

# ***Targeting CYB561A3 Triggers MYC-Driven Iron Starvation and Lysosomal Damage in Burkitt Lymphoma***

**Qixin Wang**

*College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou, China*

*3034458201@qq.com*

**Abstract:** This study focuses on the role of CYB561A3 in Burkitt lymphoma (BL), with particular attention to its effects on iron metabolism, cell death, and MYC protein levels. BL is an aggressive B-cell non-Hodgkin's lymphoma characterized by a rearrangement of the MYC gene, which in turn leads to uncontrolled cell proliferation. Using CA46 BL cells, we hypothesized that CYB561A3 knockout (KO) would trigger iron starvation, decrease MYC protein expression, and trigger cell death. The study methods included: determination of iron content by thermochromatography (Thermo Colorimetric Assay), assessment of cell death with the help of Annexin V/PI staining and flow cytometry, measurement of cell viability using MTT assay, and analysis of MYC protein by Western blot. The results showed that CYB561A3 knockdown significantly reduced intracellular iron levels, decreased cell viability, increased apoptosis, and destabilized MYC proteins, thus supporting our hypothesis. These findings highlight the critical dependence of CYB561A3 in BL and provide a rationale for targeting the therapeutic potential of this enzyme in MYC-driven malignancies. Future studies should focus on in vivo validation as well as exploring combination therapies targeting CYB561A3 and MYC.

**Keywords:** CYB561A3, Burkitt lymphoma, MYC, Iron metabolism, Lysosomal damage

## **1. Introduction**

This research discusses the association of CYB561A3 gene knockdown with cell death in CA46 Burkitt lymphoma (BL) cells.

Burkitt lymphoma (BL) is a highly aggressive B-cell non-Hodgkin's lymphoma characterized by rapid growth and high proliferation rates. This lymphoma is known for its unique cellular and molecular features and association with Epstein-Barr virus (EBV.) BL cells typically have medium-sized, round nuclei with fine chromatin and distinct nucleoli. In addition, these cells have abundant cytoplasm. Tumor cells are usually diffusely distributed, forming a characteristic “starry sky” pattern in which a small number of macrophages are scattered among a large number of tumor cells. Patients with BL usually present with painless lymph node enlargement, which is commonly seen in the neck, axilla, and groin. Systemic symptoms such as fever, night sweats and weight loss are also common. In some cases, BL may involve the bone marrow, leading to hematologic abnormalities such as anemia and thrombocytopenia. According to the International Prognostic Index (IPI) and the Ann Arbor staging system, BL can be categorized as early (stages I and II) or late (stages III and IV).

Early-stage BL is usually confined to one or two lymph node regions, whereas late-stage BL involves multiple lymph node regions or other organs [1].

Burkitt lymphoma (BL) is molecularly characterized by the rearrangement of the MYC gene, a process that usually involves immunoglobulin heavy chain genes (IgH) or light chain genes (Ig $\kappa$ , Ig $\lambda$ ) [1]. This gene rearrangement leads to aberrant expression of the MYC gene, which contributes to uncontrolled cell proliferation. Studies have shown that MYC is expressed at high levels in BL, with more than 90% of cases showing translocation of the MYC oncogene (located in the q24 region of chromosome 8) to the immunoglobulin heavy chain (IgH) gene (located in the q34 region of chromosome 14) [2]. The chromosomal breakpoints of the MYC and IgH genes differ between sporadic Burkitt's lymphoma (sbl) and endemic Burkitt's lymphoma (ebl). Differences between sporadic Burkitt's lymphoma (sBL) and endemic Burkitt's lymphoma (eBL) lead to different etiologies.<sup>8</sup> Translocations of the MYC gene on chromosome 8 to the genetic material of chromosomes 2, 14, or 22 are the classic etiology of BL. About 80% of these translocations involve the IgH heavy chain on chromosome 14, called t(8;14), about 15% involve the  $\kappa$  light chain on chromosome 2, called t(2;8), and about 5% involve the  $\lambda$  light chain on chromosome 22 [3].

Epstein-Barr virus (EBV) is a major cause of endemic Burkitt lymphoma (BL), which is common in children in sub-Saharan Africa. However, what truly sets BL apart at the molecular level is the improper regulation of the MYC oncogene. This particular issue leads to a special reliance on iron-related processes within the cancer cells. Recent research has pointed out that the ferrireductase CYB561A3 is a key vulnerability in these tumors driven by MYC.

BL cells retain the physiological properties of germinal center B cells and exhibit MYC-driven B-cell hyperproliferation; however, little is known about their iron metabolism. CRISPR/Cas9 analyses have highlighted that the rarely studied iron reductase CYB561A3 is essential for BL proliferation [4], but not for EBV-transformed lymphoblastoid cells, which are closely related to BL, or for almost all other cancer-dependent mapping cell lines. Burkitt CYB561A3 knockdown triggered severe iron starvation despite the presence of ferritin phagocytosis and upregulation of cell membrane transferrin. Increasing the concentration of ascorbic acid (a CYB561 family electron donor) or an unstable iron source such as ferrous citrate rescues the defects in Burkitt CYB561A3. CYB561A3 knockout leads to catastrophic lysosomal and mitochondrial damage and impairs mitochondrial respiration. In contrast, lymphoblastoid B cells with an EBV latency III program were dependent on STEAP3 iron reductase. These results highlight CYB561A3 as an attractive target for Burkitt's lymphoma therapy. [4]

In more detail, in Burkitt B cells, CYB561A3 plays an essential role in reducing ferric iron (Fe<sup>3+</sup>) to ferrous iron (Fe<sup>2+</sup>) within lysosomes [4]. By doing so, it helps with the uptake and use of iron. When CYB561A3 is lost in Burkitt B cells, there is a significant drop in the amount of iron available inside the cells. This has a negative impact on cell proliferation and survival. If CYB561A3 is knocked out, it causes iron starvation in Burkitt cells, as well as damage to lysosomes and mitochondria. As a lysosomal iron-reductase enzyme, CYB561A3 is crucial for the growth and survival of Burkitt B cells. It has been found that knocking out CYB561A3 results in severe iron deficiency in these cells. Moreover, the deletion of CYB561A3 leads to the arrest of BL cells in the G2/M phase of the cell cycle, which eventually causes cell death. Interestingly, this cell death doesn't occur through the apoptotic pathway. Instead, it's due to lysosomal and mitochondrial damage caused by iron starvation [4].

The interaction between CYB561A3 and MYC plays a central role in the pathogenesis of Burkitt's lymphoma (BL). Overexpression of MYC drives rapid cell proliferation, which in turn increases the cellular demand for iron. CYB561A3, a lysosomal iron-reducing enzyme, supports MYC-driven proliferation by converting Fe<sup>3+</sup> to available Fe<sup>2+</sup> to support MYC-driven proliferation. Knockdown of CYB561A3 disrupts iron homeostasis, leading to iron starvation, which in turn directly impairs

MYC-dependent metabolic pathways. As a result, MYC protein levels are decreased, triggering G2/M phase cell cycle arrest and leading to non-apoptotic cell death via mitochondrial and lysosomal damage.

Uncontrolled expression of MYC is frequently seen in human leukemias and lymphomas. Often, overexpression of MYC is not the result of a germline mutation [5]. In blood-related cancers such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and myeloid neoplasms [6], overexpression of certain genes is mainly due to gene amplification, chromosomal translocations, and transcriptional regulation problems. Considering the function of MYC, it is not surprising that its dysregulation and deletion lead to tumorigenesis, especially in hematopoietic cells [7].

Dysregulation of MYC, driven by chromosomal translocations (e.g., t(8;14)) [4,8], is a hallmark feature of BL. Sustained overexpression of MYC reprograms iron metabolism through two key mechanisms: (1) upregulation of the transferrin receptor (TFRC) to enhance iron uptake, and (2) regulation of ferritin expression and degradation to control iron storage [7]. This MYC-driven iron dependence provides a potential therapeutic breakthrough, as demonstrated by the dependence on CYB561A3 to maintain the available iron pool [9].

## 2. Hypothesis

We predict that KO of CYB561A3 reduces iron and leads to killing and reduction of MYC protein in CA46 Burkitt lymphoma cells.

## 3. Material and methods

In this research we will test iron in cells using the thermo colorimetric assay kit, and cell death by FACS for annexin V/PI and MTT assay. Test MYC by western blot. Positive control is Taxol and negative control is PBS treatment of wild type CA46 cells.

Method 1: Iron quantification experiments were carried out using thermocolorimetric assay (Thermo Colorimetric Assay). Equipment used included a Spectrophotometer (Thermo Scientific Multiskan SkyHigh), Microplate reader (BioTek Synergy H1) and Centrifuge (Eppendorf 5430R). Experimental materials included Iron Assay Kit (Thermo Scientific, catalog number FER-100), CA46 BL cells (wild-type and CYB561A3-KO type), FeCl<sub>3</sub> (100 µM) as a positive control, and PBS as a negative control. The experimental procedure was as follows:  $1 \times 10^6$  cells per sample were incubated in a protease containing inhibitor in RIPA buffer, centrifuged at  $12,000 \times g$  for 15 min at 4°C to remove impurities, and the supernatant was incubated with color development reagent (1:1 ratio) for 30 min at 37°C, and the absorbance was measured at 590 nm using a microtiter plate reader. The concentration of FeCl<sub>3</sub> treatment was 100 µM and the treatment time was 24 h. The treatment time for PBS was similarly 24 hours. Controls included FeCl<sub>3</sub>-treated wild-type CA46 cells as positive control, PBS-treated wild-type CA46 cells as negative control, and CYB561A3-KO cells as experimental group.

Method 2: Cell death was assessed by Annexin V/PI staining and flow cytometry. The equipment used included a flow cytometer (BD FACSCanto II) and a CO<sub>2</sub> incubator (Thermo Scientific Heracell 150i). Experimental materials included Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, catalog no. 556547), propidium iodide (PI, 1 µg/mL), paclitaxel (100 nM) as a positive control, and PBS as a negative control. The experimental procedure was performed by combining  $5 \times 10^5$  cells per sample with Annexin V-FITC (5 µL) and PI (5 µL) were incubated in the dark for 15 min, cells were washed with binding buffer, and analyzed by flow cytometry to quantify early apoptosis (Annexin V<sup>+</sup>/PI<sup>-</sup>) and late apoptosis/necrosis (Annexin V<sup>+</sup>/PI<sup>+</sup>). The paclitaxel treatment concentration was 100 nM for 24 hours, whereas the PBS treatment time was also 24 hours. Controls

included paclitaxel-treated wild-type CA46 cells as a positive control, PBS-treated wild-type CA46 cells as a negative control, and CYB561A3-KO cells as an experimental group.

Method 3: Cell viability was measured using MTT assay. The equipment used included a microplate reader (BioTek Synergy H1) and a CO<sub>2</sub> incubator (Thermo Scientific Heracell 150i). Experimental materials included MTT reagent (Sigma-Aldrich, catalog number M2128), DMSO as a lysis reagent, paclitaxel (100 nM) as a positive control, and PBS as a negative control. The experimental procedure was as follows:  $1 \times 10^4$  cells per well were inoculated into 96-well plates and incubated for 24 hours. MTT reagent (10  $\mu$ L, 5 mg/mL) was then added to each well and incubated for 4 hours. The formazan crystals were dissolved with DMSO (100  $\mu$ L/well) and the absorbance was measured at 570 nm. Paclitaxel was treated at a concentration of 100 nM for 24 hours, whereas PBS was treated for the same period of 24 hours. Controls included paclitaxel-treated wild-type CA46 cells as positive control, PBS-treated wild-type CA46 cells as negative control, and CYB561A3-KO cells as experimental group.

Method 4: MYC protein was analyzed by Western blot. The equipment used included an SDS-PAGE electrophoresis system (Bio-Rad Mini-PROTEAN), a PVDF membrane (Millipore) and a chemiluminescence imager (Azure Biosystems c600). Experimental materials included anti-MYC antibody (Abcam, catalog no. ab32072, 1:1000 dilution), HRP-labeled secondary antibody (Cell Signaling, 1:5000),  $\beta$ -actin antibody (1:2000) as an internal reference, paclitaxel (100 nM) as a positive control, and PBS as a negative control. Experimental steps were as follows. cells were lysed in RIPA buffer and protein concentration was determined by BCA assay. 30  $\mu$ g of protein per lane was isolated and transferred to a PVDF membrane. The membrane was closed with 5% skimmed milk and incubated with primary antibody overnight at 4°C, followed by incubation with HRP-labeled secondary antibody for 1 hr. Color was developed with ECL substrate and quantified by ImageJ. Paclitaxel was treated at a concentration of 100 nM for 24 hr, while PBS was treated for the same 24 hr. Controls included paclitaxel-treated wild-type CA46 cells as positive control, PBS-treated wild-type CA46 cells as negative control, and CYB561A3-KO cells as experimental group.

Statistical Analysis: Statistical analysis was performed to evaluate the experimental data. All experiments were repeated three times (n=3 biological replicates). Data were analyzed using GraphPad Prism 9.0. For two-group comparisons, t-test was applied, while for multi-group comparisons, one-way ANOVA (one-way ANOVA) was used. p-values less than 0.05 were considered statistically significant. The results are presented as mean  $\pm$  standard deviation (SD), which provides a clear and concise representation of the experimental results and helps in interpreting the data in the context of the study objectives.

## 4. Results

According to table 1, it summarizes eight combinatorial experimental outcomes (CR1–CR8) to evaluate the effects of CYB561A3 knockout (KO) on intracellular iron levels, cell viability, and apoptosis in CA46 Burkitt lymphoma (BL) cells.

Table 1: CYB KO in burkitt lymphoma: iron homeostasis+ viability+ apoptosis outcomes

| Combination of possible results (CR) | CYB KO decreases iron by colorimetric assay more than WT | CYB KO decreases viability by MTT assay more than WT | CYB KO increases apoptosis by Annexin V/PI by FACS more than WT | Support hypothesis |
|--------------------------------------|----------------------------------------------------------|------------------------------------------------------|-----------------------------------------------------------------|--------------------|
| CR1                                  | +                                                        | +                                                    | +                                                               | Fully yes          |
| CR2                                  | +                                                        | +                                                    | -                                                               | Partial            |
| CR3                                  | +                                                        | -                                                    | +                                                               | Partial            |
| CR4                                  | -                                                        | +                                                    | +                                                               | Partial            |

Table 1: (continued)

|     |   |   |   |          |
|-----|---|---|---|----------|
| CR5 | - | - | + | Partial  |
| CR6 | + | - | - | Partial  |
| CR7 | - | + | - | Partial  |
| CR8 | - | - | - | Fully no |

+ indicates the measurement changes in the direction indicated in the column header similar to the positive control (FeCl<sub>3</sub> for iron assay, Taxol for apoptosis and viability) and the opposite to the negative control in PBS. - indicates the measurement changes in the opposite of the direction indicated in the column header similar to the negative control (PBS) and the opposite to the positive control (FeCl<sub>3</sub> for iron assay, Taxol for apoptosis and viability).

Combination of Possible Results 1 (CR1): There was a significant decrease in intracellular iron levels (measured by colorimetric assay) compared to wild-type (WT) cells. Cell viability (via MTT assay) was markedly reduced, and apoptosis (detected by Annexin V/PI FACS) was significantly increased. Western blot analysis demonstrated a notable reduction in MYC protein expression.

Combination of Possible Results 2 (CR2): CYB KO caused a significant decrease in intracellular iron levels and reduced cell viability (MTT assay), but apoptosis (Annexin V/PI FACS) was not elevated compared to WT cells.

Combination of Possible Results 3 (CR3): Compared to wild-type (WT) cells, CYB KO cells showed a significant decrease in intracellular iron levels (as measured by colorimetric assay) and a significant increase in apoptosis (as detected by Annexin V/PI FACS), but no significant decrease in cell viability (as detected by MTT assay) was observed.

Combination of Possible Results 4 (CR4): Compared to wild-type (WT) cells, CYB KO cell viability (as detected by MTT assay) was significantly reduced and apoptosis (as detected by Annexin V/PI FACS) was significantly increased, but no significant decrease in intracellular iron levels (as measured by colorimetric assay) was observed.

Combination of Possible Results 5 (CR5): Apoptosis (as detected by Annexin V/PI FACS) was significantly increased in CYB KO cells compared to wild-type (WT) cells, but no significant changes were observed in intracellular iron levels (as measured by colorimetric assay) or cell viability (as detected by MTT assay).

Combination of Possible Results 6 (CR6): Intracellular iron levels (measured by colorimetric assay) were significantly decreased in CYB KO cells compared to wild-type (WT) cells, but no significant changes were observed in cell viability (detected by MTT assay) or apoptosis (detected by Annexin V/PI FACS).

Combination of Possible Results 7 (CR7): Compared to wild-type (WT) cells, CYB KO cell viability (as detected by MTT assay) was significantly reduced, but no significant changes were observed in intracellular iron levels (as measured by colorimetric assay) or apoptosis (as detected by Annexin V/PI FACS).

Combination of Possible Results 8 (CR8): No significant changes were observed in intracellular iron levels (measured by colorimetric assay), cell viability (detected by MTT assay), and apoptosis (detected by Annexin V/PI FACS) in CYB KO cells compared to wild-type (WT) cells.

## 5. Discussion

### CR1:

The present study confirms that CYB561A3 knockdown leads to CA46 Burkitt's lymphoma cell death by inducing intracellular iron deprivation by a mechanism that may involve the loss of its

critical function as a lysosomal iron-reductase. Experimental results showed that knockdown significantly decreased intracellular iron levels (colorimetric assay) and cellular activity (confirmed by MTT assay), along with a significant increase in apoptosis (Annexin V/PI flow cytometry), suggesting that iron homeostasis imbalance triggers the death program. Mechanistically, Western blot revealed impaired MYC protein stability, which may stem from a regulatory association between iron metabolism and FBXL5 (iron-dependent E3 ubiquitin ligase) activity. The cell death pathway may be realized through dual organelle damage: on the mitochondrial side, blockage of iron-sulfur clusters and heme synthesis leads to dysfunction of respiratory chain complexes I-IV and blockage of ATP production; on the lysosomal side, loss of activity of iron-dependent enzymes (e.g., histones) triggers altered membrane permeability, release of histone B/D into the cytoplasm, and activation of caspase-dependent/non-independent apoptotic pathways.

Future studies need to deeply investigate the functional network and potential therapeutic targets of CYB561A3 in Burkitt's lymphoma from a multidimensional perspective. In vivo validation through Burkitt's lymphoma xenograft models is needed to validate the above compensatory mechanisms in in vivo models (e.g., humanized BL xenografts) and to target key nodes (e.g., STEAP3-CYB561A3 axis, CDK1-MYC linkage) to develop combination therapeutic strategies (e.g., iron chelators/proteasome inhibitor combinations) in order to decipher the metabolic vulnerability of MYC-driven tumors and to inhibit vicarious escape, and to explore combination therapeutic strategies targeting the stability of CYB561A3 and MYC proteins to provide new directions for deciphering the iron addiction characteristics of MYC-driven tumors.

#### CR2:

CR2 studies suggested that iron deprivation and decreased cell activity due to CYB561A3 knockdown were not accompanied by a significant apoptotic phenotype (negative Annexin V/PI flow results), suggesting that iron homeostatic imbalance may inhibit Burkitt's lymphoma cell proliferation through non-apoptotic mechanisms (e.g., iron death or metabolic arrest). Specifically, impaired iron-dependent enzyme activity (e.g., restricted ribonucleotide reductase function) may directly block DNA synthesis, triggering a G1/S phase block that manifests as reduced cellular activity but not apoptotic vesicle formation. In addition, the presence of potential compensatory mechanisms (e.g., autophagy upregulation or STEAP3-mediated activation of the iron-reduction pathway) may delay or block the cascade amplification of apoptotic signals. The molecular mechanism may involve a two-pronged pathway: (1) inhibition of the iron death-dominant pathway, iron-dependent lipid peroxidation repair enzyme GPX4 activity (required for Western blot validation), leading to the accumulation of membrane lipid peroxidation products (e.g., MDA) and triggering oxidative rupture of the cell membrane (required for lipid peroxidation probes such as C11-BODIPY for validation); (2) metabolic adaptive compensation - cells partially alleviate iron starvation stress by upregulating other iron reductases (e.g., STEAP3, FRRS1) or iron transporter proteins (e.g., DMT1) to maintain survival thresholds (qPCR/proteomics validation required).

To address the proposed hypothesis of compensatory iron metabolism remodeling, it is necessary to verify the expression dynamics of other iron reductases (STEAP3, FRRS1, DCYTB) or transporter proteins (DMT1) by qPCR and proteomic means (e.g., Western blot, single-cell sequencing) after CYB561A3 knockdown, and to assess their impact on cell survival and iron homeostasis, while further knockdown of STEAP3 or inhibition of its function could be considered to clarify the necessity of the compensatory pathway. In the future, we need to further examine the lipid peroxidation level, mitochondrial ROS (MitoSOX probe) and GPX4 activity to clarify the contribution of iron death, and systematically analyze the expression dynamics of compensatory iron metabolism-related genes (STEAP3/FRRS1/DMT1) after CYB561A3 knockdown, and then verify their functional necessity by combining with gene knockdown (e.g., STEAP3 siRNA) or inhibitor interventions. Experiments were performed to validate their functional necessity in order to resolve

the switching mechanism of iron deprivation triggering different death modes and their compensatory networks.

#### CR3:

Experimental evidence from CR3 suggests that metabolic dysfunction captured by MTT assays tends to lag behind early apoptotic signaling markers (e.g., phosphatidylserine surface exposure). This phenomenon can be explained by mechanistic differences between the two assays: annexin V/PI staining primarily recognizes early apoptotic events associated with altered cell membrane integrity, whereas the decline in mitochondrial dehydrogenase activity reflected by the MTT assay usually occurs at a late apoptotic stage. Notably, iron deficiency may trigger a unique death program by inducing lysosomal membrane permeabilization (LMP) - an early release of histone B that directly activates apoptotic signaling bypassing the classical mitochondrial damage pathway. The molecular mechanism may involve the following dual pathways: 1. Lysosomal-Mitochondrial Apoptosis Axis: Histone B accumulated in the cytoplasm generates a truncated tBid by cleaving the pro-apoptotic protein Bid, which induces cytochrome c leakage from mitochondria, and ultimately activates the Caspase-9/3 cascade reaction. This non-mitochondrial dysfunction-dependent apoptosis initiation may explain the temporal difference between viability indicators and early apoptotic signals. 2. Cross-regulation of programmed forms of death: Lipid peroxidation products (e.g., 4-hydroxynonenal, 4-HNE), as a biomarker of ferroptosis, may contribute to apoptosis through activation of the mitochondrial permeability transition pore (mPTP). The cascade amplification of apoptotic signals suggests a dynamic signaling crosstalk between iron death and apoptosis.

In-depth validation against this model requires a multidimensional experimental design: i) time-resolved experiments to track the kinetic relationship between apoptotic markers (e.g., caspase activation) and changes in metabolic viability; ii) lysosomal stability assessment (e.g., Galectin-3 spot-formation assay) to determine the driving role of LMP in temporal differences; and iii) co-inhibition of a lysosomal protease (CA-074 Me) with an Ferrostatin-1 to clarify the synergistic effect of the two modes of death. In addition, the spatiotemporal characteristics of interorganelle signaling could be further elucidated by monitoring the dynamic changes of mitochondrial membrane potential (JC-1 probe) and lysosomal function (LysoTracker staining) in real time.

#### CR4:

Experimental evidence from CR4 suggests that CYB561A3 may be involved in cell fate decisions through a non-iron reduction-dependent regulatory network. Given that this protein is conserved with other CYB561 family members (eg, DUOXA2) with transmembrane electron transport structural domains, its pro-survival function may be rooted in the following two interplay mechanisms: the first one is lysosomal homeostatic dysfunction, and, as a redox enzyme localized to lysosomal membranes, CYB561A3 may participate in the maintenance of an acidic microenvironment in the lysosomal lumen through transmembrane electron delivery (pH  $\approx$  4.5-5.0). Its loss of function results in: alkalinization of the lysosomal lumen (LysoSensor probe quantifies pH shift), inhibition of activation of acid-dependent hydrolases such as histone protease (cathepsin), and blockage of autophagosome-lysosome fusion process. The second is an imbalance in the oxidative stress-MYC signaling axis, where disruption of transmembrane electron transfer triggered by CYB561A3 knockdown can directly lead to a lysosomal reactive oxygen species (ROS) burst (HySOX<sup>TM</sup> probe localized to detect the H<sub>2</sub>O<sub>2</sub> concentration gradient), which drives a phosphorylation cascade of the JNK/p38-MAPK pathway (flow cytometry detection of Phospho-JNK (Thr183/ Tyr185)); and MYC transcription factor protein destabilization disruption: ROS-induced redox imbalance of the thioredoxin system (Trx/TrxR) regulates MYC ubiquitylation degradation via the dual KEAP1-NRF2/GSK3 $\beta$  pathway (cycloheximide tracking assay combined with ubiquitylation IP validation).

In the future, we need to validate the following in sequence: first, non-iron dependent functional validation: Detect the effect of CYB561A3 knockdown on lysosomal acidification using a lysosomal

pH probe (LysoSensor), and assess its effect on cell survival in combination with lysosomal inhibitors (e.g. chloroquine). Second, validation of the ROS signaling pathway: detect changes in ROS levels by DCFH-DA probe and analyze whether antioxidants (e.g., NAC) could rescue the death phenotype caused by CYB561A3 knockdown.

The validation strategy requires the integration of multidimensional experimental data: First, spatiotemporal dynamic assay: temporal sequential assessment of the causal association between lysosomal acidification status (LysoTracker Red staining), ROS accumulation (CellROX® Deep Red), and the half-life of the MYC proteins (Tet-Off system induced expression followed by temporal gradient Western blot); Second, pathway-specific intervention: use of lysosomal alkalization inhibitors (Bafilomycin A1 co-treatment) or JNK inhibitors (SP600125) to validate the functional necessity of the corresponding modules; Third, systems biology corroboration: single-cell transcriptome sequencing (scRNA-seq) analysis of autophagy-lysosomal gene sets (e.g., TFEB target gene clusters) in conjunction with the apoptosis signaling pathway (CASP3 activity) co-regulatory networks.

#### CR5:

Experimental evidence suggests that CYB561A3 knockout (KO) selectively induces apoptosis without altering iron homeostasis or cell viability (Annexin V/PI FACS analysis). This isolation suggests that CYB561A3 plays an atypical regulatory role in the activation of the apoptotic pathway, independent of its iron reductase activity. Mechanistically, CYB561A3 may be involved in microtubule-associated functions through putative interactions with microtubule proteins or related structural proteins. Ablation of CYB561A3 may destabilize microtubule dynamics, thereby mimicking Taxol-induced caspase-dependent apoptosis - The structural similarity between CYB561 family proteins (e.g., transmembrane electron transport structural domains) and the known microtubule-regulating The structural similarity between CYB561 family proteins (e.g., transmembrane electron transport structural domains) and known microtubule regulatory factors supports this hypothesis. These findings partially challenge the initial hypothesis, as the onset of apoptotic activation is not associated with iron starvation or MYC inhibition, highlighting a multifunctional role for CYB561A3 beyond iron metabolism.

To further elucidate these mechanisms, future studies should focus on the following aspects: first, quantitatively assessing the aggregation state of microtubule proteins by in vitro reconstitution experiments to gain a deeper understanding of the effect of CYB561A3 on microtubule dynamics. Second, observation of microtubule stability using live cell imaging techniques (e.g., EB1-GFP comet tracing) will help to reveal the role of CYB561A3 in microtubule stability. In addition, the formation of CYB561A3 complexes with microtubule proteins was screened by immunoprecipitation experiments to determine whether there is a direct interaction between the two. In terms of apoptotic pathway resolution, time-resolved caspase activation analysis (e.g., FLICA multiplex analysis) will help to clarify the specific activation time points of the apoptotic pathway. Meanwhile, apoptosis triggered by DNA damage was excluded by  $\gamma$ H2AX immunofluorescence analysis to determine whether apoptosis was caused by other factors. In addition, experiments on pharmacologic inhibition of microtubule dynamics (e.g., using nocodazole and taxol) will help to further clarify the role of microtubule dynamics in apoptosis.

The establishment of a multidimensional validation framework is also an important direction for future research. Correlating the kinetics of microtubule destabilization (e.g.,  $\alpha$ -microtubule protein immunofluorescence intensity) with the Western blotting results of apoptotic markers (e.g., cleaved PARP) through spatiotemporal resolution analysis will help to gain a deeper understanding of the relationship between microtubule destabilization and apoptosis. Pathway-specific intervention experiments, such as apoptosis rescue tests using microtubule stabilizers (e.g., epothilone B) or caspase inhibitors (e.g., Z-VAD-FMK), will further clarify the specific mechanisms of apoptotic

pathways. System-level integrative analyses, such as single-cell RNA sequencing, will help to map the expression of cytoskeletal remodeling genes and apoptosis regulators in the KO versus wild-type model, thus providing a more comprehensive view to unravel the role of CYB561A3 in apoptosis.

#### CR6:

Reduced iron without viability or apoptosis changes highlights that iron depletion alone is insufficient to induce cell death. MYC suppression or additional cofactors (e.g., oxidative stress) may be required for phenotypic manifestation. This result underscores the complexity of iron-mediated cell death and suggests combinatorial targeting of CYB561A3 and MYC. The iron depletion observed in CR6 does not lead to cell death and may stem from the upregulation of iron uptake (TfR1) and inhibition of iron storage (Ferritin) by the cell through the IRP/IRE system, or activation of autophagy to recycle iron resources. In addition, redundant iron reductases (e.g. STEAP3) may compensate for CYB561A3 function. Future experiments are needed to validate these compensatory mechanisms and explore the synergistic effects of iron depletion and MYC inhibition. With these analyses, the results of CR6 can be more comprehensively interpreted as cells resisting iron depletion through multilevel adaptive mechanisms, rather than simply negating the hypothesis. This suggests that therapies targeting CYB561A3 need to be used in combination with other targets, such as MYC or autophagy, to push the limits of cellular compensation.

Future research directions should focus on delving into the function of CYB561A3 and its regulatory network in Burkitt's lymphoma (BL) with the aim of advancing the development of potential therapeutic strategies. First, further validation of the effects of CYB561A3 knockout (KO) on iron metabolism and cellular phenotype is needed. For example, the relationship between iron metabolism reprogramming and cell death signaling was clarified by culturing CYB561A3 KO cells in media with different iron concentrations and determining the iron concentration thresholds for their functional exertion. In addition, cellular heterogeneity at different time points (e.g., 24 h, 48 h, 72 h) was analyzed in conjunction with single-cell sequencing technology to reveal the evolutionary relationship between iron metabolic reprogramming and death signaling. In terms of validation of molecular mechanisms and compensatory pathways, multiple possible results (e.g., CR2, CR6, CR8) suggest that cells may compensate for CYB561A3 deficiency by upregulating other iron reductases (e.g., STEAP3, FRRS1, DCYTB) or iron transport proteins (DMT1). Changes in the expression of these genes need to be validated by qPCR, Western blot, or single-cell sequencing, and their effects on the phenotype assessed in combination with iron chelator/overload experiments. In addition, the compensatory mechanism was further clarified by knocking down STEAP3 in CYB561A3 KO cells to see whether it exacerbated the iron starvation or cell death phenotype.

#### CR7:

In CR7 (unchanged iron and apoptosis with reduced viability), the viability loss likely stems from non-iron-related mechanisms, such as cell cycle arrest or metabolic disruption. CYB561A3 may interact with cell cycle regulators (e.g., CDK1) or glycolytic enzymes. This result suggests that CYB561A3 may affect cell survival by regulating cell proliferation or energy metabolism pathways rather than directly relying on iron metabolism or classical apoptosis pathways. KO may interfere with the expression or activity of key regulatory proteins of the cell cycle (e.g., CDK1, Cyclin B1), leading to G2/M phase arrest; KO may inhibit glycolysis or mitochondrial oxidative phosphorylation and trigger ATP depletion and CR7 results contradict the hypothesis, suggesting that its functional diversity needs to be revisited and may involve synergistic regulation of multiple pathways.

For the exploration of non-iron-dependent functions, CR4, CR5, and CR7 suggest that CYB561A3 may affect cell survival by regulating lysosomal pH, ROS signaling, or cell cycle-associated proteins (e.g., CDK1). These hypotheses need to be validated by lysosomal pH probes (e.g., LysoSensor), ROS assays (DCFH-DA), and cell cycle analysis (PI staining). These studies will allow a more comprehensive understanding of the mechanism of CYB561A3's role in cell survival and death.

Future experiments should analyze cell cycle distribution (PI staining) and glycolytic flux (extracellular acidification rate assay) to identify alternative pathways. For example, screen CYB561A3 knockout-sensitive genes by CRISPR libraries to verify whether they lead to cycle block by inhibiting glycolysis or nucleotide metabolism (e.g., RRM2). Meanwhile, lysosomal-mitochondrial functional coupling was dynamically monitored using dual fluorescent reporter systems (e.g., mito-LysoTracker) to further reveal its role in cellular metabolism and energy supply.

CR8:

CR8 fully contradicts the hypothesis, suggesting CYB561A3 is dispensable in CA46 cells under these conditions. Compensatory ferrireductases (e.g., STEAP3) or experimental variability (e.g., incomplete KO) may explain this outcome. CA46 cells may express other iron reductases (e.g., STEAP3, FRRS1, or DCYTB) that are compensatorily up-regulated in the absence of CYB561A3 to maintain iron-reducing functions. Validation method: qPCR/WB assay for the expression level of candidate iron reductases. If the medium contains high levels of iron (e.g., saturated with transferrin), the effect of CYB KO on iron uptake may be masked. Phenotypes under iron-limiting conditions need to be tested. If the phenotype appears late, the observation period needs to be extended or an inducible KO model needs to be used. Also, CA46 cells (Burkitt lymphoma origin) may have specific metabolic patterns (e.g., high glycolytic activity) that are less sensitive to iron-dependent pathways.

In the future, for compensatory pathway exclusion and validation, candidate compensatory genes such as DCYTB can be knocked down in CYB561A3 knockdown cells, and the compensatory effect can be assessed in combination with iron metabolism markers (e.g., ferritin, transferrin receptor); and for validation of the specificity of the technology, whole genome sequencing or CRISPR off-targeting analyses (e.g., GUIDE-seq) can be performed to confirm the CYB561A3 knockdown specificity and exclude clonal heterogeneity interference. Finally, in terms of technology validation and functional diversity exploration, for negative results of CR8, it is necessary to confirm the specificity of CYB561A3 KO by whole-genome sequencing or CRISPR off-target analysis and to exclude experimental variability (e.g. incomplete knockdown or clonal heterogeneity). Identification of CYB561A3 intercalating proteins (e.g., microtubule-associated proteins) by IP-MS or neighbor-tagging technology (BioID) can reveal its non-classical functions. These studies will contribute to an in-depth understanding of the multiple roles of CYB561A3 in iron metabolism, death signaling, and the cell cycle, and will inform the development of combined therapeutic strategies based on its functional weaknesses. Through multi-level validation (molecular, cellular, in vivo) and dynamic analysis, it is expected to reveal new therapeutic targets for BL and provide a reference for other MYC-driven tumors.

## 6. Conclusion

In conclusion, this study identifies a critical dependence of CYB561A3 in Burkitt's lymphoma (BL), and knockdown of CYB561A3 induces iron starvation, lysosomal dysfunction, and destabilization of MYC proteins, which ultimately leads to non-apoptotic cell death. These findings directly fill the knowledge gap identified in the "Introduction" regarding the mechanistic interplay between iron metabolism and MYC-driven proliferation in BL. By demonstrating that CYB561A3-mediated lysosomal iron reduction is indispensable for maintaining iron homeostasis and supporting MYC stability, this study sheds light on the metabolic vulnerabilities specific to MYC-dependent BL cells. In particular, the iron-dependent phenotype observed in CR1 defines CYB561A3 as metabolically critical in BL pathogenesis.

These results highlight the therapeutic potential of targeting CYB561A3 in MYC-driven malignancies, which may reduce off-target effects given its minimal expression in non-malignant cells. However, partially supported scenarios (e.g., CR2, CR6) highlight the complexity of compensatory pathways, such as STEAP3 upregulation or autophagy-mediated iron recycling, which

may attenuate CYB561A3 loss. This emphasizes the need for combinatorial strategies, such as pairing CYB561A3 inhibitors with MYC-targeting drugs or iron chelators, to overcome resistance and extend efficacy.

Going forward, future studies will have to validate the mechanistic hypotheses proposed for each CR regimen through molecular-cellular-animal multilevel experiments. Integration of dynamic analyses (e.g., time-resolved iron flux measurements), multi-omics techniques (e.g., proteomics for mapping surrogate iron reductases), and targeted interventions (e.g., CRISPR screens for identifying synthetic lethal partners) will systematically parse the functional network of CYB561A3 in BL. In addition, in vivo validation using BL xenograft models and development of small molecule inhibitors targeting CYB561A3 are critical next steps. Ultimately, this study not only deepens our understanding of iron addiction in MYC-driven cancers, but also paves the way for innovative therapies targeting the iron metabolism-death signaling axis, bringing new hope to patients with aggressive lymphomas.

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