every 3-5 days. Subsequently, 800 μg/ml was found to be the optimal G418 screening concentration since this concentration killed all cells within 14 days. Following transfection of H1299 cells with the overexpression plasmids, the cells were incubated with 800 μg/ml G418 for 14 days. Screening was then terminated for clone amplification, whereby a cell suspension of each clone was prepared by diluting the cells to 1 cell/10 μl with RPMI-1640 medium to a 96-well plate. Cells were divided into four groups: pEX-3, pEX-3-PYCR1, pEX-3+TRAIL and pEX-3-PYCR1+TRAIL. In pEX-3-PYCR1+TRAIL group, the PYCR1 overexpression stable transgenic cell line was treated with 50 ng/ml TRAIL at 37˚C for 48 h. Then the expression of PYCR1, DR4 and DR5 gene were detected by RT-qPCR or western blotting.

Detection of PYCR1,DR4 and DR5 gene expression by reverse transcription-quantitative PCR (RT-qPCR). At first, the difference expression of PYCR1 gene in HBE, H1299 and H460 cells were detected by RT-qPCR. Total RNA of those cells were extracted using TRIzol (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.) respectively, before the RNA concentration and integrity of each sample was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.). The first-strand synthesis of cDNA was conducted using the Revert Aid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc. and reacted in gene amplification PCR apparatus (Biometra Tone 96, Jena, Germany) at 42˚C for 60 min and then at 70˚C for 10 min. qPCR was then performed using the cDNA as a template and a HiScript II One Step qRT-PCR SYBR Green Kit (cat. no. Q221-01; Vazyme Biotech Co., Ltd.) on a CFX96 qRT-PCR detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol (pre denaturation for 5 min at 94˚C; denaturation for 30 sec at 94˚C, annealing for 30 sec at 55˚C, extension for 45 sec at 72˚C, 35 cycles; final extension for 10 min). The sequences of the primers used are listed in Table SII. A total of three replicate wells were set up for each sample. The $2^{-\Delta\Delta Cq}$ method was used to analyze the fold-change relative to that of *β-actin* (20). Following the same experimental procedure as aforementioned, the expression of PYCR1, DR4 and DR5 gene after knockdown and overexpression of PYCR1 were detested by RT-qPCR.

Western blotting analysis. Expression of *PYCR1* in HBE, H1299 and H460 cells were detected by Western blotting analysis. The whole cell lysates of those three cells were extracted using RIPA (cat. no. G2002; Wuhan Servicebio Technology Co., Ltd.) supplemented with Protease Inhibitor Cocktail (cat. no. B14001; Selleck Chemicals). The concentration of proteins was determined using the BCA Protein Quantification Kit (cat. no. E112; Vazyme Biotech Co., Ltd.). In total, 20-30 μg protein per lane was separated by SDS-PAGE (10% gel), before transfer onto PVDF membranes. After blocking the membranes for 2 h with TBST (TBS+0.1% Tween) containing 5% skimmed milk at room temperature, the membranes were incubated with primary antibodies (Table SIII) diluted with the same solution at 4˚C overnight. After washing with TBST three times to remove unbound primary antibody, IgG (H + L)-HRP secondary antibody was incubated with the membrane at 37˚C for 1 h (Table SIV). Enhanced chemiluminescence reagent (cat. no. MA0186; Dalian Meilun Biology Technology Co., Ltd.) was then used to visualize proteins, which was performed on a ChemiScope 6100 gel imaging system (Shanghai Clinx Scientific Instrument Co., LTD) and analyzed by ChemiScope11.14.0 software (Shanghai Clinx Scientific Instrument Co., LTD) and Image J 1.48 software(National Institutes of Health). β-actin was used as the internal reference protein.

TRAIL can specifically induce apoptosis in tumor cells, whereas the overexpression of anti-apoptotic proteins or downregulation of pro-apoptotic proteins will lead to TRAIL resistance (21). Therefore, the expression of apoptosis-related proteins were measured by western blotting analysis after *PYCR1* knockdown or overexpression and following TRAIL treatment. Finally, western blotting analysis were conducted to test the expression *DR4* and *DR5*. The detailed experimental western blotting procedure is similar to aforementioned.

TRAIL sensitivity detection by Cell Counting Kit-8 (CCK-8) assay. The viability of cells was monitored using a CCK-8 kit (cat. no. CK04; Dojindo Laboratories, Inc.) following the manufacturer's protocols. After *PYCR1* knockdown or over-expression, single-cell suspensions were inoculated in 96-well overnight after cell counting, ensuring that each well contained 200 μl medium and $1x10^4$ cells. Different concentrations of TRAIL solution (0, 200, 400, 600, 800 and 1,000 ng/ml) were prepared and added to the cells in each well. The 96-well plates were then further incubated at 37˚C for 48 h. A microplate reader (Bio-Rad Laboratories, Inc.) was then used to measure the optical density at 450 nm of each well following a 1 h incubation with 10% (v/v) CCK-8 reagent at 37˚C. The relative cell viability was calculated using the following formula: Cell viability (%)=[optical density $(OD)_{treated}/OD_{untreated}$) x100%. Each experiment was conducted three times with five replicates.

Flow cytometry to detect apoptosis and the expression of DR4/5 proteins on the cell membrane surface. Following transfection with NC or siRNA1 for 48 h, H1299 cells were treated with 50 ng/ml TRAIL for 48 h at 37˚C. The Annexin-V-FITC/PI staining method was used to detect the effect of PYCR1 knockdown on apoptosis. The cells were harvested using trypsin (EDTA-free) and washed twice with PBS. Annexin-V-FITC/PI kit (cat. no. BB4102; BestBio) was used to measure the extent of apoptosis according to the manufacturer's protocols. A single cell suspension was created by adding 400 μl binding buffer to $1x10^6$ cells/ml, then 5 μl Annexin-V-FITC fluorescent probe was incubated with the cell suspension for 20 min at 4˚C whilst being protected from light. Subsequently, 3 μl 20 mg/ml PI was added, followed by 5 min incubation at room temperature protected from light. A blank control is unstained cell with no Annexin V-FITC and no PI and used to regulate voltage. And control groups containing only Annexin-V-FITC or PI was set up to adjust the compensation. The prepared samples were then assayed separately on

BD FACSVerse flow cytometer (BD Biosciences), analysed by BD FACSuite™ 1.0.3 Software(BD Biosciences)and FlowJo 10 software (FlowJo LLC).

The binding of TRAIL to DR4 and DR5 on the cell membrane is a key step in the initiation of programmed cell death. Therefore, the degree of DR membrane expression can be directly measured as an indicator of sensitivity to TRAIL (22). Following transfection with siRNA, each sample was collected and treated with TRAIL, before the expression of DRs on the cell membranes was detected by flow cytometry. A total of $1x10^6$ cells were resuspended in 50 μl PBS containing 1% goat serum (cat. no. G1208-5ML; Wuhan Servicebio Technology Co., Ltd.) and incubated for 30 min at room temperature. After three washes with PBS, the cells were incubated with primary antibodies (Table SIII) at 4˚C overnight. The cells were then washed three times with PBS and incubated with the anti-Rabbit-IgG(H+L)-Cy3 secondary antibody (Table SIV) at room temperature for 30 min. An additional negative control was established to set up the instrument until spontaneous fluorescence was detected in the range of the negative gate. For this additional negative control, cells were collected following transfection with NC siRNA and treated as aforementioned but without the use of primary antibody. Fluorescence intensity was then detected using flow cytometry (BD FACSVerse, BD Biosciences). Each set of experiments was repeated three times. The remaining samples were then tested sequentially under the same instrument voltage and gating strategy. The fluorescent intensity of samples was considered to be directly proportional to the density of DR. All histograms of the treatment samples were overlayed using the FlowJo 10 software (FlowJo LLC) to compare the expression of DRs on the membrane surface. The proportion of positive cells in each treated sample was analyzed statistically and visualized by column diagrams.

To investigate the effect of PYCR1 overexpression on cell apoptosis and the cytomembrane expression of DR4/5, the same experiment was performed according to the aforementioned method following the establishment of an PYCR1 overexpression stable transgenic cell line through transfecting cells with pEX-3 control or pEX-3-PYCR1 vectors and screening with G418. These cells were then treated with TRAIL and the results analyzed by flow cytometry. For the additional negative control to set up the instrument in cytomembrane expression of DR4/5, cells collected following transfection with the pEX-3 control vector were treated as aforementioned but without the use of primary antibody.

Statistical analysis. Each experiment was repeated three times under the same conditions. The results are presented as the mean ± standard deviation. SPSS (version 24; IBM SPSS Advanced Statistics 24 Software; IBM Corp.) and GraphPad Prism (version 7.01; GraphPad Software; Dotmatics) were used for data analyses. One-way ANOVA followed by LSD post-hoc comparison was used to compare the difference among three groups. Two-way ANOVA followed by Bonferroni post-hoc test was used for cell viability analysis, to reflect the influence of *PYCR1* gene overexpression or knockdown on TRAIL sensitivity. Differences between two groups were analyzed with unpaired Student's t-tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PYCR1 may regulate TRAIL sensitivity. To evaluate whether PYCR1 can regulate TRAIL resistance in NSCLC cells, the PYCR1 protein expression levels in HBE, H460 and H1299 cells were measured using western blotting. In previous studies, H460 was reported to be a TRAIL sensitive cell line, whilst H1299 was reported to be a TRAIL resistant cell line (17,22,23). PYCR1 expression was found to be increased in the two NSCLC cell lines tested when compared with the HBE human bronchial epithelial cell line (Fig. 1A and B). In addition, PYCR1 expression was found to be increased in the TRAIL-resistant cell line, H1299, when compared with the TRAIL-sensitive cell line, H460. These observations suggested that PYCR1 may be associated with NSCLC tumorigenesis and TRAIL resistance. Therefore, PYCR1 expression was subsequently overexpressed or knocked down to determine the effects on TRAIL sensitivity in NSCLC cells.

Knocking down PYCR1 by siRNA enhances TRAIL sensitivity in H1299 cells. To clarify the effects of siRNA transfection on PYCR1 mRNA and protein expression in H1299 cells, the cells were first transfected with siRNA1 and siRNA2 before the expression levels of PYCR1 mRNA and protein were detected by RT-qPCR and western blotting. The results showed that siRNA1 transfection reduced ~95% *PYCR1* mRNA expression and ~81% PYCR1 protein level in H1299 cells after 48 h, with its inhibitory effects more pronounced compared with those mediated by siRNA2 (Fig. 1C-E). Therefore, siRNA1 was chosen for subsequent experiments. Following transfection, the cells were treated with various doses of TRAIL for 48 h. Knocking down PYCR1 expression resulted in an increased sensitivity of H1299 to TRAIL after 48 h (Fig. 1F). In our previous study (23), it was found that 50 ng/ml TRAIL can induce apoptosis in H460 (TRAIL-sensitive cell), while the effect was less obvious on H1299 (TRAIL-resistant cell line) at the same concentration. Therefore, if H1299 cells respond to 50 ng/ml TRAIL after knockdown of PYCR1 gene, it indicates that the PYCR1 gene may be involved in TRAIL resistance. So the degree of apoptosis induced by TRAIL was also observed to be significantly increased after PYCR1 knockdown followed by 50 ng/ml TRAIL treatment for 48 h (Fig. 1G and I) compared with NC cells. These findings suggested that PYCR1 may mediate TRAIL resistance in a PYCR1 knockdown-reversible manner. Changes in the expression levels of proteins associated with the DR pathway (exogenous apoptotic pathway) and the mitochondrial pathway (endogenous apoptotic pathway) were then measured by western blotting as markers of sensitivity to TRAIL. After PYCR1 knockdown by siRNA1 and treatment with 50 ng/ml TRAIL for 48 h, the expression levels of apoptosis markers, Caspase 3, Caspase 8, Bax and Bcl-2, were detected. The results showed that knocking down PYCR1 expression resulted in the significant activation of the executioner apoptotic protein, Caspase 3, with the simultaneous activation of the exogenous apoptotic marker, Caspase 8 (Fig. 1H and J). In addition, expression of Bax, which belongs to the endogenous apoptotic pathway, was increased whereas Bcl-2 was downregulated following PYCR1 knockdown. The changes of apoptotic rate and protein in siRNA1+TRAIL group were significant compared with NC+TRAIL group.

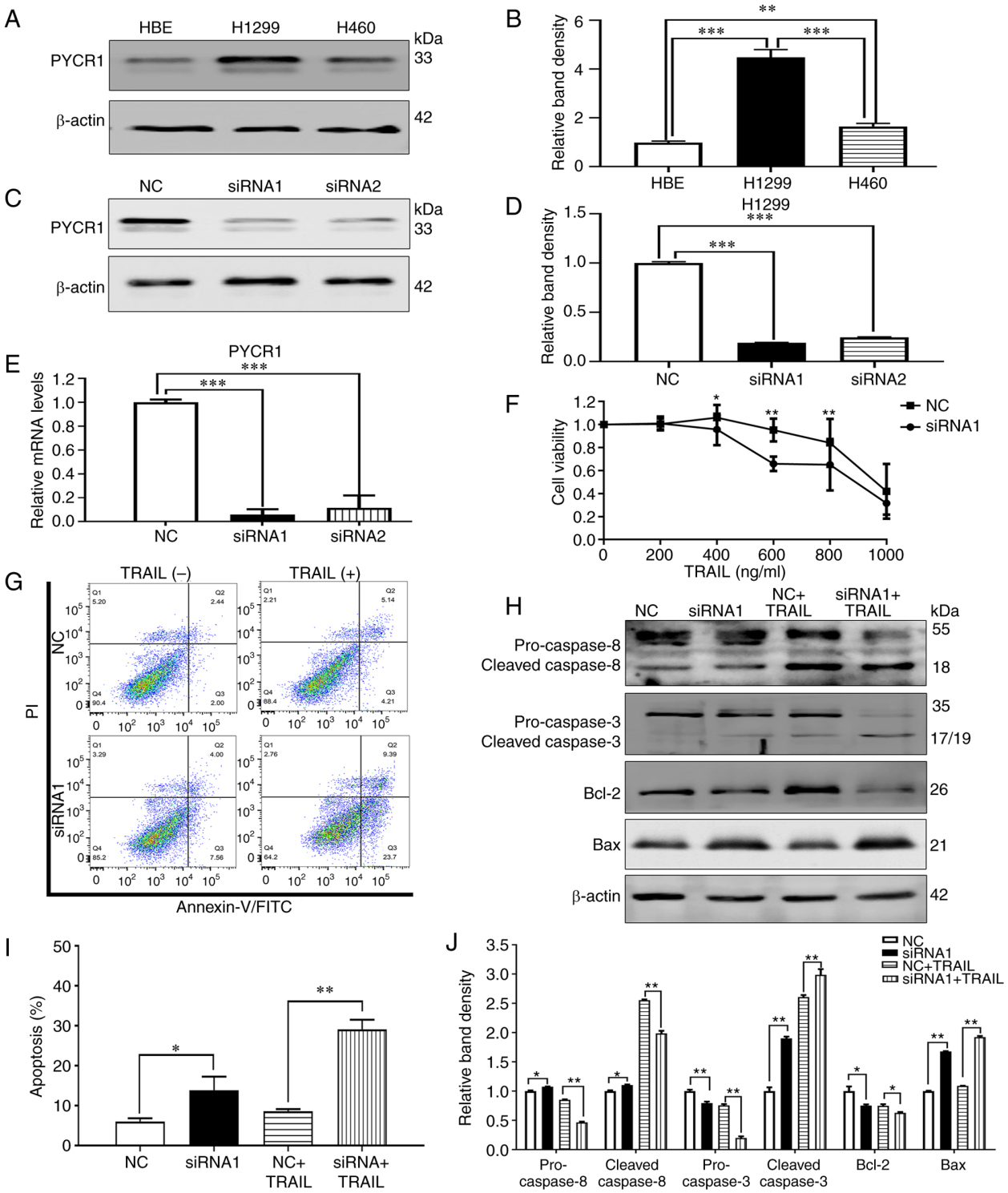

Figure 1. Knockdown of PYCR1 enhances TRAIL sensitivity in TRAIL-resistant H1299 cells. (A) PYCR1 protein expression levels were detected by western blotting in HBE, H1299 and H460 cells, which were the (B) quantified and normalized to β-actin. (C) Western blotting was used to detect the PYCR1 protein expression levels in H1299 cells transfected with siRNA1/2 and NC, which were then (D) quantified and normalized to β-actin. (E) Relative PYCR1 mRNA expression levels in H1299 cells transfected with siRNA1/2 or NC were detected by reverse transcription-quantitative PCR. (F) The viability of H1299 cells transfected with siRNA1 or NC for 48 h was measured using a Cell Counting Kit-8 assay. (G) Representative Annexin V-FITC/PI flow cytometry plots of H1299 cells transfected with siRNA1 or NC and treated with 50 ng/ml TRAIL, which were then (I) quantified. (H) Apoptosis marker protein expression levels in H1299 cells transfected with siRNA1 or NC and treated with 50 ng/ml TRAIL treatment were detected using western blotting, which were (J) quantified and normalized to β-actin. Data are presented as the mean ± SD from three different experiments. *P<0.05, **P<0.01, ***P<0.001. PYCR1, pyrroline-5-carboxylate reductase 1; TRAIL, TNF-related apoptosis-inducing ligand; NC, negative control; siRNA, small interfering RNA.

These results suggested that knocking down PYCR1 expression enhances TRAIL sensitivity by activating both the exogenous and endogenous apoptotic pathways.

Overexpression of PYCR1 promotes TRAIL resistance in H1299 cells. To analyze the relationship between PYCR1 and TRAIL sensitivity, TRAIL-resistant H1299 cells were

transfected with pEX-3-PYCR1. After transfection and G418 screening, the PYCR1 mRNA and protein expression levels were found to be significantly upregulated in H1299 cells (Fig. 2A-C). According to the results of the CCK-8 assay, overexpression of PYCR1 promoted TRAIL resistance in H1299 cells (Fig. 2D). The apoptosis rate of H1299 cells was also found to be reduced after PYCR1 overexpression (Fig. 2E and G). In addition, the protein expression levels of cleaved-caspase 8 and cleaved-caspase 3 were significantly decreased following PYCR1 overexpression (Fig. 2F and H). Expression of the pro-apoptotic protein, Bax, was found to be downregulated, whereas the anti-apoptotic protein, Bcl-2, was upregulated. The changes of apoptotic rate and protein in pEX-3-PYCR1+TRAIL group were significant compared with pEX-3-PYCR1 group. These findings suggested that PYCR1 expression is negatively associated with TRAIL sensitivity, such that downregulation of PYCR1 expression using siRNA transfection increased TRAIL sensitivity by activating the apoptotic pathway. Conversely, NSCLC cells may 'escape' from TRAIL-induced apoptosis by PYCR1 gene upregulation, resulting in TRAIL resistance.

PYCR1 reduces DR4/5 expression in NSCLC cells. A number of studies have previously demonstrated that TRAIL triggers apoptosis after binding to DR4/5, which are located on the plasma membrane (14,15). Therefore, to assess the effects of PYCR1 on DR4/5 in the context of TRAIL sensitivity, the membrane expression of these DRs was measured following PYCR1 knockdown or overexpression. According to the RT-qPCR and western blotting results, the DR4/5 mRNA and protein levels were increased following the knockdown of PYCR1 (Fig. 3A-D). The changes of DR4/5 protein in siRNA1+TRAIL group were significant compared with NC+TRAIL group. Subsequent flow cytometry assays revealed that knocking down PYCR1 expression increased the cell membrane levels of DR4/5 (Fig. 3E-H). In addition, knocking down PYCR1 promoted the cell membrane expression of DR4/5, where they can bind to TRAIL more efficiently to induce apoptosis by activating pro-apoptotic caspase proteins, thereby reversing TRAIL resistance .

By contrast, compared with pEX-3-PYCR1 group, the DR4/5 mRNA and protein expression levels were decreased in pEX-3-PYCR1+TRAIL group (Fig. 4A-D). Decreased DR4/5 expression in the cell membrane was also observed by flow cytometry following overexpression of PYCR1(Fig. 4E-H). These observations suggested that increased expression of PYCR1 not only decreased the expression of DR4/5, but also inhibited translocation of DR4/5 to the cytomembrane, thereby promoting resistance to TRAIL.

Discussion

The lung cancer incidence and mortality rates have been increasing rapidly, with >85% of lung cancer cases being diagnosed as NSCLC and ~75% of cases being diagnosed at the advanced stages on first presentation (24). At present, chemotherapy and targeted therapy are the main treatment strategies for advanced lung cancer. However, the majority of the currently available chemotherapeutic reagents function by inducing tumor cell death through blocking DNA replication and cell division, which causes DNA damage and cellular stress. Therefore, these agents can also kill normal cells and cause adverse effects. Although drugs targeting EGFR and anaplastic lymphoma kinase (ALK) mutations are available, the number of patients who can benefit from such treatments is limited. Ceritinib targets patients with lung cancer harboring ALK mutations, which only represent 3-5% of all patients with lung cancer (25). Therefore, a demand remains to identify novel therapeutic strategies to selectively kill tumor cells in patients with lung cancer.

TRAIL has the ability to induce cancer cell apoptosis whilst selectively preserving normal cells (14,15). Mechanistically, TRAIL binding to DR4/5 induces the aggregation of Fas-associated death domain and Caspase-8, which induces apoptosis in the cell of interest through the DR pathway (exogenous apoptotic pathway) and the mitochondrial pathway (endogenous apoptotic pathway) (26). In addition, TRAIL also binds to decoy receptors, DcR1/2, but DcR1/2 is only expressed in normal cells and since its intracellular segment lacks an intact structural death domain, apoptotic signaling will not be activated following binding (27,28). Therefore, TRAIL specifically induces apoptosis in tumor cells but the DcR1/2 expressed on the surfaces of normal cells will protect normal cells from TRAIL-mediated killing (29). However, the long-term clinical efficacy of TRAIL is restricted by drug resistance, the mechanism of which has remained elusive. TRAIL resistance has been documented to be caused by various factors, such as protein synthesis disorders (30), decreased DR expression and increased anti-apoptotic protein expression (21). The binding of TRAIL to its receptor is the first step in TRAIL-induced apoptotic signaling. The cell membrane localization of DR4/5, but not the absolute expression of these DRs, is the main determinant of TRAIL sensitivity (16). Previous studies have shown that TRAIL can induce the translocation of DRs to the lipid rafts of sensitive cells, which does not occur in drug-resistant cells (16,21). The present study reported that PYCR1 expression is negatively associated with TRAIL sensitivity by reducing the plasma membrane expression of DR4/5 in NSCLC cells.

PYCR1 may be an important molecule in the regulation of TRAIL resistance in H1299 cells. PYCR1, a key enzyme in proline biosynthesis (5), has been reported to be highly expressed in lung cancer cells and tissues compared with normal cells and adjacent tissues (18,31,32). High levels of PYCR1 expression were found to be associated with a poorer prognosis (31). Furthermore, PYCR1 overexpression was previously found to promote tumor cell resistance to cisplatin (18) and 5-fluorouracil (19). Knocking down PYCR1 expression was also reported to reverse fluorouracil resistance by downregulating the expression of the anti-apoptotic protein, Bcl-2 (19,20), multidrug resistance-associated protein and p-glycoprotein (18). The present study also observed increased PYCR1 expression in TRAIL-resistant H1299 cells. Knocking down PYCR1 expression using siRNA reversed this TRAIL resistance suggesting that increased PYCR1 expression may promote TRAIL resistance. This effect was likely achieved by regulating the expression of anti-apoptotic proteins and/or DRs. Oudaert *et al* (33) previously found that multiple myeloma cells from patients with relapsed/refractory cancer had significantly upregulated PYCR1 expression, whereas

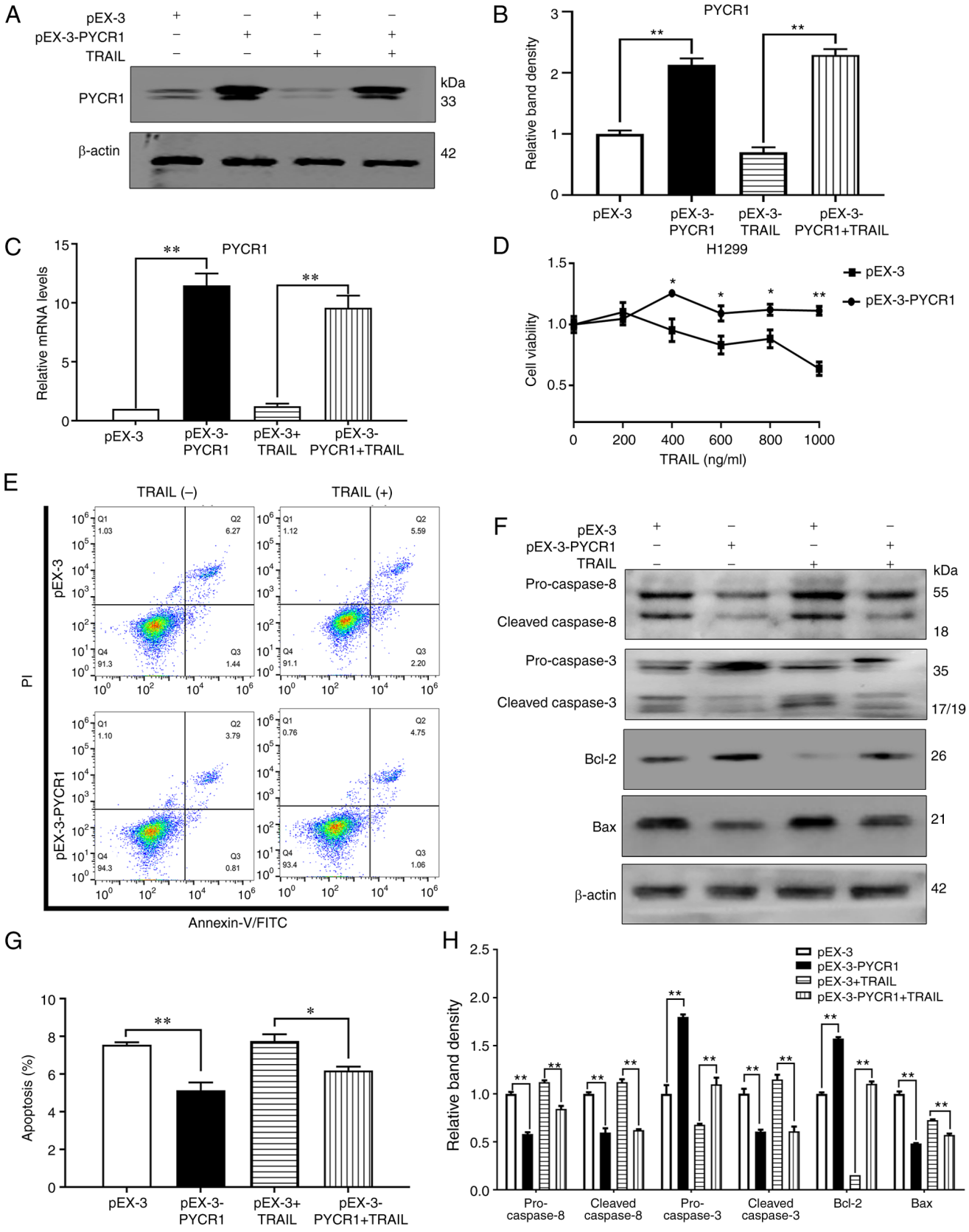

Figure 2. Overexpression of PYCR1 downregulates drug sensitivity in H1299 cells. (A) Western blotting was used to detect PYCR1 expression levels in H1299 cells transfected with the pEX-3-PYCR1 or control pEX-3 plasmid, which were then (B) quantified and normalized to β-actin. (C) Relative PYCR1 mRNA expression levels in H1299 cells transfected with the pEX-3-PYCR1 or control pEX-3 plasmid were detected by reverse transcription-quantitative PCR. (D) The viability of H1299 cells transfected with the pEX-3-PYCR1 or control pEX-3 plasmid for 48 h was measured using a Cell Counting Kit-8 assay. (E) Representative Annexin V-FITC/PI flow cytometry plots of H1299 cells transfected with pEX-3-PYCR1 or control pEX-3 and treated with 50 ng/ml TRAIL, which were then (G) quantified. (F) Apoptosis-associated protein expression levels in H1299 cells following pEX-3-PYCR1 or control pEX-3 transfection and 50 ng/ml TRAIL treatment were detected by western blotting, which were then (H) quantified and normalized to β-actin. Data are presented as the mean ± SD from three different experiments. *P<0.05, **P<0.01. PYCR1, pyrroline-5-carboxylate reductase 1; TRAIL, TNF-related apoptosis-inducing ligand; NC, negative control.

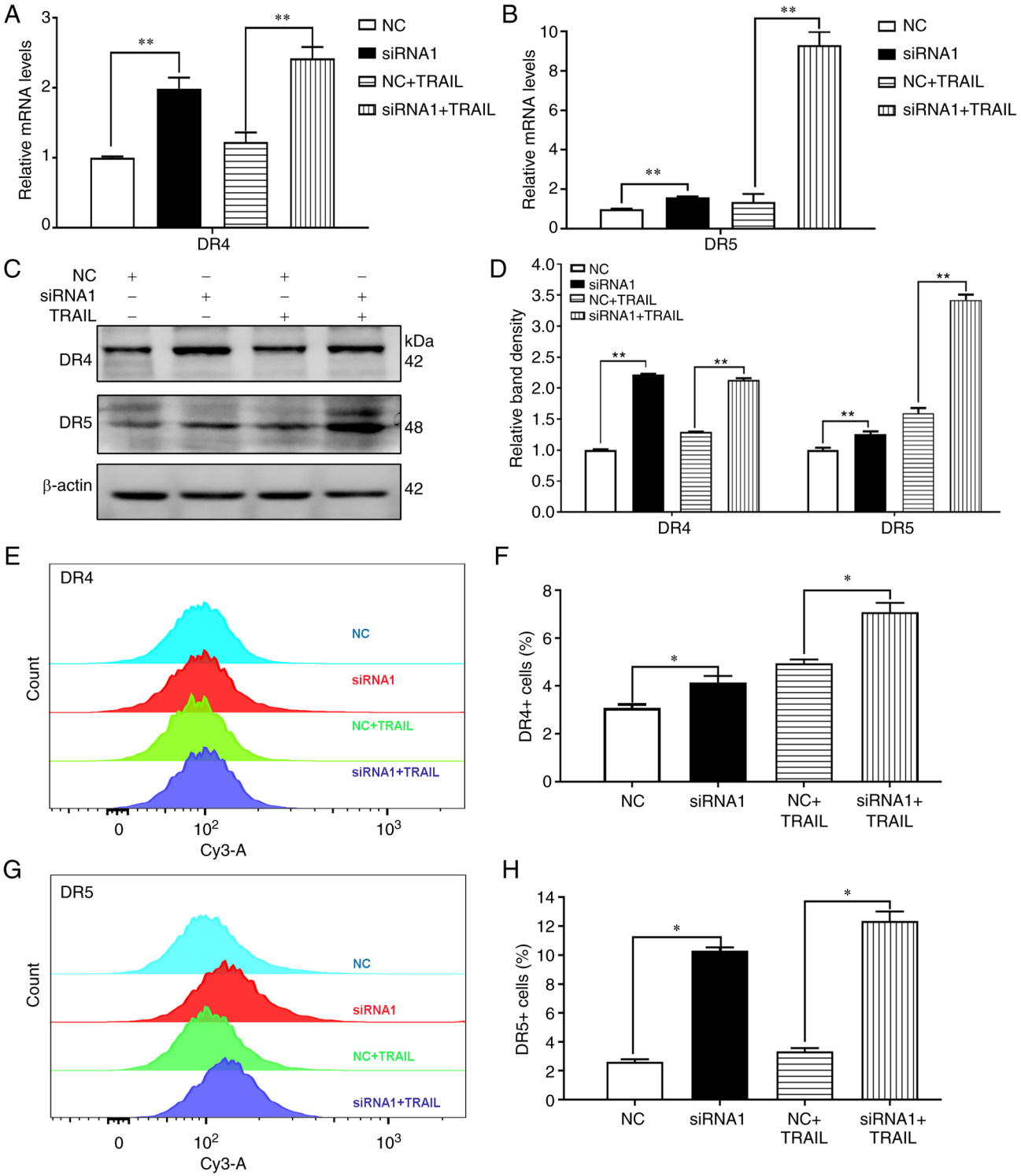

Figure 3. PYCR1 knockdown upregulates DR4/5 expression in non-small cell lung cancer cells. The relative (A) DR4 and (B) DR5 mRNA expression levels in H1299 cells following siRNA transfection and TRAIL treatment were detected by reverse transcription-quantitative PCR. (C) The DR4/5 protein expression levels in H1299 cells following siRNA transfection and TRAIL treatment were detected by western blotting, which were then (D) quantified and normalized to β-actin. The plasma membrane levels of (E) DR4 and (G) DR5 in H1299 cells following siRNA transfection and TRAIL treatment were detected by flow cytometry, which were then (F,H) quantified. Data are presented as the mean ± SD from three different experiments. *P<0.05 and **P<0.01. PYCR1, pyrroline-5-carboxylate reductase 1; TRAIL, TNF-related apoptosis-inducing ligand; NC, negative control; DR, death receptor; siRNA, small interfering RNA.

knockdown of PYCR1 expression reduced cell viability and inhibited cell proliferation by decreasing AKT, p42/44 MAPK, c-MYC and mTOR activation. In addition, Cai *et al* (31) found that PYCR1 was highly expressed in patients with NSCLC, which was in turn associated with a poorer overall survival rate and higher TNM stages. Furthermore, overexpression of PYCR1 promoted cell proliferation and inhibited apoptosis by increasing Cyclin D1 and Bcl-xl expression. PYCR1 knockdown also resulted in cell cycle arrest in the G1 phase, which inhibited cell proliferation and induced apoptosis. In another

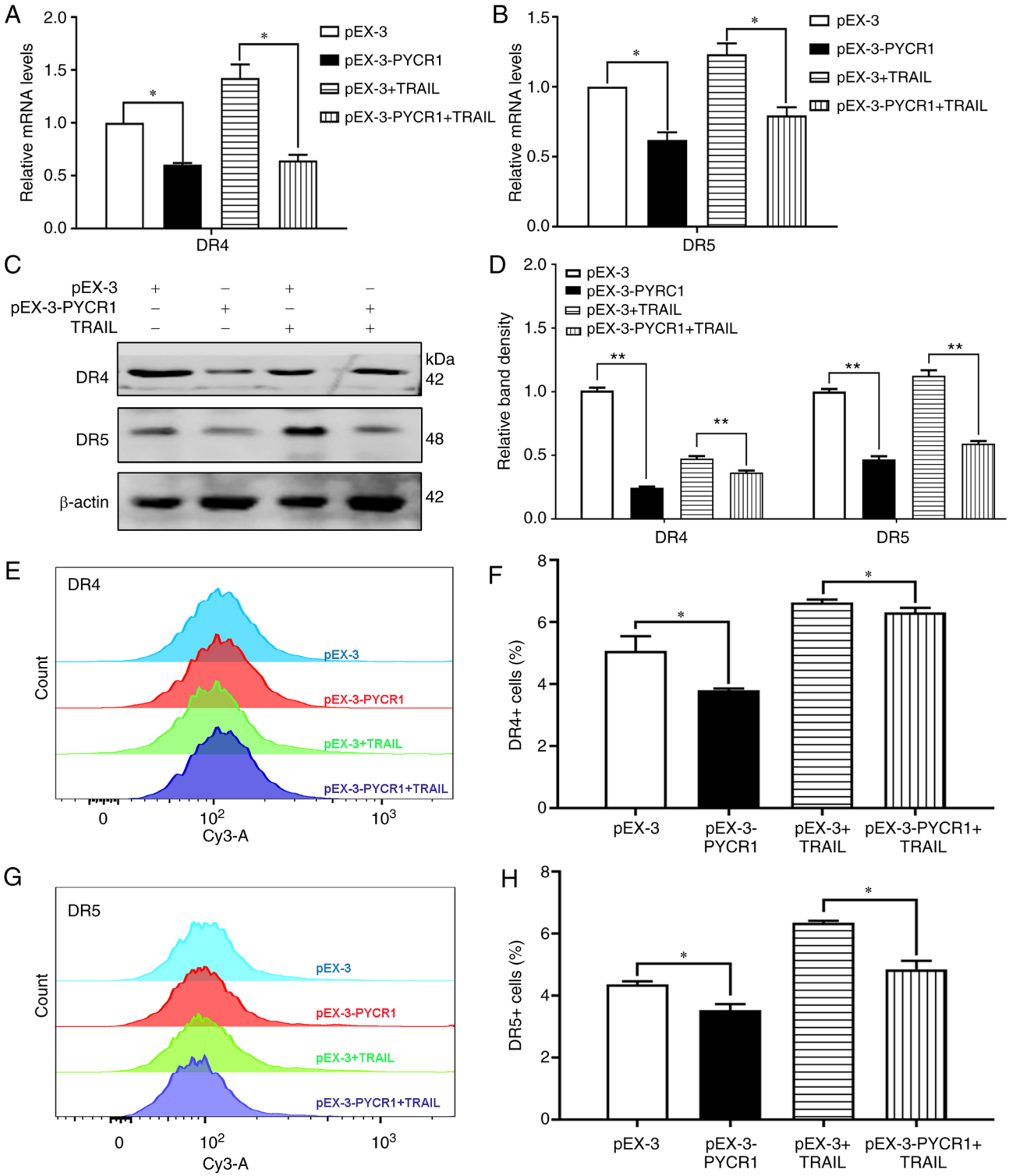

Figure 4. PYCR1 overexpression downregulates DR4/5 expression in non-small cell lung cancer cells. The relative (A) DR4 and (B) DR5 mRNA expression levels in H1299 cells transfected with the pEX-3-PYCR1 or control pEX-3 plasmid and treated with TRAIL were detected by reverse transcription-quantitative PCR. (C) The DR4/5 protein expression levels in H1299 cells transfected with pEX-3-PYCR1 or control pEX-3 plasmid and treated with TRAIL were detected by western blotting, which were then (D) quantified and normalized to β-actin. The plasma membrane levels of (E) DR4 and (G) DR5 in H1299 cells transfected with pEX-3-PYCR1 or control pEX-3 plasmid and treated with TRAIL were detected by flow cytometry, which were then (F,H) quantified. Data are presented as the mean ± SD from three different experiments. *P<0.05 and **P<0.01. PYCR1, pyrroline-5-carboxylate reductase 1; TRAIL, TNF-related apoptosis-inducing ligand; NC, negative control; DR, death receptor.

study, Nilsson *et al* (34) hypothesized that cancer cells may require high levels of PYCR1 expression to sustain growth and provide oxidative stress capacity. As the housekeeping enzyme that catalyzes the last step in proline biosynthesis, PYCR1 is mainly localized in the mitochondria and utilizes NAD(P)H as a cofactor to catalyze the conversion of 5-pyrrolinecarboxylic acid to proline (5). Schwörer *et al* (35) also previously found that TGF-β treatment elevated the expression of PYCR1 in

the mitochondrial proline biosynthetic pathway in mouse NIH-3T3 fibroblasts. Liu *et al* (36) demonstrated that PYCR1 is a target of the oncogene, c-MYC, which promotes the proliferation of P493 human Burkitt lymphoma cells and PC3 human prostate cancer cells through the regulation of proline metabolism proliferation. Knocking down c-MYC expression resulted in a decrease in PYCR1 expression and an increase in other proline metabolizing enzymes.

Previous reports concluded that PYCR1 promotes the development of lung cancer and that high PYCR1 expression was associated with poor prognosis (31,32). In the present study, an association between PYCR1 expression and TRAIL sensitivity in NSCLC was found via PYCR1 gene overexpression or knockdown experiments. The results of the present study verified that PYCR1 mediates TRAIL resistance. PYCR1 may inhibit the membrane expression of DRs for more efficient binding to TRAIL and activate the downstream cysteine protease family of pro-apoptotic molecules to induce apoptosis and reverse TRAIL resistance. In conclusion, down-regulation of PYCR1 may therefore be an effective therapeutic strategy for promoting TRAIL sensitivity in NSCLC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CYo, JH, YS and YH conceived and designed the research. CYo, JH, LW, ZH, XZ and CYi performed the experiments. CYo, CC, DS and CYi interpreted the results. CYo, JH, DS ZH and XZ analyzed the data. CYo, DS, LW, ZH, and XZ prepared the figures. CYo, JH, YS and YH searched the literature and drafted the manuscript. YS and YH edited and revised the manuscript. All authors have read and approved the final manuscript. CYo, JH and CYi confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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