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Acetylation suppresses breast cancer progression by sustaining CLYBL stability

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Abstract

Background The incidence of breast cancer remains high and it remains the leading cause of cancer-related deaths in women. A better understanding of the molecular mechanisms of breast cancer and identifying novel biomarkers will help improve therapeutic strategies. Citrate lyase beta like (CLYBL) is expressed at low levels in breast cancer tissues and is associated with low patient survival rates. In this study, we explored the regulatory mechanisms of CLYBL and its acetylation in breast cancer.

Methods CLYBL expression patterns in breast cancer were assessed using a breast cancer tissue microarray, immunohistochemistry, and publicly available datasets. The acetylation patterns of CLYBL and the related regulatory functions were detected by high resolution mass spectrometry, immunoprecipitation assays, and western blot analysis. The potential effects of CLYBL and its acetylation on breast cancer were determined using both in vitro and in vivo assays.

Results CLYBL was expressed at lower levels in breast cancer samples compared with normal tissues. This low CLYBL expression was associated with poor patient survival rates. Overexpressing CLYBL could inhibit breast cancer and reduce NRF2 pathway-mediated antioxidants. We identified two acetylated lysine sites in CLYBL, K57 and K82, using acetylated peptide affinity enrichment and high-resolution mass spectrometry. Our results suggest that K82 is the main acetylation site. Further work showed that the p300/CBP associated factor (PCAF) and histone deacetylase 3 (HDAC3) as the CLYBL acetyltransferase and deacetylase, respectively. Additionally, CLYBL acetylation facilitates its own protein stability by reducing it affinity for ubiquitin, thus enhancing the anti-breast cancer effects.

Conclusion We revealed the role of CLYBL overexpression and its acetylation in breast cancer. Our study suggests that CLYBL is a potential molecular target for breast cancer therapy.

Highlights

- Low expression of CLYBL in BC is associated with poor prognosis.
- CLYBL is acetylated at K82, which facilitates its own stability by inhibiting CLYBL ubiquitination.

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- PCAF promotes, whereas HDAC3 decreases CLYBL acetylation.

Keywords CLYBL, Acetylation, Breast cancer (BC), PCAF, HDAC3

Introduction

The incidence of breast cancer has increased over time, with this disease remaining the most common cause of female cancer-related deaths [1]. About 2 million breast cancer cases were diagnosed every year [2]. Despite advances in early diagnostic and treatment methods, the postoperative recurrence and metastasis rates in breast cancer patients are still high. Therefore, exploring new molecular targets will provide important strategies for breast cancer therapy.

Metabolic enzymes are involved in multiple processes related to cancer development and progression and have become a focus of oncology research. TCA cycle intermediates can impact tumor biology by modulating various metabolic signaling pathways [3]. Citrate lyase beta like (CLYBL) is a soluble matrix enzyme that is widely expressed in mitochondria [4]. Because CLYBL converts the TCA cycle metabolite itaconate through the citramalyl-CoA intermediate to acetyl-CoA, increased CLYBL expression levels may lead to itaconate depletion. Itaconate can inhibit Succinate dehydrogenase (SDH) and activate nuclear factor E2-related factor 2 (NRF2) via alkylation of Kelch-like ECH-associated protein 1 (KEAP1), leading to an enhanced antioxidative stress response [5, 6]. Because the cellular redox state and mitochondria both have critical effects on cell fate, there has been increased interest in their involvement in processes related to inflammation, metabolism, and carcinogenesis [7]. Therefore, we aimed to investigate the role of CLYBL and its redox metabolic regulation in cancer cells.

Post-translational modifications (PTMs), such as acetylation, phosphorylation, methylation, and ubiquitination, play important roles in regulating multiple cellular physiological processes [8, 9]. Recent studies have shown that lysine acetylation is critically involved in the regulation of various metabolic enzymes [10, 11]. Regulation of protein functions by acetylation can occur in multiple ways, including through conformational changes, protein–protein interactions, protein–DNA interactions, protein activity, and protein stability. Previous studies have suggested that acetylation and ubiquitination may compete at the same lysine residue to regulate protein stabilization and degradation [12–14]. Consequently, such PTMs can induce aberrant oncogene activation [15, 16]. Additionally, acetyltransferases, such as CREB-binding protein (CBP), p300, General Control Non-repressed 5 protein (GCN5), P300/CBP-associating factor (PCAF), and lysine acetyltransferases 5 (TIP60/KAT5), or histone deacetylases (HDACs) can catalyze both histone and non-histone acetylation or deacetylation [17]. Therefore, regulating the acetylation status of targeted proteins could potentially change its activity or stability.

In this study, we report that low CLYBL expression occurs in BC and predicts poor prognosis in breast cancer patients. Acetylation of CLYBL promotes the protein stability by regulating Kac at the CLYBL-K82 site, thereby inhibiting the proliferation of breast cancer.

Materials and methods

Inhibitors and antibodies

The following reagents were purchased from the indicated manufacturers: Inhibitors: Chidamide (HY-109015), MG132 (HY-13259) and Cycloheximide (CHX, HY-12320) were purchased from MedChemExpress. Antibodies: Flag tag (D6W5B, CST), HA tag (C29F4, CST), CLYBL (ab235434, Abcam), (17314-1-AP, Proteintech), Nrf2 (ab62352, Abcam), Myc (ab32072, Abcam), Acetylated-Lysine (9441S, CST), β-actin (AC038, Abclonal), Lamin (A11495, Abclonal) and α-Tublin (AC007, Abclonal).

Plasmids construction and transfection

Human CLYBL and HDAC3 were amplified from a MDA-MB231 cDNA library and subcloned into pLVX-Puro. Other plasmids were constructed by Sangon Biotech Co., Ltd (Shanghai, China). Each plasmid was transfected by Lipofectamine™ 3000 transfection reagent at a dose recommended by the manufacturer instructions.

Cell culture and treatment

MDA-MB231、Hs578T、SUM159 and BT549 cells were grown in DMEM supplemented with 10% FBS. For establishing stable transfectants with CLYBL expression, BC cells were transfected with pLVX-CLYBL lentivirus and stable expressed cells were selected with puromycin (100 ng/ml) for three times. The cells were grown in a cell culture incubator at 37 °C and 5% CO_2. The treatment of Chidamide, MG132, and CHX is described in the figure legends.

Quantitative RT-PCR

Total RNA was isolated using PureLink RNA Mini kit (Thermofisher) according to the manufacturer's instructions. Specific quantitative RT-PCR experiments were performed using Power SYBR™ Green PCR Master Mix following manufacturer's protocol (Thermofisher). Gene expression level was normalized to GAPDH level in respective samples as an internal control, and the experiments were performed at least three times. The primers used for RT-qPCR were: 5'-TGCACCACCAACTGCTT

AGC-3'(forward) and 5'-GGCATGGACTGTGGTCATG AG-3'(reverse) for GAPDH; 5'-GGGCACAGGTAAAA CCAAATAG-3'(forward) and 5'-TTTTCACAATGACC GAATACCG-3'(reverse) for GCLM; 5'-ACCAGCGTGC CATAGAGAATGAG-3'(forward) and 5'-CAGCCTTCG GTCTTGGTCCAG-3'(reverse) for GSS; 5'-CCTCCCT GTACCACATCTATGT-3'(forward) and 5'-GCTCTTC TGGGAAGTAGACAG – 3'(reverse) for HMOX; 5'-AG TATCCTGCCGAGTCTGTTCTGG-3'(forward) and 5'-AATATCACAAGGTCTGCGGCTTCC – 3'(reverse) for NQO-1.

Colony formation assay

Colony formation assay was performed using double-layer agar in 24-well plates. The top layer is 0.35% agar and the bottom layer is 0.7% agar. The two type of breast cancer cells were seeded in desired medium with proper cell counts and the colonies were stained and counted after cultivation for about 20 days.

Mouse experiments

Animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee at the Zhejiang University. Balb/c mice (about 6 wk old) were injected with 1×10^6 4T1 cells. After the tumor formation, the Chidamide(50 mg/kg)was injected to the mice, every two days for two weeks. Then the tumor were isolated from mice and the acetylation proteome were applied.

To examine the effect of CLYBL acetylation on tumorigenesis, second mammary fat pad of nude mice (5–8 wk old) were injected with 1×10^6 exogenous wild type CLYBL overexpressing MDA-MB231 cells on the left flank and exogenous CLYBL mutant K82Q cells on the right flank. Tumor formation was monitored every 2 days for 23 days. Tumors size and weight were measured.

Peptide immunoprecipitation

Proteins from mouse tumor were extracted and subjected to trypsin digestion. Pan anti-Kac antibodies were first conjugated to Protein A Sepharose beads and then incubated with digested peptides with gentle agitation overnight at 4 °C. The beads were then washed three times with wash buffer (20 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40). Peptides were eluted from the beads with 0.1% TFA and the eluents were vacuum-dried and the C18 ZipTips were used to remove the salts for mass analysis.

HPLC–MS/MS analysis

Peptides were separated and eluted with a gradient of 3–85% HPLC buffer A (0.1% formic acid in acetonitrile, v/v) in buffer B (0.1% formic acid in water, v/v) at a flow rate of 200 nl min – 1 over 70 min (35 min for coelution studies). Then a Q-Exactive mass spectrometer (Thermo Fisher Scientific) was used to ionize and analysis the eluted peptides. Full mass spectrometry over the range m/z 300 to 1,500 with a resolution of 70,000 at m/z 200 was acquired in the Orbitrap mass analyser. The 13 most intense ions with charge ≥ 2 were fragmented with normalized collision energy of 27 and tandem mass spectra were acquired with a mass resolution of 17,600 at m/z 200.

Bioinformatics analysis

Amino acid sequences from the mass spectra data were searched against mouse protein sequences from the UniProt database for the identification of certain proteins. MaxQuant (V1.6.15.0) was used to analyze secondary mass spectrometry data in this research. The signal intensity of each peptide was obtained by searching the mass spectrum data. The relative quantification of protein was carried out according to the signal intensity. Subcellular localization was assessed by WolF PSORT software for the annotation of identified proteins.

Gene Ontology (GO) annotation of the identified proteins in this study were carried out based on DAVID Bioinformatics Resource 6.8 (NIAID/NIH). GO terms enriched in the annotation results were extracted, and the proteins were then functionally classified according to cellular component, molecular function and biological process.

Isolation of nuclear and cytoplasmic extract

The nuclear extraction was prepared using an NE-PER Nuclear Cytoplasmic Extraction Reagent kit (Invent Biotechnologies, MN, USA) according to the manufacturer's instruction.

Immunoprecipitation and western blotting

Cells were lysed in 1% NP40 buffer (150 mM NaCl and 25 mM Tris-HCl, pH 7.2 ~ 7.4) containing protease inhibitor cocktail. The cell lysates were incubated with Flag/HA beads at 4 °C overnight after cell debris was removed by centrifugation at 16,000 g for 10 min and 4 °C. After incubation, the samples were washed with TBS buffer three times. Then, the beads were boiled in 2× SDS loading buffer and centrifuged at 4 °C in preparation for the western blot analysis. The adjusted protein samples were mixed with loading buffer and electrophoresed on 4–20% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels. After electrophoresis, the proteins were transferred to PVDF membranes. Blocking the membranes with 5% fat-free milk followed by overnight incubation with the indicated primary antibody at 4 °C. Following incubation with secondary antibody on the next day, the blots were visualized using ECL assay.

Tissue microarray and immunohistochemical (IHC) staining

Tissue microarray containing specimens obtained from 140 cases of breast cancer patients was purchased from Shanghai Outdo Biotech Inc (HBreD140Su03). All clinical samples were collected with informed consent under the Health Insurance Portability and Accountability Act (HIPAA) approved protocols.

The endogenous peroxidase blocker H_2O_2 was added to block non-specific antigens after dewaxing and hydrating the tissue samples. After overnight incubation with primary antibodies at 4 °C, secondary antibodies were added to the samples. Following the addition of Diaminobenzidine (DAB), the nucleus was stained with hematoxylin. The pictures were scanned using an Olympus Microscope.

Statistical analysis

Results are expressed as mean ± SD or SEM as indicated. Comparisons were made by the two-tailed Student's t test or one-way ANOVA. Survival curves were performed using the Kaplan-Meier method, and differences were analyzed by the log rank test. In all statistical tests, $P < 0.05$ was considered statistically significant.

Results

CLYBL is downregulated in breast cancer, which correlates with poor patient survival rates

Identifying additional target molecules will provide potential new treatment approaches for breast cancer. Firstly, we analyzed the overall survival (OS) and relapse-free survival (RFS) of breast cancer patients and according to CLYBL expression status, we found that low CLYBL expression was associated with poor patient survival rates (Fig. 1A-C). CLYBL mRNA expression patterns from TCGA dataset also showed CLYBL was downregulated in breast cancer samples (Fig. 1D). We also compared CLYBL expression levels in human breast cancer tissues from 140 patients to corresponding adjacent samples by immunohistochemical (IHC) staining. The IHC results showed that the CLYBL protein expression patterns were consistent with the above analysis at the mRNA level (Fig. 2A–C). We further investigated the associations between CLYBL expression levels and various clinicopathological features of breast cancer patients. Certain features tended to be associated with low CLYBL expression, but the differences were not statistically significant (Fig. 2D). Univariate and multivariate analyses suggested that breast cancer patient overall survival correlated with CLYBL expression, grade, age, TNM stage, ER expression, and P53 expression. Among these factors, CLYBL expression, ER expression, and age (≥60) were independent clinicopathological factors for poor overall survival in these patients (Fig. 2E). These data suggest that low CLYBL expression levels may predict poor prognosis in breast cancer patients.

Overexpression of CLYBL can inhibit breast cancer and reduce NRF2 pathway-mediated antioxidants

To further determine the role of CLYBL in breast cancer, we examined the impact of CLYBL expression on MDA-MB231、Hs578T、SUM159 and BT549 cell proliferation. We evaluated the tumorigenicity in vitro using soft agar assays, finding that overexpressing CLYBL significantly repressed colony formation of these cells (Fig. 3A and Supplementary Fig. S1A). Next, we assessed the tumorigenicity using xenograft models. Consistent with our previous results, MDA-MB231 cells with CLYBL overexpression showed markedly reduced tumor growth in vivo compared with wild-type cells (Fig. 3B). CLYBL converts the TCA cycle metabolite itaconate through the citramalyl-CoA intermediate to acetyl-CoA, with increased CLYBL levels potentially leading to itaconate depletion. Itaconate was previously demonstrated to regulate antioxidant production in a Nrf2-dependent manner [18]. Because redox status is emerging as an anti-cancer treatment approach and oxidative metabolism is involved in cancer cell fate determination [19], we next evaluated if CLYBL can affect the antioxidant pathway. As expected, CLYBL overexpression resulted in decreased expression levels of Nrf2 in the nucleus and mRNA expression of GSS, GCLM, NQO-1 and HMOX in the Nrf2 pathway (Fig. 3C/D and Supplementary Fig. S2A). These results illustrated the anti-tumor effects of CLYBL overexpression in breast cancer, which may be caused by the reduced antioxidant levels mediated by Nrf2.

CLYBL can be acetylated at residue K82

PTMs are vital for maintaining cellular homeostasis and modulating the conformation, activity, interactions, and stability of proteins [20, 21]. Acetylation is one of the major PTMs and often is involved in crosstalk with ubiquitination. In our study, CLYBL acetylation modification was observed using co-immunoprecipitation assays. This acetylation was increased after treatment with Chidamide (CHI), a benzamide-based class l histone deacetylase inhibitor (Fig. 4A/B). Furthermore, we established tumor xenografts by inoculating 4T1 cells in BALB/C mice. Tumor tissues were collected 14 days after CHI treatment and sent for profiling of the lysine acetylation proteome. The subcellular localization of the identified proteins displayed a wide distribution among the nucleus, cytoplasm, and mitochondria (Fig. 4C). Proteins with only one modification site accounted for 35.5% of the total identified proteins, while proteins with seven or more modification sites accounted for 16.3% (Fig. 4D). We performed gene ontology (GO) analyses of the proteins

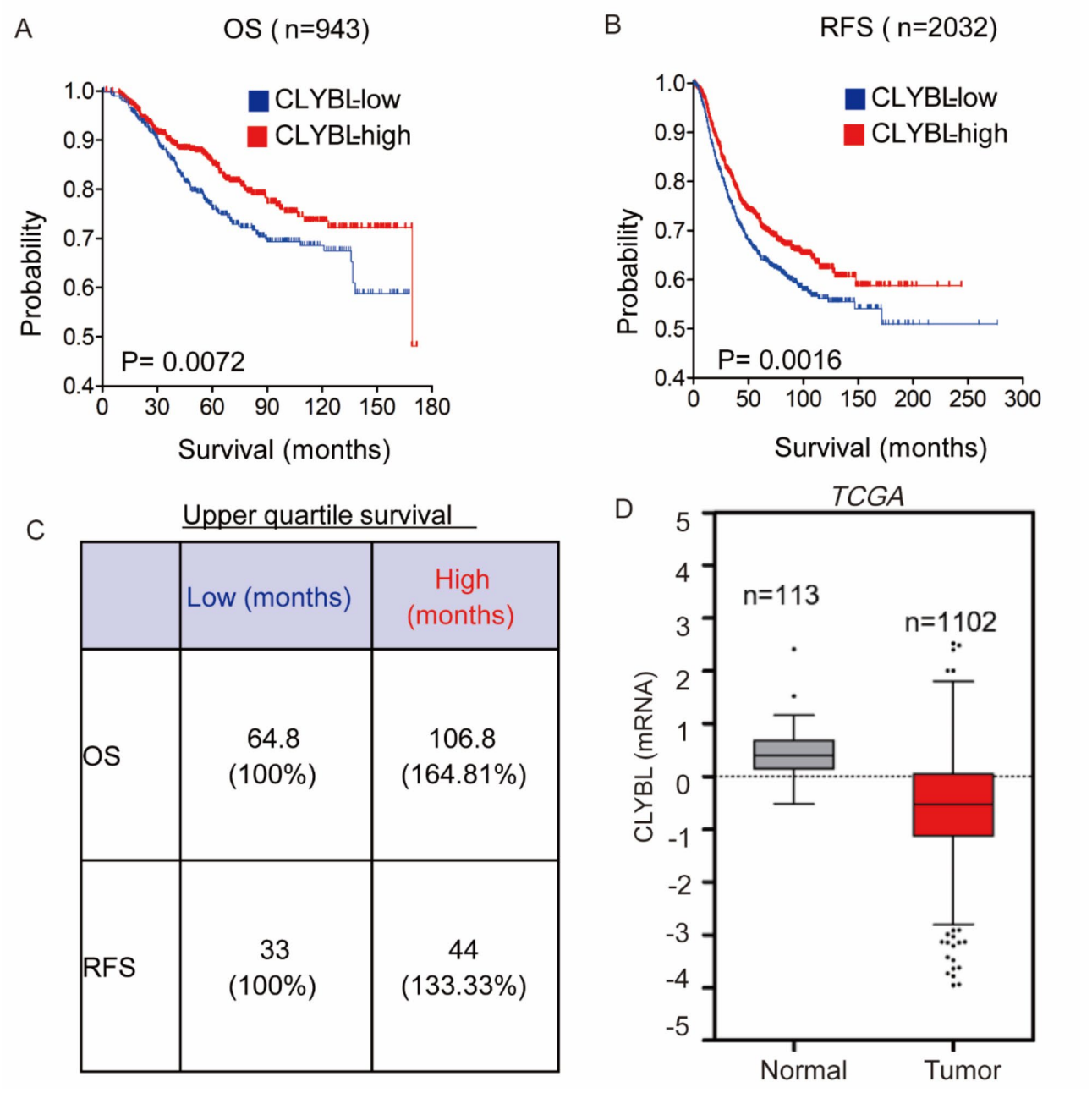
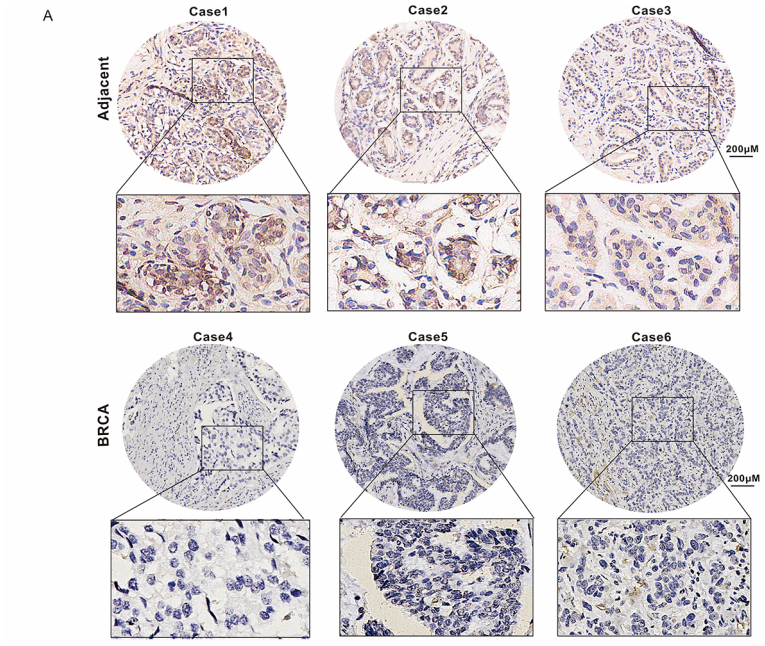


Fig. 1 Breast cancer has low CLYBL expression level that is negatively associated with survival rates. (**A-C**) Kaplan–Meier survival analysis for OS and RFS of breast cancer patients according to CLYBL expression status. The *P*-value was determined using the log-rank test. (**D**) Box plots showing CLYBL mRNA expression patterns in breast cancer samples from the TCGA dataset

with downregulated and upregulated acetylation levels following CHI treatment (Fig. 4E). Among these proteins, mass spectrometry analysis identified two potential acetylation sites in CLYBL, K57 and K82, both of which are highly conserved among species (Fig. 4F/G). We then tested if these two lysine sites are the main acetylation sites on CLYBL. Flag-tagged CLYBL mutants, in which each of the two lysine residues was changed to arginine via site-directed mutagenesis, were transfected into MDA-MB231 and Hs578T cells. Compared with CLYBL-K57R, CLYBL-K82R revealed significantly reduced acetylation levels, but the double-mutant CLYBL-K57R/82R did not show any further decreased acetylation (Fig. 4H Supplementary Fig. S2B). Thus, CLYBL can be regulated by acetylation, with K82 being the main site of this PTM.



D

Correlation between CLYBL expression and clinicopathological characteristics

	variables	CLYBL expression		total	χ2	p value
		low	High			
Age (year)	< 50	43	23	66	0.060	0.806
	>= 50	45	22	67		
Age (year)	< 60	56	32	88	0.743	0.389
	>= 60	32	13	45		
Grade	II	60	31	91	0.007	0.934
	III	28	14	42		
TNM stage	TNM I	22	10	32	0.797	0.671
	TNM II	34	21	55		
	TNM III	32	14	46		
HER2	negative	62	36	98	1.176	0.278
	positive	18	6	24		
ER	negative	30	10	40	2.360	0.124
	positive	55	35	90		
PR	negative	39	22	61	0.149	0.700
	positive	47	23	70		
	positive	10	8	18		
P53	negative	36	27	63	0.316	0.574
	positive	11	6	17		

* Statistically significant(p<0.05)

E

Variables in the Equation

Variables	Univariate analysis			Multivariate analysis		
	HR	95%CI	p value	HR	95%CI	p value
Expression	0.301	0.104-0.866	0.026	0.185	0.049-0.701	0.013
Grade	2.170	1.044-4.514	0.038	1.677	0.585-4.806	0.336
Age (>=60)	2.205	1.063-4.574	0.034	3.879	1.206-12.476	0.023
T stage	1.312	0.610-2.822	0.487			
N stage	1.835	1.296-2.598	0.001	2.749	0.799-9.461	0.109
TNM stage	2.357	1.343-4.135	0.003	0.395	0.083-1.876	9.461
ER	0.319	0.153-0.662	0.002	0.220	0.072-.665	0.007
PR	0.724	0.348-1.504	0.386			
P53	2.682	1.064-6.761	0.036	1.928	.668-5.566	0.225

* Statistically significant(p<0.05)

Fig. 2 CLYBL expression in breast cancer tissues. (**A**) Immunohistochemical staining of CLYBL protein in adjacent and matched breast cancer tissues. Scale bars: 200 μM. (**B**) CLYBL expression levels were quantified in both breast cancer tissues and adjacent tissues using the IRS score (P<0.001). Statistical analysis was performed using the unpaired t-test. (**C**) Kaplan-Meier survival curve showing a significant correlation between low CLYBL expression levels and decreased survival in human breast cancer. The P-value was determined using the log-rank test. (**D**) The correlations between CLYBL expression levels and various clinicopathological characteristics. (**E**) Univariate and multivariate analyses of the factors correlated with overall survival of breast cancer patients. The P-value was determined using the log-rank test

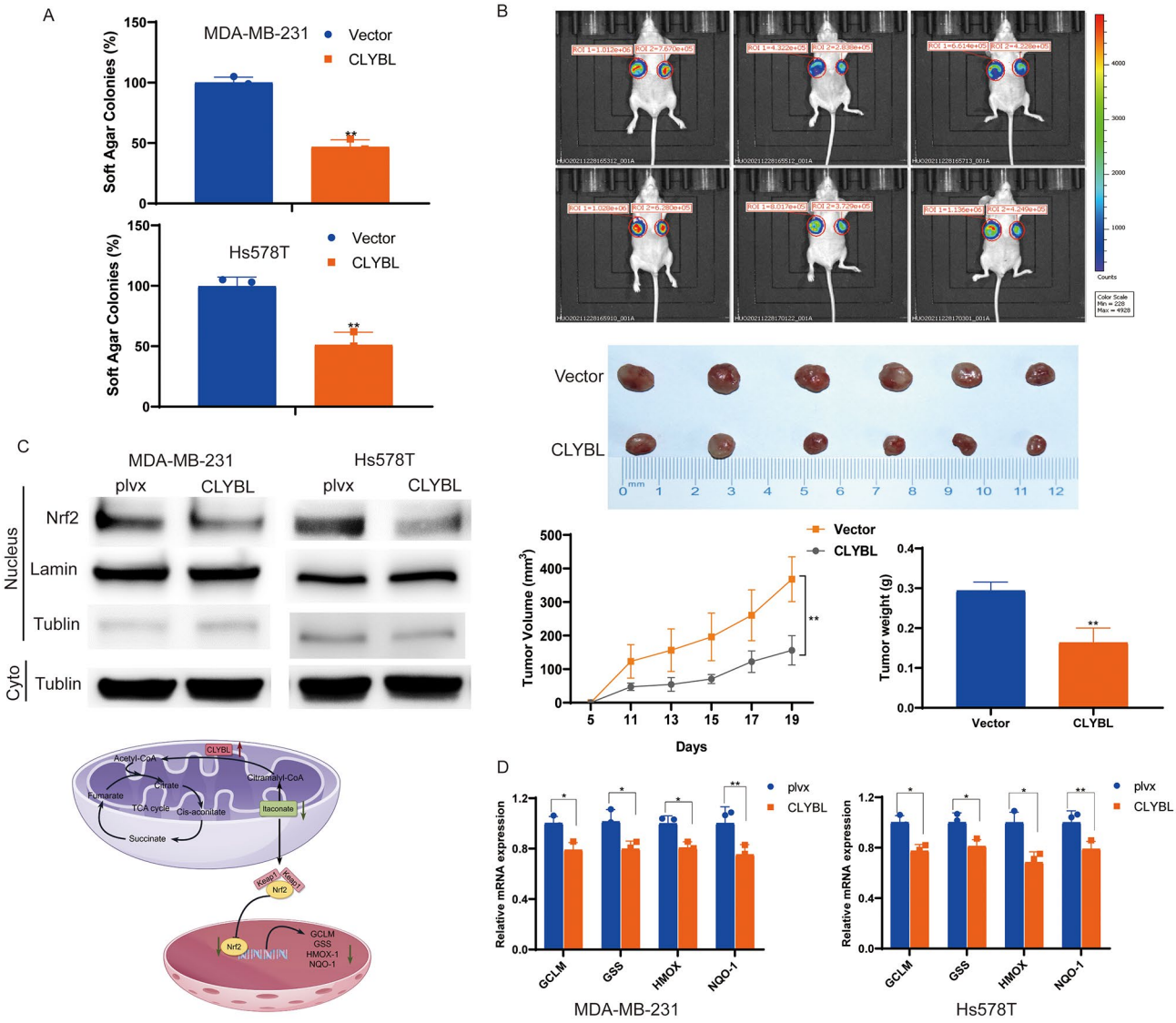


Fig. 3 Overexpression of CLYBL can inhibit breast cancer and reduce NRF2 pathway-mediated antioxidants. (**A**) Soft agar assays were performed using MDA-MB231 and Hs578T cells with or without CLYBL overexpression. Data are presented as the mean ± SD, $n = 3$, $^{**}P < 0.01$. (**B**) MDA-MB231-Luc cells with or without CLYBL overexpression were injected into the second mammary fat pad of nude mice. The tumor volume was recorded every 2 days. After 19 days, the mice were sacrificed and the wet weight of each tumor was determined at autopsy. Data are presented as the mean ± SD of six mice, $^{**}P < 0.01$ by Student's t-test. (**C**) The nuclear proteins of MDA-MB231 and Hs578T cells were collected with or without CLYBL overexpression. (**D**) RT-qPCR was used to detect the mRNA expression levels of *GCLM*, *GSS*, *HMOX*, and *NQO-1*. Data are presented as the mean ± SD, $n = 3$, $*P < 0.05$, $**P < 0.01$

CLYBL is acetylated by PCAF and deacetylated by HDAC3

Lysine acetylation is a reversible PTM typically controlled by lysine acetyltransferases (KATs) and lysine deacetylase (KDACs), also referred to as histone deacetylases (HDACs). To identify the specific upstream enzyme responsible for CLYBL acetylation, we ectopically co-expressed CLYBL with various KATs: P300, CBP, PCAF, Tip60, and GCN5. Among these acetyltransferases, we found that PCAF dramatically increased CLYBL acetylation levels and had an interaction with CLYBL, suggesting that PCAF is the predominant CLYBL acetyltransferase (Fig. 5A/B and Supplementary Fig. S2C). Next, we aimed to identify the specific CLYBL deacetylase. Treatment with CHI, which selectively inhibits HDAC1, HDAC2, HDAC3, and HDAC10, increased CLYBL acetylation levels (Fig. 4B). Interestingly, CLYBL is a mitochondrial enzyme and HDAC3 can translocate to the mitochondria to deacetylate non-histone proteins [22]. We then detected if HDAC3 can influence CLYBL acetylation. Overexpression of HDAC3 in HEK293T cells markedly reduced CLYBL acetylation levels (Fig. 5C and Supplementary Fig. S2D). Furthermore, co-immunoprecipitation analysis identified a specific interaction between CLYBL and HDAC3 in these cells (Fig. 5D/E). Taken together, these data suggest that CLYBL is acetylated by PCAF and deacetylated by HDAC3.

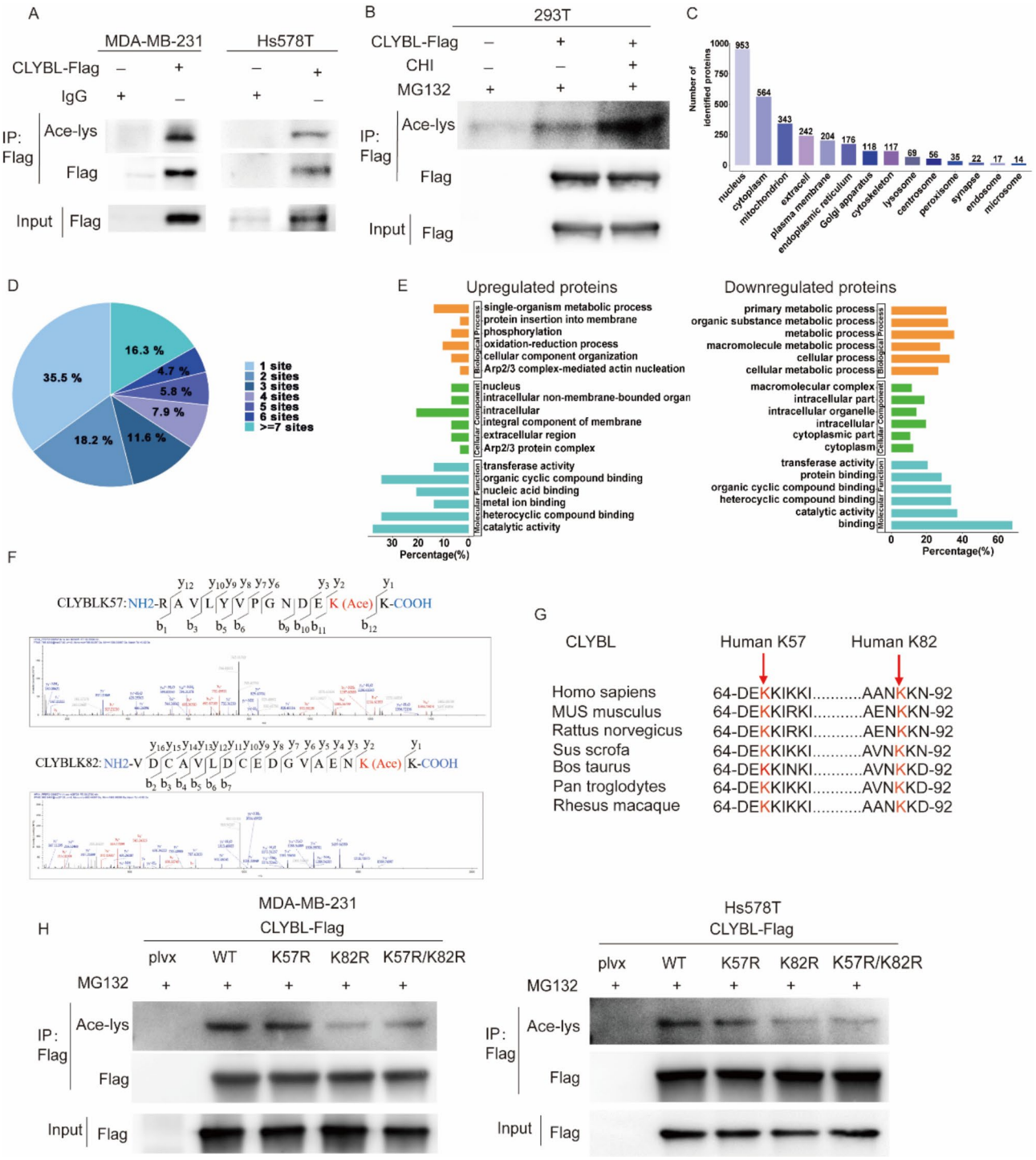


Fig. 4 (See legend on next page.)

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Fig. 4 CLYBL is acetylated at its lysine 82 site. (**A**) Acetylation of exogenous CLYBL-Flag in MDA-MB231 and Hs578T cells. Immunoprecipitation of whole cell lysates with an anti-Flag antibody or IgG was followed by western blot analysis with the anti-acetyl-lysine and anti-Flag antibodies. (**B**) Acetylation of exogenous CLYBL-Flag in HEK293T cells treated with deacetylase inhibitors CHI (3.2 μM) for 24 h. CLYBL-Flag was immunoprecipitated with the anti-Flag antibody, and then the precipitates were analyzed using an anti-acetyl-lysine antibody (Ace-lys). 4T1 cells were injected into the second mammary fat pad of BALB/C mice and tumor tissues were collected 14 days after CHI treatment, and then sent for profiling of the lysine acetylation proteome. (**C**) The bar diagram shows the subcellular localization of proteins modified by acetylation determined with WolF PSORT software. (**D**) The pie chart shows the distributions of the number of sites in the modified proteins. (**E**) The left and right sides of the bar chart represent the GO terms enriched in the proteins with upregulated or downregulated acetylation levels. (**F**) The tissues lysates and isotopically-labeled lysates were mixed and immunoprecipitated with anti-acetyl-lysine antibody-conjugated beads, and then were analyzed by mass spectrometry (MS). (**G**) Alignment of the CLYBL amino acid sequences from various species. Red shading indicates the conserved K57 and K82 residues. (**H**) CLYBL-Flag (K57R), CLYBL-Flag (K82R), CLYBL-Flag (K57R/K82R), or CLYBL-Flag (WT) were transfected into MDA-MB231 and Hs578T cells. The lysates were extracted, and then CLYBL acetylation was detected by co-IP and western blot analysis with the indicated antibodies

K82 acetylation inhibits CLYBL degradation via the K48-linked polyubiquitination–proteasome pathway

The interaction between two lysine modifications, acetylation and ubiquitination, can affect the stability of multiple cellular proteins [23]. We therefore evaluated if acetylation at K82 could affect CLYBL–ubiquitin binding. Firstly, immunoprecipitation of CLYBL-Flag showed that CLYBL and ubiquitin could form conjugates (Fig. 6A). Next, we constructed acetylation-mimetic mutants CLYBL-K82Q and determined its association with ubiquitylated proteins. Compared with CLYBL-WT, CLYBL-K82Q co-precipitated fewer ubiquitylated proteins (Fig. 6B). Because K48-linked polyubiquitination mainly mediates protein degradation through the proteasome, subsequent immunoprecipitation assays demonstrated that K48-linked ubiquitination was co-precipitated with CLYBL (Fig. 6C). Finally, we used cycloheximide assays to detect the degradation rate of CLYBL, showing that CLYBL-K82Q or treated with Chidamide (CHI) had a much longer half-life than CLYBL-WT (Fig. 6D and Supplementary Fig. S3B). Additionally, we evaluated the tumorigenicity in vivo using xenograft models, finding that MDA-MB231 cells with CLYBL-K82Q overexpression had significantly reduced tumor growth compared with CLYBL-WT (Fig. 6E). Collectively, these results revealed that acetylation of CLYBL at K82 could repress K48-linked ubiquitination, which stabilizes the expression of CLYBL and further inhibiting breast cancer.

Discussion

During the past several decades, molecular-targeted therapies were being in progress in various cancers [24–27]. Breast cancer is the leading cause of cancer-related deaths in women, and new predictive biomarkers are urgent to shape the future therapeutic landscape. CLYBL is an intermediate of the C5-di-carboxylate metabolic pathway and was reported to be a citramalyl-CoA lyase [28]. Recent studies have found that CLYBL is upregulated in COVID-19 patients, with increased CLYBL levels potentially leading to the depletion of itaconate, which plays an important role in anti-oxidation and anti-inflammation [29]. However, the role of CLYBL in cancer is still unclear. In this study, we observed downregulated CLYBL expression levels in human breast cancer tissues and that these low levels were associated with low patient survival rates. Overexpression of CLYBL could inhibit breast cancer growth in both in vivo and in vitro assays. Therefore, our research identified CLYBL as a potential tumor suppressor in breast cancer.

Abnormal antioxidant activity in cancer cells is thought to be a contributing mechanism to tumorigenesis [30], with regulation of redox status emerging as an anticancer treatment. In this study, we observed decreased antioxidant activity after CLYBL overexpression. CLYBL can convert itaconate to acetyl-CoA, and the accumulation of CLYBL leads to the loss of upstream metabolites, thus resulting in decreased itaconate levels. Itaconate was demonstrated to regulate antioxidant production in a Nrf2-dependent manner [18]. As a pivotal regulator of antioxidant defense, Nrf2 was downregulated in the nucleus after CLYBL overexpression. These data suggest that CLYBL can possibly inhibit breast cancer via reduced antioxidant production mediated by Nrf2.

PTMs are critical for the functions of various proteins. Acetylation is a major PTM and has been reported to be essential in tumorigenesis as protein acetylation plays crucial roles in cell metabolism, oncogenic activity, immune response and so on [31]. Although acetylation modification is a main PTM in histones, growing evidence has indicated that acetylation of non-histone proteins can regulate several cellular processes that are crucial for maintaining cellular homeostasis [32, 33]. P53 was one of the first non-histone proteins recognized to be regulated by acetylation, with this PTM promoting its tumor suppressive function [34]. In colorectal cancer, Wang et al. found that deacetylation of IDH1 can regulates cellular metabolism and inhibit tumor metastasis [35]. In our research, we found that CLYBL could be regulated by acetylation after treatment with CHI, a benzamide-based class l HDAC inhibitor. Furthermore, we identified two acetylation sites using acetylated peptide affinity enrichment and high-resolution mass spectrometry, verifying that K82 was the main site. There are multiple regulatory roles of acetylation in proteins, one

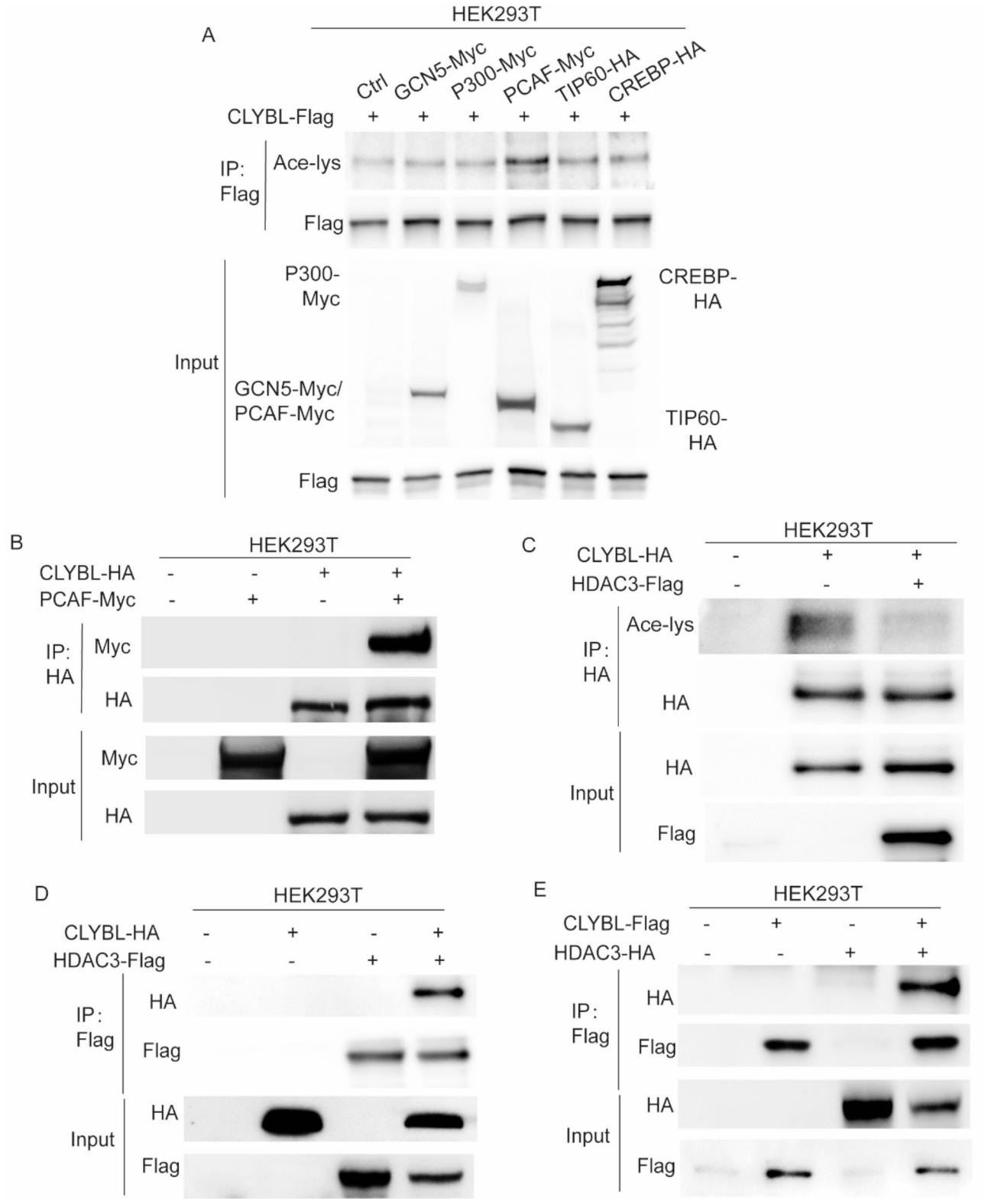

Fig. 5 CLYBL is acetylated by PCAF and deacetylated by HDAC3. (**A**) HEK293T cells were transfected with CLYBL-Flag and a serious of acetyltransferases, GCN5-Myc, P300-Myc, PCAF-Myc, TIP60-HA, and CREBP-HA, to detect CLYBL acetylation levels by immunoprecipitation. (**B**) Exogenous co-IP was used to detect the interactions between PCAF and CLYBL in HEK293T cells. (**C**) HEK293T cells co-transfected with CLYBL-HA and HDAC3-Flag were used to detect CLYBL acetylation levels by immunoprecipitation. (**D**, **E**) Co-immunoprecipitation of HDAC3 with CLYBL was performed using HEK293T cells co-transfected with CLYBL-HA and HDAC3-Flag or CLYBL-Flag and HDAC3-HA

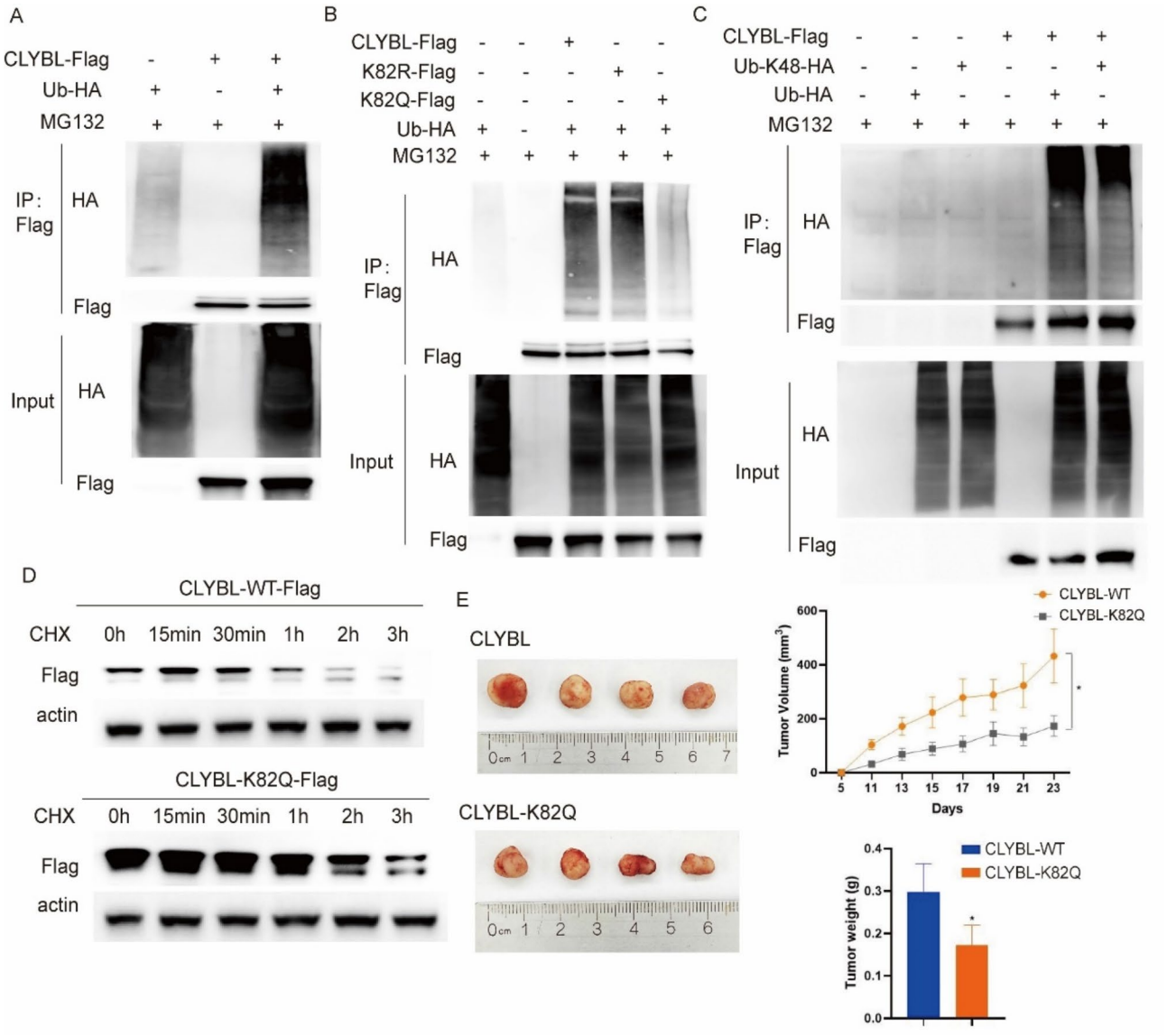

Fig. 6 K82 acetylation inhibits CLYBL degradation and further represses breast cancer. (**A**) HEK293T cells were transfected with CLYBL-Flag and/or Ub-HA vectors, followed by treatment with MG132 (100 μM). The lysates were extracted and CLYBL ubiquitination was detected by co-IP and western blot analysis with the indicated antibodies. (**B**) CLYBL-Flag (K82Q), CLYBL-Flag (K82R), CLYBL-Flag (WT), or Ub-HA were transfected in HEK293T cells. The lysates were extracted and CLYBL ubiquitination was detected by co-IP and western blot analysis with the indicated antibodies. (**C**) CLYBL-Flag, Ub-K48-HA, or Ub-HA were transfected in HEK293T cells. The lysates were extracted and K48-linked ubiquitination of CLYBL was detected by co-IP and western blot analysis with the indicated antibodies. (**D**) HEK293T cells were transfected with CLYBL-Flag (WT) and CLYBL-Flag (K82Q) for 36 h, then incubated with 20 μg/mL cycloheximide (CHX) for the indicated times and analyzed by western blot analysis. (**E**) MDA-MB231 cells with CLYBL (WT) or CLYBL (K82Q) overexpression were injected into the second mammary fat pad of nude mice. The tumor volume was recorded every 2 days. After 23 days, the mice were sacrificed and the wet weight of each tumor was determined at autopsy. Data are presented as the mean ± SD of six mice, **P* < 0.05 by Student's t-test

of which is to mediate proteasomal degradation of proteins by competing with ubiquitination at lysine residues. Our study found that acetylation of CLYBL at K82 could enhance its own stability by reducing K48-linked polyubiquitination and degradation. This was consistent with previous work that acetylation can influence the stability of proteins such as ZEB1, Slug, LATS1, and DOT1L [17, 36–38]. Thus, we demonstrated a tumor suppressive effect of CLYBL acetylation in breast cancer, with competition between ubiquitination and acetylation at K82 being the possible underlying mechanism for acetylation-dependent CLYBL stabilization.

Acetylation is mainly regulated by KATs and KDACs. The main canonical mammalian KATs include GCN5, P300, PCAF, Tip60, and CREBP [39]. Here, we found that expressing PCAF resulted in clear increases in CLYBL acetylation levels. HDACs are enzymes that catalyze the removal of acetyl-functional groups from lysine

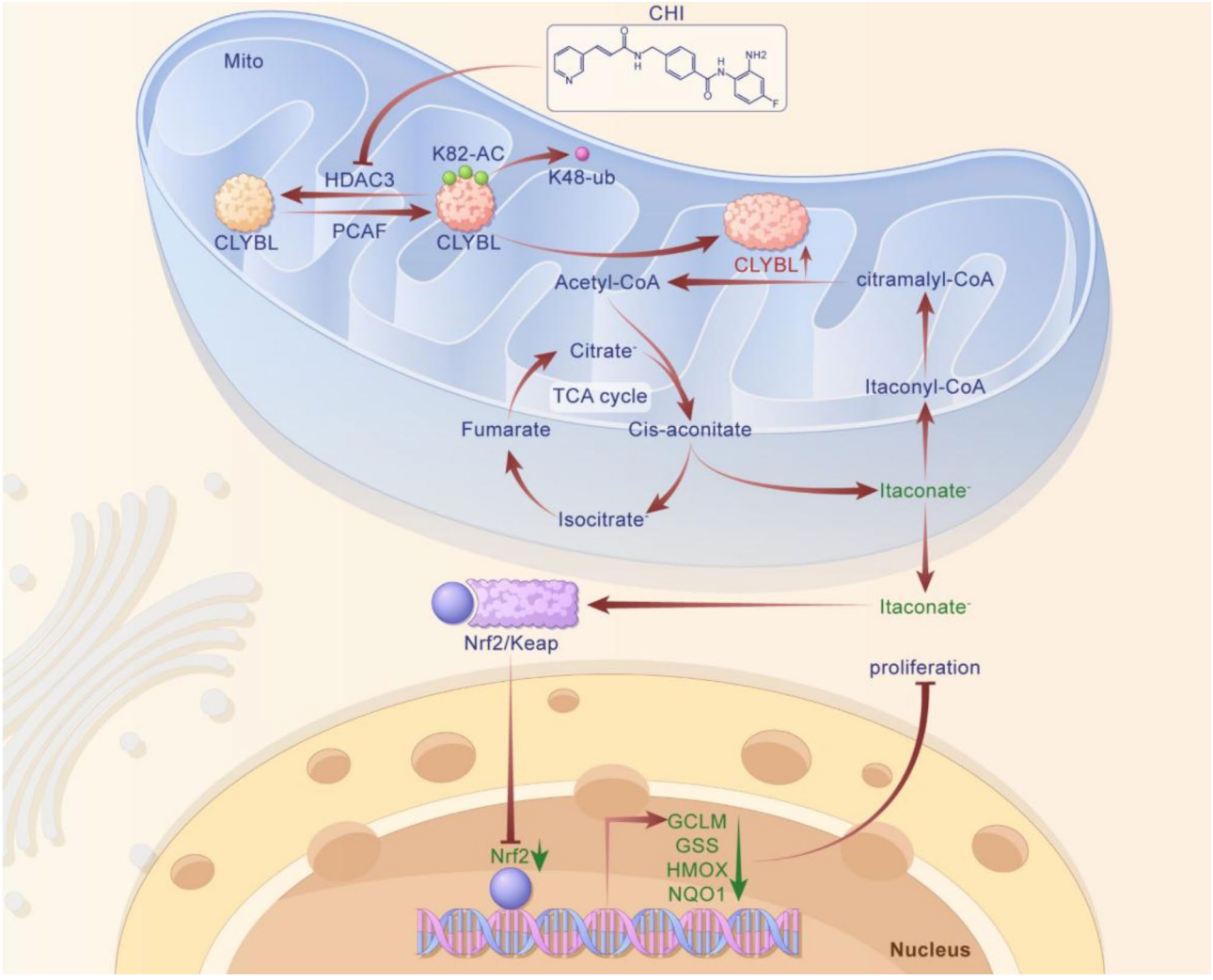

Fig. 7 Schematic depicting CLYBL and its acetylation regulation in BC. CLYBL is modified by acetylation at K82. PCAF contributes to its acetylation and HDAC3 promotes its deacetylation. Acetylation of CLYBL facilitates its own stability by reducing the affinity of CLYBL for K48-linked ubiquitin, which could inhibit breast cancer and reduce NRF2 pathway-mediated antioxidants

residues of histone and non-histone proteins. Among them, HDAC3 is reportedly localized to the mitochondria to deacetylate non-histones [22]. Chi et al. found that HDAC3 could translocate to the mitochondria to deacetylate and inactivate HADHA, a fatty acid oxidation-related enzyme [22]. In our research, HDAC3 could catalyze the removal of the acetyl group from CLYBL. Additionally, both PCDF and HDAC3 can directly interact with CLYBL. These results suggested that PCAF is the predominant CLYBL acetyltransferase and HDAC3 is deacetyltransferase.

In summary, we have identified the TCA metabolic enzyme CLYBL as a tumor suppressor in breast cancer that can be used to predict poor prognosis in clinical patients. In the future exploration, the development of CLYBL acetylated modified antibody may further help the clinical diagnosis of breast cancer pathogenesis and prognosis. Mechanistically, CLYBL can be modified by acetylation at its K82 site, with PCAF catalyzing CLYBL acetylation and HDAC3 catalyzing CLYBL deacetylation. Furthermore, acetylation of CLYBL increases its own protein stability to further enhance the tumor suppression effect, which may be associated with reduced Nrf2-dependent antioxidant levels (Fig. 7). Our results can help provide new molecular targets and theoretical bases for clinically treating breast cancer.

Abbreviations

CLYBL	Citrate lyase beta like
BC	Breast cancer
SDH	Succinate dehydrogenase
NRF2	Nuclear factor E2-related factor 2
KEAP1	Kelch-like ECH-associated protein 1
PTMs	Post-translational modifications
CBP	CREB-binding protein
GCN5	General Control Non-repressed 5 protein

PCAF	P300/CBP-associating factor
TIP60 (KAT5)	Lysine acetyltransferases 5
HDACs	Histone deacetylases
OS	Overall survival
RFS	Relapse-free survival

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-025-06200-3 .

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Author contributions

ZJD and CFD designed the research and supervised this study. XYD, CLM and XYC designed and performed most of the research; MY and QHC participated in the sequence alignment; RCL and XYL performed data curation; XYD wrote the manuscript; LCB, YNW and BZ performed the animal experiments; ZYS and LJW reviewed and edited the draft. All authors have read and approved the final manuscript.

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Data availability

The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The experiments were performed according to the approved guidelines established by the institutional review board at Zhejiang University, Hangzhou, China. Animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee at the Zhejiang University, Hangzhou, China.

Consent for publication

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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References

1. Garrido-Castro AC, Lin NU, Polyak K. Insights into Molecular classifications of Triple-negative breast Cancer: improving patient selection for treatment. Cancer Discov. 2019;9(2):176–98.
2. Tari DU. Breast Cancer: a Multi-disciplinary Approach from Imaging to Therapy. Curr Oncol. 2024;31(1):598–602.
3. Eniafe J, Jiang S. The functional roles of TCA cycle metabolites in cancer. Oncogene. 2021;40(19):3351–63.
4. Laura S, Yang L, Nakatsuka NJ, Calvo SE, Zenon G, Mootha VK. CLYBL is a polymorphic human enzyme with malate synthase and β-methylmalate synthase activity. Hum Mol Genet. 2014;23(9):2313–23.
5. Itaconate is an. Anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1. Nature. 2018;556(7699):113–7.
6. Li R, Zhang P, Wang Y, Tao K. Itaconate: a metabolite regulates inflammation response and oxidative stress. Oxid Med Cell Longev. 2020;2020:5404780.
7. Boese AC, Kang S. Mitochondrial metabolism-mediated redox regulation in cancer progression. Redox Biol. 2021;42(4):101870.
8. Regulation of Cellular. Metabolism by Protein Lysine Acetylation. Science. 2010;327(5968):1000–4.
9. Chunaram C, Chanchal, Kumar, Florian G, et al. Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science. 2009;325(5942):834–40.
10. Zhao Q, Zhang Z, Li J, Xu F, Zhang J. Lysine Acetylome Study of Human Hepatocellular Carcinoma Tissues for Biomarkers and therapeutic targets Discovery. Front Genet. 2020;11:572663.
11. Gil J, Ramírez-Torres A, Encarnación-Guevara S. Lysine acetylation and cancer: a proteomics perspective. J Proteom. 2016;150:297–309.
12. Caron C, Boyault C, Khochbin S. Regulatory cross-talk between lysine acetylation and ubiquitination: role in the control of protein stability. BioEssays: News Reviews Mol Cell Dev Biology. 2005;27(4):408–15.
13. Li H, Wittwer T, Weber A, et al. Regulation of NF-κB activity by competition between RelA acetylation and ubiquitination. Oncogene. 2012;31(5):611–23.
14. Wang G, Li S, Gilbert J, Gritton HJ, Wang Z, Li Z, et al. Crucial roles for SIRT2 and AMPA receptor acetylation in synaptic plasticity and memory. Cell Rep. 2017;20(6):1335–47.
15. Wang M, Lin H. Understanding the function of mammalian sirtuins and Protein Lysine Acylation. Annu Rev Biochem. 2021;90:245–85.
16. Figlia G, Willnow P, Teleman AA. Metabolites regulate Cell Signaling and Growth via Covalent Modification of proteins - ScienceDirect. Dev Cell. 2020;54(2):156–70.
17. Liu C, Yang Q, Zhu Q, Lu X, Li M, Hou T, et al. CBP mediated DOT1L acetylation confers DOT1L stability and promotes cancer metastasis. Theranostics. 2020;10(4):1758–76.
18. He R, Liu B, Xiong R, Geng B, Meng H, et al. Itaconate inhibits ferroptosis of macrophage via Nrf2 pathways against sepsis-induced acute lung injury. Cell Death Discovery. 2022;8(1):43.
19. Cruz-Gregorio A, Aranda-Rivera AK, Sciutto E, Fragoso G, Pedraza-Chaverri J. Redox state associated with antitumor and immunomodulatory peptides in cancer. Arch Biochem Biophys. 2022;730:109414.
20. Zhu G, Jin L, Sun W, Wang S, Liu N. Proteomics of post-translational modifications in colorectal cancer: Discovery of new biomarkers. Biochim Biophys Acta Rev Cancer. 2022;1877(4):188735.
21. Hsu CY, Fu SH, Chien MW, Liu YW, Chen SJ, Sytwu HK. Post-translational modifications of transcription factors harnessing the etiology and pathophysiology in Colonic diseases. Int J Mol Sci. 2020;21(9):3207.
22. Chi Z, Chen S, Xu T, Zhen W, Yu W, Jiang D, et al. Histone Deacetylase 3 couples Mitochondria to drive IL-1β-Dependent inflammation by configuring fatty acid oxidation. Mol Cell. 2020;80(1):43–58.
23. Shimizu K, Gi M, Suzuki S, North BJ, Watahiki A, Fukumoto S, et al. Interplay between protein acetylation and ubiquitination controls MCL1 protein stability. Cell Rep. 2021;37(6):109988.
24. Pagano E, Romano B, Cicia D, Iannotti FA, Venneri T, Lucariello G, et al. TRPM8 indicates poor prognosis in colorectal cancer patients and its pharmacological targeting reduces tumour growth in mice by inhibiting Wnt/beta-catenin signalling. Br J Pharmacol. 2023;180(2):235–51.
25. Romano B, Pagano E, Iannotti FA, Piscitelli F, Brancaleone V, Lucariello G, et al. N-Acylethanolamine acid amidase (NAAA) is dysregulated in colorectal cancer patients and its inhibition reduces experimental cancer growth. Br J Pharmacol. 2022;179(8):1679–94.
26. Ahmed S, Khan H, Aschner M, Mirzaei H, Capasso R. Anticancer potential of Furanocoumarins: mechanistic and therapeutic aspects. Int J Mol Sci. 2020;21(16):5622.

27. Ahin TZ, Ylmaz B, Capasso NYCSAA. Recent insights into the nutritional immunomodulation of cancer-related microRNAs. Phytother Res. 2023;37(10):4375–97.
28. Shen H, Campanello GC, Flicker D, Grabarek Z, Hu J, Luo C, et al. The human knockout gene CLYBL connects itaconate to vitamin B-12. Cell. 2017(4):171.
29. Liu Y, Song L, Zheng N, Shi J, Wu H, Yang X, et al. A urinary proteomic landscape of COVID-19 progression identifies signaling pathways and therapeutic options. Sci China Life Sci. 2022;65(9):1866–80.
30. Hayes JD, Dinkova-Kostova AT, Tew KD. Oxidative stress in Cancer. Cancer Cell. 2020;38(2):167–97.
31. Dang F, Wei W. Targeting the acetylation signaling pathway in cancer therapy. Sem Cancer Biol. 2021;85:209–18.
32. Narita T, Weinert BT, Choudhary C. Functions and mechanisms of non-histone protein acetylation. Nat Reviews: Mol cell Biology. 2019;20(3):156–74.
33. Verdin E, Ott M. 50 years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. Nat Rev Mol Cell Biol. 2015;16(4):258–64.
34. Xia Z, Kon N, Gu AP, Tavana O, Gu W. Deciphering the acetylation code of p53 in transcription regulation and tumor suppression. Oncogene. 2022;41(22):3039–50.
35. Wang B, Ye Y, Yang X, Liu B, Shen Z. SIRT 2-dependent IDH1 deacetylation inhibits colorectal cancer and liver metastases. EMBO Rep. 2020;21(4):e48183.
36. Perez-Oquendo M, Manshouri R, Tian Y, Fradette JJ, Rodriguez BL, Kundu S, et al. ZEB1 is regulated by K811 Acetylation to Promote Stability, NuRD Complex Interactions, EMT, and NSCLC Metastasis. Mol Cancer Res. 2023;21(8):779–94.
37. Dai X, Xin Y, Xu W, Tian X, Wei X, Zhang H. CBP-mediated slug acetylation stabilizes slug and promotes EMT and migration of breast cancer cells. Sci China Life Sci. 2021;64(4):563–74.
38. Yang S, Xu W, Liu C, Jin J, Li X, Jiang Y. LATS1 K751 acetylation blocks activation of Hippo signalling and switches LATS1 from a tumor suppressor to an oncoprotein. Sci China Life Sci. 2022;65(1):129–41.
39. Shvedunova M, Akhtar A. Modulation of cellular processes by histone and non-histone protein acetylation. Nat Rev Mol Cell Biol. 2022;23(5):329–49.

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