

Cytoglobin attenuates melanoma malignancy but protects melanoma cells from ferroptosis

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Abstract. Cutaneous malignant melanoma is the most aggressive and the deadliest form of skin cancer. There are two types of limitations which universally exist in current melanoma therapy: Adverse effects and reduced efficiency. Cytoglobin (CYGB), an iron hexacoordinated globin, is highly enriched in melanocytes and frequently epigenetically silenced during melanoma genesis. The present study aimed to explore its potential role as a biomarker for ferroptosis treatment. It was observed that B16F10 and A375 melanoma cells with loss of CYGB expression were highly sensitive to ferroptosis inducers RSL3 and erastin, whereas G361 melanoma cells with highly enriched CYGB were resistant to RSL3 or erastin. Ectopically overexpressed CYGB rendered B16F10 and A375 cells resistant to RSL3 or erastin, accompanied by decreased proliferation and epithelial-mesenchymal transition (EMT). By contrast, knockdown of CYGB expression made G361 cells sensitive to ferroptosis induction but induced proliferation and EMT progression of G361 cells. Mechanistically, CYGB-induced resistance of melanoma cells to ferroptosis may have been associated, in part, with i) Suppression of EMT; ii) upregulation of glutathione peroxidase 4 expression; iii) decrease of labile iron pool. *In vivo* study also demonstrated that CYGB

overexpression rendered xenograft melanoma much more resist to RSL3 treatment. Based on these findings, CYGB is a potential therapeutic biomarker to screen the melanoma patients who are most likely benefit from ferroptosis treatment.

Introduction

Melanoma is the deadliest form of skin malignancy; although it is less common than other types of skin cancer, it causes ~75% of skin cancer-related mortality due to its high metastatic potential (1). Surgery is the most effective therapeutic strategy at the localized stage; however, treatment options are limited when patients are diagnosed with advanced or metastatic melanoma (2). Although targeted therapy and checkpoint immunotherapy have improved survival in some cases, most patients do not benefit from this combination therapy at all and other patients relapse after an initial treatment response (3,4). Therefore, future therapeutic efficacy will depend on novel therapy targets or method to overcome resistance to existing targeted and immunotherapy.

Ferroptosis is a newly identified mode of regulated cell death that is triggered by iron-dependent lipid reactive oxygen species (ROS) accumulation (5-7). It is well known that decreased activity of the cysteine-glutamate antiporter (System Xc⁻), inhibition of glutathione peroxidase 4 (GPX4) and increased labile iron promote cell ferroptosis (8). Ferroptosis inducers have strong therapeutic potential in cancer as single agents or in combination with targeted therapies or immunotherapies (9). Therefore, it is critical to identify predictive biomarkers that can accurately predict the responses of patients with melanoma to ferroptosis induction.

Cytoglobin (CYGB) was discovered more than two decades ago and classified as a member of the globin family due to its phylogenetic and structural similarities with other globins (myoglobin, hemoglobin and neuroglobin) (10). Unlike myoglobin, hemoglobin and neuroglobin which are specifically expressed in muscle cells, red blood cells and cells of the central nervous system, respectively, CYGB is ubiquitously present in various organs, suggesting that CYGB

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plays a more universal role than the other three globins (11). CYGB serves a respiratory role in normal cells via intrinsic oxygen-binding capacity, nitric oxide dioxygenase activity and ROS scavenging activity (12-14). CYGB is endogenously expressed at a high level in melanocytes, the primary origin of melanoma. Notably, although CYGB is also highly enriched in several melanoma cell lines such as G361, P22 and C32TG, most melanomas lose their CYGB expression during the melanocyte-to-melanoma transition via promoter methylation (15). Generally, CYGB overexpression predicts resistance to ferroptosis due to its ROS scavenging activity and labile iron regulation via its haem, a coordination complex of a porphyrin ring with an iron ion. However, Ye *et al* (16) found that CYGB overexpression promotes sensitivity of colon cancer cells to ferroptosis. Although a recent study reported that CYGB silencing induced sensitivity of melanoma cells to ferroptosis, the underlying mechanisms remained elusive (17). As CYGB is expressed differently among melanoma cells, it is critical to clarify the relationship between melanoma ferroptosis sensitivity and CYGB expression and its underlying mechanisms, which will determine whether CYGB could be screened as the sensitive biomarker to ferroptosis treatment among melanoma patients.

In the present study, the results showed that CYGB expression levels significantly influenced the vulnerability of melanoma cells to ferroptosis. Although CYGB overexpression inhibited melanoma cell proliferation and blocked epithelial-mesenchymal transition (EMT), it induced resistance of melanoma cells to ferroptosis via reduced labile iron pool (LIP), ROS and increased GPX4 expression. CYGB knockdown, however, played opposite biological roles.

Materials and methods

Cell culture and viability assay. All cell lines were purchased from the American Type Culture Collection and maintained at 37°C with 5% CO₂. B16F10, A375 and G361 cells were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin (MilliporeSigma). MTT assay was used to test the effects of RSL3 or erastin (MedChemExpress) on changes in cell viability. Melanoma cells were treated with RSL3 or erastin or vehicle (DMSO) for 24 h. MTT reagent (at 5 mg/ml) was added to each well at final concentration of 0.5 mg/ml and the plate was incubated at 37°C for 4 h. Subsequently, supernatant was then carefully removed and DMSO (100 µl) was added. The absorbance of the lysates was assessed via a microplate reader (Bio-Rad Laboratories, Inc.) at 490 nm. The relative cell viability was expressed as a percentage of the control well.

Generation of stable knockdown and overexpressed melanoma cell lines. To produce constitutive expression or knockdown of CYGB, a lentivirus carrying the CYGB CDS (protein coding region) or short hairpin RNA (shRNA) sequences (CTCAAC ACTGTCGTGGAGAACCTGCATGA) targeting human CYGB as described previously (13) fused with FLAG were constructed by and purchased from GeneChem Biotechnology. The lentiviral plasmid for overexpression (cat. no. #GV341)

with the Ubi-MCS-3FLAG-SV40-puromycin system, and knockdown (#GV112) with U6-MCS-CMV-puromycin system. Lentivirus particles were produced in HEK293T cells (American Type Culture Collection) by co-transfection of the respective transfer vector (10 µg) with the packaging plasmids (2nd generation packaging system), PSPAX (10 µg) and pMD2G (5 µg, all from Novobio Scientific, Inc.) using PEI at for 48 h at 37°C. Lentivirus particles were collected 48 h after co-transfection. A375, B16F10 and G361 cell lines were transduced with lentivirus (MOI: 1, 5 and 10) for 12 h at 37 °C. These cells were then replaced with a fresh culture medium and followed by at least two-week puromycin (10 µg/ml) selection to obtain stable cell lines. At the same time, the non-infected cells as the negative control were also incubated the puromycin selection and more than 95% cells died within 3 days. The concentration of puromycin to maintain stable cell lines is 1 µg/ml.

Measurement of lipid ROS. Cells were seeded in 6-well plates with 2.5x10⁵ cells/well. After treatment of RSL3 or erastin (12 h), cells were stained with 5 µM of BODIPY 581/591 C11 (cat. no. D3861; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at 37°C in DMEM. Lipid peroxidation was analyzed by the detection of a shift in peak fluorescence emission from 590-510 nm. The fluorescence intensities of cells per sample were analyzed using the BD FACS Aria cytometer (BD Biosciences).

Measurement of mitochondrial ROS. To analyze the formation of mitochondrial ROS, MitoSOX red staining (Invitrogen; Thermo Fisher Scientific, Inc.) was used. Cells were seeded in 6-well plates at 2.5x10⁵ per well. Following treatment with RSL3 or erastin (12 h), cells were stained with 10 µM MitoSOX Red for 30 min at 37°C and then washed twice with PBS and images captured using confocal laser-scanning microscopy (Leica Microsystems GmbH).

Measurement of mitochondrial membrane potential (MMP). To detect changes of MMP, 5,6,6-dichloro-1,1,3,3-tetraethyl-imidacarbocyanine iodide (JC-1) staining was used according to manufacturer's instructions (Beyotime Institute of Biotechnology). Cells were seeded in 6-well plates at 2.5x10⁵ per well. Following treatment with RSL3 or erastin (12 h), cells were stained with 10 µM JC-1 for 30 min at 37°C and then washed twice with PBS and visualized images captured using confocal laser-scanning microscopy (Leica Microsystems GmbH). With high MMP, JC-1 remains in the aggregate form in the mitochondria and emits red fluorescence. Green emission of the dye represented the monomeric form of JC-1, appearing in the cytosol following mitochondrial membrane depolarization.

LIP assay. Intracellular LIP levels were determined using calcein acetoxymethyl ester (Corning Inc.) and iron chelator, deferoxamine (Abcam) according to the manufacturer's instructions. Briefly, melanoma cells were seeded in 6-well plates at 2.5x10⁵ per well and grown overnight. Then the cells were loaded with calcein acetoxymethyl ester (8 µg/ml) at 37°C for 30 min and then washed twice with PBS. Deferoxamine was added at a final concentration of 100 µM to remove iron

from calcein acetoxymethyl ester. The change in mean fluorescence between chelator-treated and untreated cells was used as an indirect measure of the LIP. Fluorescence was measured at 485 nm excitation and 535 nm emission with a VICTOR X3 microplate reader (PerkinElmer, Inc.).

Transwell invasion assay. Transwell chambers (BD Biosciences) were used to assay cell invasion ability. B16F10 MOCK (1×10^4 cells/well), B16F10 CYGB+ (1×10^4 cells/well), A375 MOCK (2.5×10^4 cells/well), A375 CYGB+ (2.5×10^4 cells/well), G361 shCtrl (5×10^4 cells/well) and G361 shCYGB (5×10^4 cells/well) were seeded into the upper chamber and cultured in serum-free RPMI medium with complete medium containing 10% FBS was added to the bottom chambers. After incubation of the 24-well plates at 37°C for 24 (B16F10 MOCK and B16F10 CYGB+), 36 (A375 MOCK and A375 CYGB+) and 48 h (G361 shCtrl and shCYGB), respectively, cells were fixed with 4% paraformaldehyde for 30 min at room temperature and stained with 0.1% crystal violet for 20 min. The migrated cells across the chamber were washed with PBS and then images captured using an Olympus IX83 inverted light microscope (Olympus Corporation).

Western blotting. Cultured cells or tumor tissues were homogenized and lysed in ice-cold RIPA buffer containing protease inhibitor PMSF (1 nM) for 50 min on ice. The extracts were and centrifuged at $12,000 \times g$ and 4°C for 10 min. Protein amount was determined by a bicinchoninic acid protein assay kit (Shanghai Biyuntian Biotechnology Co., Ltd.). Each protein sample (50 μ g/lane) was separated by 10% SDS-PAGE and then transfected to nitrocellulose membranes (MilliporeSigma). Membranes were blocked in 5% fat-free milk at room temperature for 1 h and incubated with primary antibodies overnight at 4°C, followed by incubation with the HRP-conjugated secondary antibodies for 1 h at room temperature. Proteins were visualized by chemiluminescence reagent ECL. The protein band densities were quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.). Primary antibodies against cytoglobin (cat. no. 60228; Proteintech Group, Inc.), zinc finger E-box-binding homeobox 1 (ZEB1; cat. no. 3396; CST Biological Reagents Co., Ltd.), E-cadherin (cat. no. 3195; CST Biological Reagents Co., Ltd.), N-cadherin (cat. no. 13116; CST Biological Reagents Co., Ltd.), GPX4 (cat. no. 59735, CST), heme oxygenase-1 (HO-1; cat. no. 82206, CST), GAPDH (cat. no. 92310; CST Biological Reagents Co., Ltd.) and horseradish peroxidase-linked secondary antibody (cat. no. 51332; CST Biological Reagents Co., Ltd.) was used.

Tumor xenograft model. All animal studies were performed in accordance with the use and care of laboratory animals and approved by the Ethics Committee of Ningbo University (Ningbo, China; approval no. AEWC2023067). A total of 40 8-week-old C57BL/6 male mice (20~23 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd and kept under specific pathogen-free conditions in standard cages in temperature (20–26°C)- and humidity (40–70%)-controlled conditions with a 12 h light/dark cycle. The control murine B16F10 (B16F10 MOCK) cells (2×10^6 cells per mouse) or

CYGB-overexpression B16F10 CYGB+ cells (2×10^6 cells per mouse) were subcutaneously injected into the right flank of the mice. From the day tumor were initially established in tumor implants, tumor volume was measured every day and tumor volume was calculated by the modified ellipsoidal formula (tumor volume=(length $\times$ width²)/2). When tumor volume reached ~ 100 mm³, vehicle or RSL3 (100 mg/kg in 20 μ l DMSO plus 80 μ l corn oil, every other day) was delivered daily to the subcutaneous site where cancer cells were injected until sacrifice with carbon dioxide. The flow rate of carbon dioxide displaced $\leq 30\%$ of the chamber volume/min.

Statistical analysis. Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical differences of two groups were evaluated by one-way analysis of variance (ANOVA) and Statistical differences of three groups were evaluated by One-way ANOVA followed by Newman-Keuls test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Endogenously expressed CYGB is implicated in controlling the sensitivity of melanoma cells to ferroptosis induction. As shown in Fig. 1A, CYGB expression was significantly different in murine B16F10 melanoma cells, human A375 and G361 melanoma cells. CYGB was abundantly expressed in G361 cell line but not detected in B16F10 and A375 cells. It was next examined whether CYGB affects the sensitivity of melanoma cells to ferroptosis induction. As shown in Fig. 1B, three melanoma cells showed diverse vulnerability to ferroptosis inducer RSL3. B16F10 and A375 cells were highly sensitive to RSL3 treatment compared with G361 cells. In addition, B16F10 cells were more sensitive to RSL3 treatment compared with A375 cells. As expected, the three melanoma cells showed similar vulnerability to another ferroptosis inducer erastin compared with RSL3, despite erastin induced weaker ferroptosis than RSL3. In the following functional study of CYGB, 0.5 μ M RSL3 and 2.5 μ M erastin was used.

Ectopically expressed CYGB regulates the induction of ferroptosis in melanoma cells. To test whether genetic alteration affects the sensitivity of melanoma cells to ferroptosis induction, CYGB expression was selectively enhanced using CYGB-specific overexpression lentivirus in B16F10 and A375 cells, and CYGB expression was knocked down using CYGB-specific shRNAs (Figs. 2A, B and S1A). CYGB overexpression induced a significant increase in cell viability upon RSL3 (0.5 μ M) or erastin (2.5 μ M) treatment in B16F10 CYGB+ (Fig. 2C) or A375 CYGB+ cells (Fig. S1B), respectively, whereas knockdown of CYGB expression led to a significant decrease in cell viability following RSL3 or erastin treatment in G361 shCYGB cells (Fig. 2D). In addition, the decreased cell viability by CYGB overexpression was accompanied by decreased lipid peroxidation as evidenced by reduced lipid ROS in B16F10 CYGB+ (Fig. 2E–G) and A375 CYGB+ cells (Fig. S1C–E) exposed to RSL3 or erastin, respectively. By contrast, CYGB silencing resulted in increased lipid peroxidation as evidenced by increased lipid ROS levels in G361 shCYGB cells following exposure to RSL3 or erastin (Fig. 2H–J). It was next examined whether CYGB expression

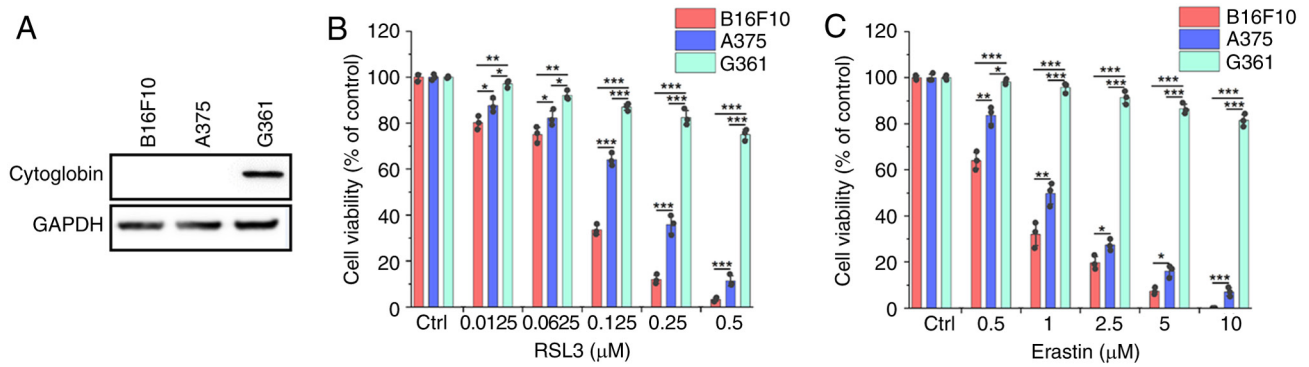


Figure 1. CYGB negatively regulates ferroptosis in melanoma cells. (A) Immunoblot analysis of CYGB protein in B16F10, A375 and G361 cell lines. (B) MTT assay used to detect cell viability of three melanoma cells following exposure to RSL3. (C) MTT assay used to detect cell viability of three melanoma cells following exposure to erastin. Results are presented as mean $\pm$ SEM from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001. CYGB, cytoglobin.

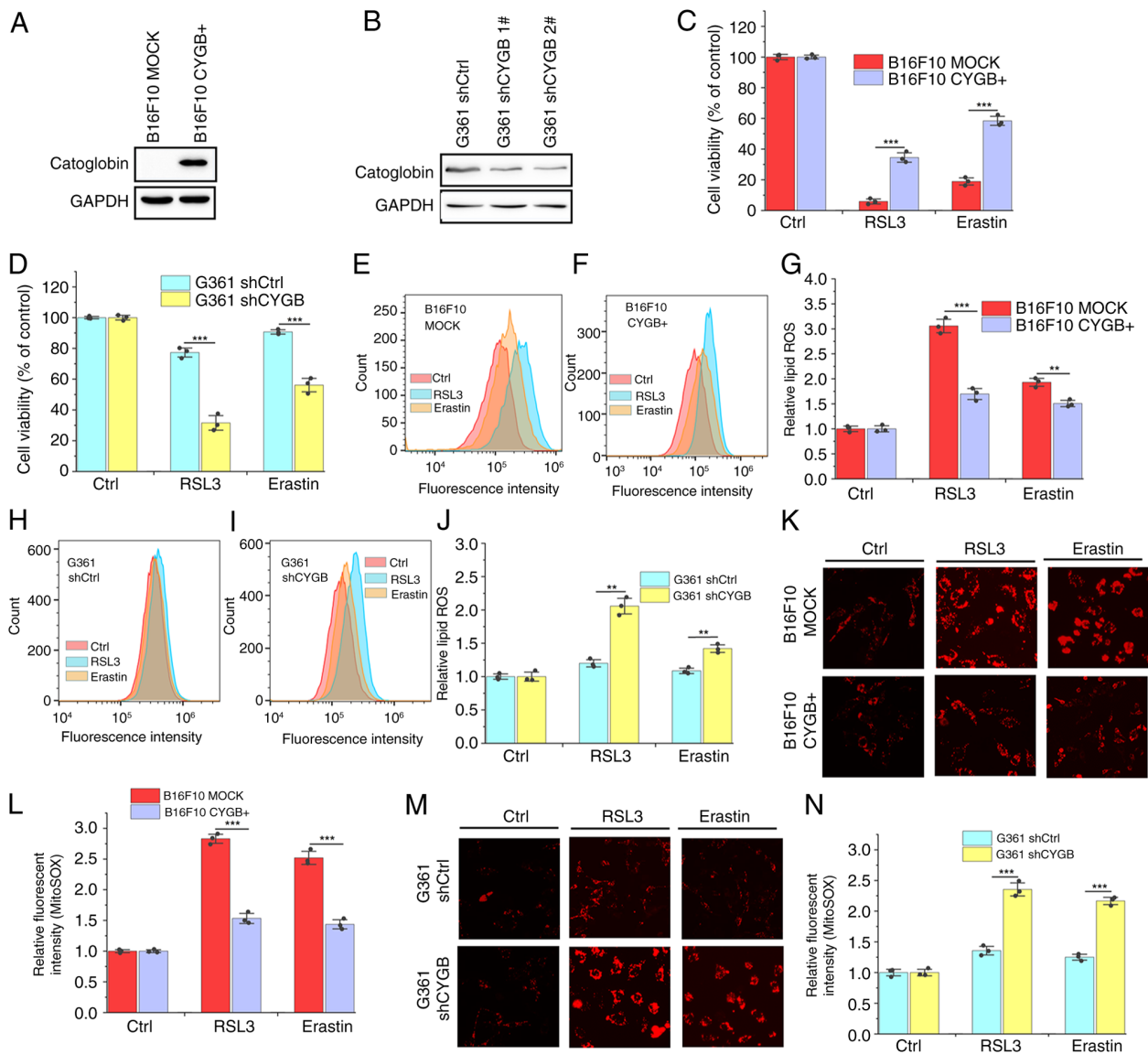


Figure 2. Ectopically expressed CYGB affected cell viability, cellular lipid ROS and mitochondria ROS in melanoma cells. Immunoblotting was used to detect CYGB protein in (A) B16F10 cells transfected with CYGB-overexpression or MOCK viruses or (B) G361 cells transfected with CYGB shRNA or Ctrl shRNA viruses. MTT assay was performed to detect cell viability in (C) B16F10 cells transfected with CYGB-overexpression or MOCK viruses or (D) G361 cells transfected with CYGB shRNA or Ctrl shRNA viruses following exposure to RSL3 or erastin. Lipid ROS was detected in (E-G) B16F10 cells transfected with CYGB-overexpression or MOCK viruses or (H-J) G361 cells transfected with CYGB shRNA or Ctrl shRNA virus following exposure to RSL3 or erastin using BODIPY-C11. Mitochondrial ROS was detected in (K and L) B16F10 cells transfected with CYGB-overexpression or MOCK viruses or (M and N) G361 cells transfected with CYGB shRNA or Ctrl shRNA viruses following exposure to RSL3 or erastin using MitoSOX Red (magnification, $\times 20$). Results are presented as mean $\pm$ SEM, **P<0.01 and ***P<0.001. CYGB, cytoglobin; ROS, reactive oxygen species; sh, short hairpin; Ctrl, control.

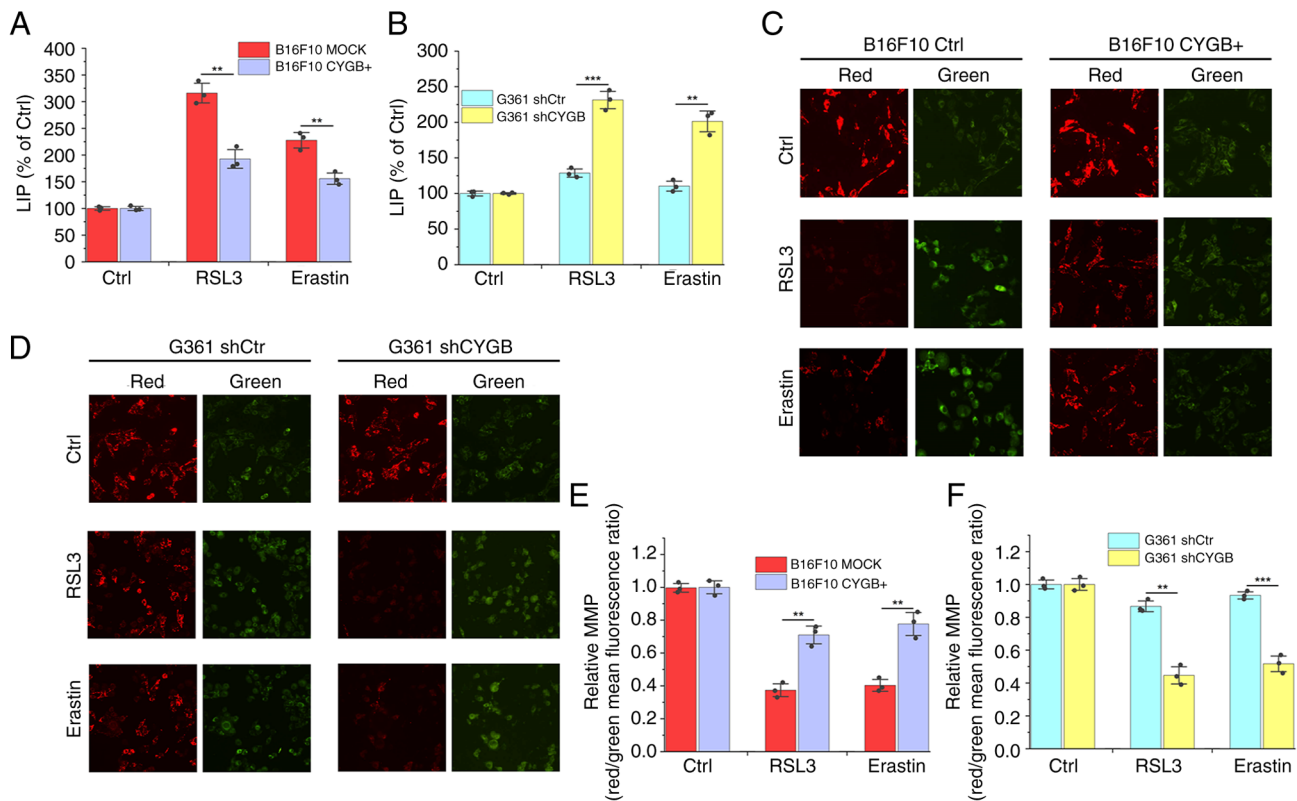


Figure 3. Ectopically expressed CYGB affected cellular LIP and MMP in melanoma cells. Intracellular LIP levels were specifically detected in (A) B16F10 cells transfected with CYGB-overexpression or MOCK viruses or (B) G361 cells transfected with CYGB shRNA or Ctrl shRNA viruses following exposure to RSL3 or erastin using calcein acetoxymethyl ester and deferoxamine. (C) MMP was determined in B16F10 cells transfected with CYGB-overexpression or MOCK viruses following exposure to RSL3 or erastin using JC-1 staining (magnification, x20). (D) MMP was determined in G361 cells transfected with CYGB shRNA or Ctrl shRNA viruses following exposure to RSL3 or erastin using JC-1 staining (magnification, x20). (E) Quantitative evaluation of MMP in B16F10 cells transfected with CYGB-overexpression or MOCK virus following exposure to RSL3 or erastin using JC-1 staining. (F) Quantitative evaluation of MMP G361 cells transfected with CYGB shRNA or Ctrl shRNA viruses following exposure to RSL3 or erastin using JC-1 staining. Results are presented as mean $\pm$ SEM, ** P <0.01 and *** P <0.001. CYGB, cytoglobin; LIP, labile iron pool; MMP, mitochondrial membrane potential; sh, short hairpin; Ctrl, control.

also affected ROS in mitochondria. As shown in Figs. 2K, L and S1F and G, CYGB overexpression suppressed mitochondria ROS levels in B16F10 CYGB+ and A375 CYGB+ cells compared with their vector control upon treatment of RSL3 or erastin. As expected, the inhibition of CYGB expression induced mitochondria ROS levels in G361 shCYGB cells following exposure to RSL3 or erastin (Fig. 2M and N). CYGB is implicated in regulating iron levels due to its haem structure (18,19). Therefore, the present study further examined the labile iron concentration in melanoma cells with ectopically expressed CYGB following ferroptosis induction. CYGB overexpression significantly decreased labile iron concentration in B16F10 CYGB+ (Fig. 3A) and A375 CYGB+ cells (Fig. S1H) following exposure to RSL3 or erastin, respectively. By contrast, it was observed that CYGB knockdown led to increased intracellular labile iron levels in G361 shCYGB cells following exposure to RSL3 or erastin (Fig. 3B). Finally, it was further examined whether ectopically expressed CYGB affected mitochondria function upon ferroptosis induction in melanoma cells. It was found that MMP was significantly decreased after treatment with RSL3 or erastin for over 12 h in melanoma cells (Figs. 3C and D, S1I), although RSL3 or erastin temporarily induced MMP hyperpolarization (data not shown). Enforced overexpression of CYGB could stabilize the MMP and attenuate depolarization in B16F10 CYGB+

(Fig. 3C and E) and A375 CYGB+ cells (Fig. S1I and J). By contrast, CYGB knockdown enforced RSL3 or erastin-mediated decrease of MMP in G361 shCYGB cells (Fig. 3D and F).

Ectopic expression of CYGB affects melanoma malignancy. CYGB serves as a tumor suppressor and to possess an oncogene role in lung cancer cell line (19). The present study then evaluated whether ectopically expressed CYGB affects melanoma cell function in tumor biology. Over 5 days of the experiment, diminished proliferation was observed in CYGB-overexpressing melanoma cell line B16F10 CYGB+ (Fig. 4A) and A375 CYGB+ (Fig. 4B). By contrast, CYGB knockdown significantly promoted the proliferation of G361 shCYGB cells (Fig. 4C). It was further examined whether ectopic expression of CYGB affected melanoma cell invasion. In comparison with the control cell line, CYGB overexpression markedly decreased cellular invasion in B16F10 CYGB+ and A375 CYGB+ cells (Fig. 4D). By contrast, CYGB knockdown significantly enhanced cellular invasive potential in G361 shCYGB cells (Fig. 4D).

CYGB expression influences EMT and GPX4 expression. A previous study showed that cancer cells undergoing EMT are vulnerable to ferroptosis induction (20). The present study tested whether CYGB expression affects cellular EMT process

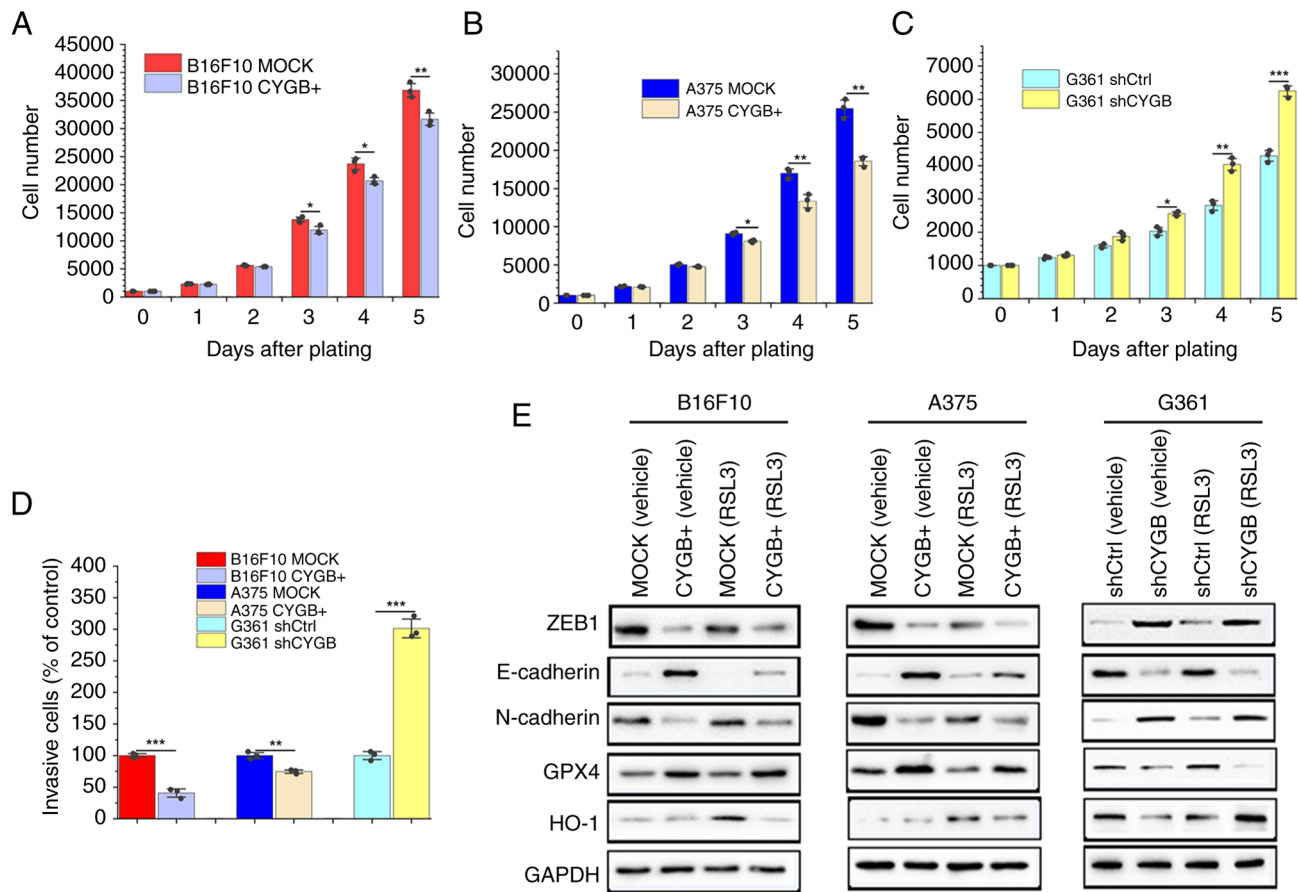


Figure 4. Ectopically expressed CYGB attenuated cell proliferation and invasive potential in melanoma cells. MTT was used to determine the proliferation of (A) B16F10 and (B) A375 cells transfected with CYGB-overexpression or MOCK viruses or (C) the proliferation of G361 cells transfected with CYGB shRNA or Ctrl shRNA viruses. (D) Transwell chambers were used to detect the invasive potential in B16F10 and A375 cells transfected with CYGB-overexpression or MOCK viruses or G361 cells transfected with CYGB shRNA or Ctrl shRNA viruses. (E) Cell lysates were analyzed by immunoblotting with indicated antibodies. Results are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. CYGB, cytoglobin; ZEB1, zinc finger E-box-binding homeobox 1; GPX4, glutathione peroxidase 4; HO-1, heme oxygenase-1.

in melanoma cells. As shown in Fig. 4E, CYGB overexpression significantly upregulated the expression of E-cadherin, an epithelial marker and downregulated the expression of N-cadherin, a mesenchymal marker in B16F10 CYGB+ and A375 CYGB+ cells (21), suggesting that CYGB overexpression impaired the EMT process of melanoma cells. By contrast, CYGB knockdown markedly decreased E-cadherin expression accompanied by increased N-cadherin expression in G361 shCYGB cells, which suggests that lower expression of CYGB promotes EMT status of melanoma cells. In addition, ZEB1, the E-cadherin transcriptional repressor, was significantly downregulated in CYGB-overexpressing B16F10 CYGB+, A375 CYGB+ cells and upregulated in CYGB-silencing G361 shCYGB cells, respectively (Fig. 4E). GPX4, an essential regulator of ferroptosis, is reported to be transcriptionally regulated by ZEB1 (22). In the present study, it was observed that high expression level of ZEB1 was associated with low GPX4 expression, suggesting that CYGB expression influences GPX4 expression, which determines the sensitivity of melanoma cells to ferroptosis induction. In addition, it was also found that HO-1, a stress response marker, was found to be significantly upregulated in B16F10 MOCK and A375 MOCK with loss of CYGB expression and in G361 shCYGB cells with low CYGB expression following exposure to RSL3 (Fig. 4E).

CYGB overexpression limits the growth of the melanoma xenografts but induces resistance to RSL3 treatment. To determine whether CYGB overexpression inhibits the growth of subcutaneous melanoma, the control murine B16F10 (B16F10 MOCK) cells and CYGB-overexpression B16F10 CYGB+ cells were subcutaneously transplanted into C57BL mice. As shown in Fig. 5A and B, it was observed that B16F10 CYGB+ cells developed a smaller tumor volume compared with B16F10 MOCK cells. In addition, a significant delay in tumor growth was observed in RSL3-treated group compared with the vehicle-treated group. Notably, compared with xenograft tumors derived from B16F10 CYGB+ cells, xenograft tumors derived from B16F10 MOCK cells were more sensitive to RSL3 treatment.

Discussion

In recent decades, targeted therapy and immunotherapy have been explored as two effective cancer treatments. However, the development of intrinsic or acquired resistance is a challenge for cancer targeted therapy or immunotherapy, especially in solid tumor therapy (23). Ferroptosis is a recently-identified form of programmed cell death that is different from apoptosis, autophagy, pyroptosis and necrosis in terms of morphology, physiology and

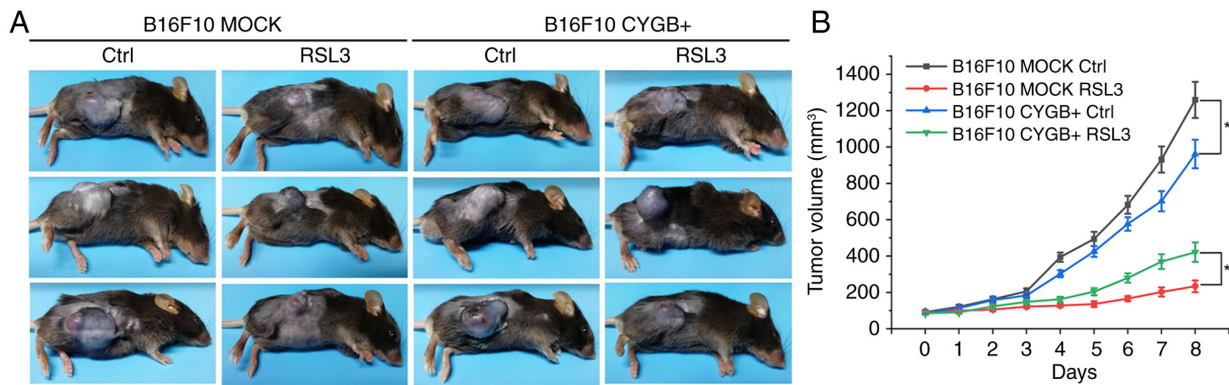


Figure 5. Ectopically overexpressed CYGB attenuated xenograft melanoma growth but induced resistance to RSL3 treatment. (A) Representative images of tumors in live mice from each treatment group. (B) The 8-week-old C57BL/6 (eight mice per group) were injected subcutaneously with indicated control B16F10 (B16F10 MOCK) cells (2×10^6 cells per mouse) or CYGB-overexpression B16F10 CYGB+ cells (2×10^6 cells per mouse) and treated with RSL3 (100 mg per kg, every other day; intratumorally injected,) when the tumor volume reached 100 mm³. Tumor volume was calculated every day. * $P < 0.05$, CYGB, cytoglobin; Ctrl, control.

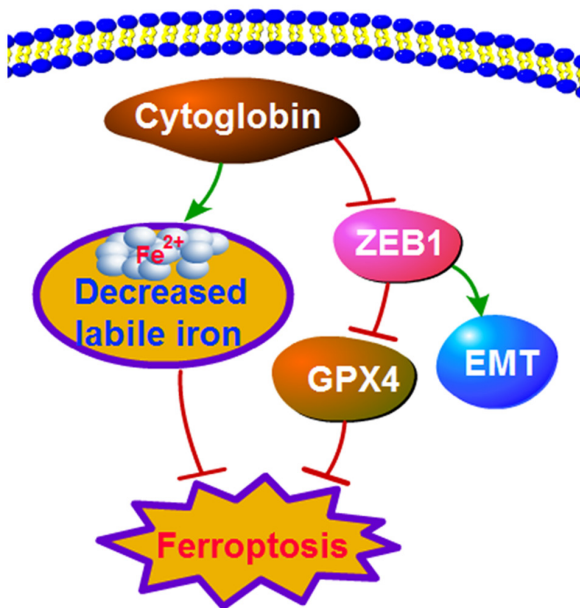


Figure 6. Schematic depicting the underlying mechanism by which CYGB induces resistance of melanoma cells to ferroptosis. Mechanistically, on one hand, CYGB decreases intracellular labile iron. On the other hand, CYGB induces GPX4 expression and blocks EMT via suppression of ZEB1 expression. The aforementioned biological functions of CYGB induce resistance of melanoma cells to ferroptosis. CYGB, cytoglobin; GPX4, glutathione peroxidase 4; EMT, epithelial-mesenchymal transition; ZEB1, zinc finger E-box-binding homeobox 1.

biochemistry (24). Substantial genetic and biochemical evidence has demonstrated that ferroptosis is involved in anti-tumor activity of cancer radiotherapy, targeted therapy and immunotherapy (25-27). On one hand, ferroptosis inducers have shown promise in treating specific tumor types (5); on the other hand, ferroptosis induction may render drug-resistant cancer cells more sensitive to the aforementioned tumor therapies (28,29). Given that the sensitivity of ferroptosis inducers varies greatly across types of cancer cells, highlighting identification of ferroptosis-sensitive biomarkers for cancer therapy.

CYGB, a new member of the globin family, has been proposed to be involved in regulation of iron equilibrium,

reversibly binding oxygen, cellular response to oxidative stress (10). Therefore, CYGB could be a potential ferroptosis-sensitive biomarker. CYGB is expressed at high levels in melanocytes and some melanoma cell lines, whereas loss of CYGB expression occurs during the melanocyte-to-melanoma transition (15). In the present study, CYGB was observed to be highly enriched in G361 melanoma cell line. By contrast, loss of CYGB expression was detected in B16F10 and A375 melanoma cell lines. Notably, B16F10 and A375 cells were highly sensitive to RSL3 or erastin treatment compared with G361 cells. These results were in line with previous studies (30,31). In addition, ectopic expression demonstrated that CYGB overexpression made B16F10 CYGB+ and A375 CYGB+ melanoma cells resistant to ferroptosis induction, whereas CYGB knockdown rendered G361 shCYGB melanoma cells vulnerable to ferroptosis induction. In agreement, De Backer *et al* (17) also found that CYGB knockdown increased the sensitivity of G361 cells to RSL3 treatment. The results of the present study suggested that CYGB played a crucial role in influencing the sensitivity of melanoma cell to ferroptosis induction. The influence of CYGB on the ferroptosis sensitivity of melanoma cells was tightly linked to corresponding changes of cellular lipid ROS, mitochondria ROS, MMP and, especially, labile iron concentration. Cellular iron plays a unique role in promoting lipid peroxide generation during ferroptosis (32,33). CYGB, as a hemeprotein, could potentially be involved in the regulation of iron abundance and Fe²⁺-Fe³⁺ transformation (34) and finally inducing resistance to ferroptosis induction.

Accumulating evidence indicates that CYGB plays a tumor suppressor role because most cancers have a decreased expression of CYGB as observed in tylosis with esophageal cancer as well as melanoma (13,35,36). In agreement, the present study found that CYGB overexpression inhibited the proliferation and invasion of melanoma cells, whereas CYGB knockdown promoted melanoma cell proliferation and invasion. The present study also observed a slow growth of xenograft melanoma derived from CYGB-overexpressing melanoma cells. Notably, CYGB overexpression antagonized RSL3 or erastin-mediated ferroptosis in cultured melanoma cells and significantly decreased the sensitivity of xenograft melanoma to RSL3 *in vivo*. In line with the present results,

Oleksiewicz *et al* (19) reported that CYGB shows a tumor suppressor function in normoxia but promotes tumorigenic potential of lung cancer cells when exposed to stress, suggesting a bimodal function in tumorigenesis, depending on cellular microenvironmental conditions.

In the present study, CYGB overexpression impaired EMT progression via inhibition of E-cadherin transcriptional repressor ZEB1, whereas CYGB knockdown induced EMT via upregulation of ZEB1. Studies have shown that aggressive cancers that have undergone EMT are more susceptible to ferroptosis induction (37-39). It is also found that drug-tolerant persister cells in high mesenchymal state are selectively sensitive to ferroptosis (40). In agreement, the present study found that B16F10 and A375 cells with high metastatic potential, but not low metastatic G361 cells, were highly sensitive to ferroptosis inducers. A recent study reported that ZEB1 directly inhibits GPX4 transcription in breast cancer cells (22). The present study found that CYGB induced GPX4 expression via suppression of ZEB1 expression, which further explained why CYGB-overexpressing melanoma cells are resistant to ferroptosis inducers. However, the detailed mechanism of how CYGB regulates ZEB1 expression needs to be further investigated. HO-1 is a cytoprotective enzyme induced in response to cellular stress (41). The present study found that CYGB overexpression suppressed RSL3 or erastin-induced HO-1 expression, whereas CYGB knockdown promoted its expression when melanoma cells exposed to RSL3 or erastin. It may largely be explained by the cytoprotective role for CYGB, which inhibits RSL3 or erastin-induced ferroptosis and correspondingly blocks stress-induced HO-1 expression.

As summarized in Fig. 6, the results of the present study demonstrated that CYGB expression was involved in regulation of ZEB1 expression, which finally influences GPX4 expression and cellular EMT progression. In addition, CYGB was also implicated in regulation of LIP. The aforementioned CYGB function explained why low or loss of CYGB expression renders melanoma cells highly sensitive to ferroptosis induction. Given that the sensitivity of ferroptosis inducers varies greatly across melanoma cell lines, it is necessary to screen the patients with melanoma and low, or especially loss, of CYGB expression who are most likely to benefit from ferroptosis inducer treatment.

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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

ZZ designed the experiments and supervised the study. QY and JZ performed the experiments and analyzed the data. JZ wrote the original manuscript. XZ wrote the manuscript and analyzed and interpreted data. PZ, FW and YY analyzed data and revising the manuscript. ZZ and JZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics committee of Ningbo University (Ningbo, China; approval no. AEWC2023067). All methods were performed in accordance with relevant guidelines and regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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