

# Large-Scale Identification of Lysine Crotonylation Reveals Its Potential Role in Oral Squamous Cell Carcinoma

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**Purpose:** Lysine crotonylation, an emerging posttranslational modification, has been implicated in the regulation of diverse biological processes. However, its involvement in oral squamous cell carcinoma (OSCC) remains elusive. This study aims to reveal the global crotonylome in OSCC under hypoxic conditions and explore the potential regulatory mechanism of crotonylation in OSCC.

**Methods:** Liquid-chromatography fractionation, affinity enrichment of crotonylated peptides, and high-resolution mass spectrometry were employed to detect differential crotonylation in CAL27 cells cultured under hypoxia. The obtained data were further subjected to bioinformatics analysis to uncover the involved biological processes and pathways of the dysregulated crotonylated proteins. A site-mutated plasmid was utilized to investigate the effect of crotonylation on Heat Shock Protein 90 Alpha Family Class B Member 1 (HSP90AB1) function.

**Results:** A large-scale crotonylome analysis revealed 1563 crotonylated modification sites on 605 proteins in CAL27 cells under hypoxia. Bioinformatics analysis revealed a significant decrease in histone crotonylation levels, while up-regulated crotonylated proteins were mainly concentrated in non-histone proteins. Notably, glycolysis-related proteins exhibited prominent up-regulation among the identified crotonylated proteins, with HSP90AB1 displaying the most significant changes. Subsequent experimental findings confirmed that mutating lysine 265 of HSP90AB1 into a silent arginine impaired its function in promoting glycolysis.

**Conclusion:** Our study provides insights into the crotonylation modification of proteins in OSCC under hypoxic conditions and elucidates the associated biological processes and pathways. Crotonylation of HSP90AB1 in hypoxic conditions may enhance the glycolysis regulation ability in OSCC, offering novel perspectives on the regulatory mechanism of crotonylation in hypoxic OSCC and potential therapeutic targets for OSCC treatment.

**Keywords:** crotonylation, hypoxia, oral squamous cell carcinoma, glycolysis, heat shock protein 90 alpha family class B member 1

## Introduction

Protein post-translational modifications (PTMs) represent a crucial set of epigenetic regulatory mechanisms involved in diverse biological functions. PTMs, such as phosphorylation, acetylation, ubiquitination, methylation, crotonylation, and others, play vital roles in processes like DNA replication, transcription, cell differentiation, and organismal development.<sup>1–3</sup> With the advancement of high-resolution liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), numerous novel amino acid modifications have been identified, expanding our understanding of how these modifications can impact protein activity, stability, localization, and interactions.<sup>4–6</sup> Dysregulation of PTMs has been associated with the occurrence and development of various diseases, including neoplastic disease,

neuropsychiatric disease, tissue injury, and so on.<sup>7–9</sup> Unraveling the intricate involvement of PTMs in biological processes and diseases holds promise for identifying potential therapeutic targets.

Lysine crotonylation (Kcr) is a newly discovered posttranslational modification, first reported in 2011. This demonstrates specific enrichment of active gene promoters or potential enhancers within mammalian cell genomes.<sup>10</sup> Structurally, the crotonyl group comprises a four-carbon, planar chemical moiety containing a carbon–carbon (C–C)  $\pi$ -bond, resembling acetylation. Crotonylation modifies histones by reducing their positive charge and loosening their binding to negatively charged DNA, thereby facilitating the binding of transcription factors.<sup>11</sup> In addition to histones, non-histone proteins have also been found to undergo crotonylation, which has demonstrated involvement in nearly all major biological processes.<sup>12–14</sup> Evidence suggests that Kcr plays a role in various physiological and pathological processes, including spermatogenesis, neuropsychiatric disease, inflammation, and carcinogenesis.<sup>15,16</sup> However, the precise regulatory mechanisms of Kcr in cancer remain largely unknown, necessitating further investigation to comprehend its contribution to cancer development and progression.

Oral squamous cell carcinoma (OSCC) is a prevalent malignancy in the head and neck region, characterized by its heterogeneity and aggressive phenotypes.<sup>17</sup> Despite the identification of several hotspot biomarkers such as DUXAP9, FSCN1, and YAP1, our comprehension of OSCC pathogenesis remains limited.<sup>18–20</sup> Gaining a deeper understanding of the intricate processes involved in OSCC holds promise for advancing treatment strategies and enhancing patient prognosis. Tumor hypoxia is recognized as a significant characteristic of the neoplastic microenvironment, exerting profound effects on tumor behavior by influencing metabolic pathways, stimulating angiogenesis, and conferring resistance to chemotherapy.<sup>21</sup> Our previous investigations have substantiated the presence of a hypoxic environment in OSCC and its contribution to malignant progression and enhanced chemoresistance.<sup>22–24</sup> Nevertheless, the association between crotonylation and hypoxia in OSCC remains largely unexplored.

Heat shock protein 90 (HSP90) is an ATP-dependent molecular chaperone that belongs to a highly conserved protein family. The model consists of four subtypes: heat shock protein 90 alpha family class A member 1 (HSP90AA1), heat shock protein 90 alpha family class B member 1 (HSP90AB1), glucose-regulated protein 94 (GRP94), and TNF receptor-associated protein 1 (TRAP1). Recent investigations have emphasized the upregulation of HSP90AB1 in diverse cancer types, demonstrating a significant correlation with prognostic outcomes.<sup>25–28</sup> In our previous study, we observed elevated expression of HSP90AB1 in head and neck squamous cell carcinoma (HNSCC) correlated with T grade, lymph node metastasis, and prognosis. Experimental knockdown of HSP90AB1 resulted in suppressed proliferation, migration, and glycolytic activity in HNSCC cells.<sup>29</sup> Nonetheless, the precise underlying mechanism by which HSP90AB1 regulates glycolysis remains incompletely elucidated.

In the present study, we employed liquid-chromatography fractionation to comprehensively map the crotonylation modification of proteins in OSCC under hypoxic conditions. Furthermore, we conducted a bioinformatics analysis to investigate the biological processes and pathways associated with dysregulated crotonylated proteins. Our research endeavors to shed light on the regulatory mechanisms of crotonylation in OSCC under hypoxia, with the ultimate goal of identifying potential therapeutic targets for the treatment of OSCC.

## Materials and Methods

### Cell Culture

The human OSCC cell line CAL27 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and possessed official cell authentication reports ([Supplementary File 1](#)). Hence, our study is exempt from ethical approval. CAL27 was cultured in DMEM (KeyGEN Biotech, Nanjing, China) supplemented with 10% FBS (ExCell Bio, Suzhou, China), 100  $\mu$ g/mL streptomycin, and 100 U/mL penicillin. The cells were cultured in a humidified incubator under either normoxic conditions (5% CO<sub>2</sub>/20% O<sub>2</sub>) or hypoxic conditions (5% CO<sub>2</sub>/1% O<sub>2</sub>/94% N<sub>2</sub>) at 37°C (Thermo Scientific™ Forma Series II Water Jacket CO<sub>2</sub> Incubator). Confluent cells were trypsinized with 0.05% trypsin containing 0.02% EDTA (KeyGEN Biotech, Nanjing, China).

## Protein Extraction and Trypsin Digestion

To extract proteins, each sample was sonicated three times on ice using a high-intensity ultrasonic processor (Scientz-IID) in a lysis buffer (8 M urea, 1% protease inhibitor cocktail, Selleck, B14001). For PTM experiments, inhibitors such as 3  $\mu$ M TSA (Selleck, S1045) and 50 mM NAM (Selleck, S1899) were also added to the lysis buffer. The remaining debris was removed by centrifugation at 12,000 g at 4°C for 10 min (Thermo Scientific™ Pico 17). Finally, we collected the supernatant and determined the protein concentration with a BCA kit according to the manufacturer's instructions (KeyGEN Biotech, Nanjing, China).

## TMT/iTRAQ Labeling

After trypsin digestion, peptides were desalted using a Strata X C18 SPE column (Phenomenex) and then vacuum-dried. The peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for the TMT/iTRAQ kit (Thermo Scientific™). Briefly, we thawed and reconstituted one unit of TMT/iTRAQ reagent in acetonitrile, and the peptide mixtures were incubated with the reagent for 2 hr at room temperature. The samples were then pooled, desalted, and dried by vacuum centrifugation.

## Antipan-Lysine Crotonylation Antibody Enrichment

To enrich modified peptides, tryptic peptides were dissolved in a NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0) and incubated with pre-washed antibody beads (PTM Bio, Lot number 001) at 4°C overnight with gentle shaking. Afterward, we washed the beads four times with NETN buffer and twice with H<sub>2</sub>O. The bound peptides were eluted from the beads with 0.1% trifluoroacetic acid. Finally, we combine the eluted fractions and vacuum-dry them. For LC-MS/MS analysis, we desalted the resulting peptides with C18 ZipTips (Millipore) following the manufacturer's instructions. In the case of bio-material-based PTM enrichment for phosphorylation, we first incubated peptide mixtures with IMAC microsphere suspension in loading buffer (50% acetonitrile/6% trifluoroacetic acid) with vibration. The enriched phosphopeptides were collected by centrifugation, and the supernatant was removed. To remove nonspecifically adsorbed peptides, we washed the IMAC microspheres sequentially with 50% acetonitrile/6% trifluoroacetic acid and 30% acetonitrile/0.1% trifluoroacetic acid. We then added an elution buffer containing 10% NH<sub>4</sub>OH to elute the enriched phosphopeptides from the IMAC microspheres, which were subsequently collected with vibration. The supernatant containing phosphopeptides was lyophilized and prepared for LC-MS/MS analysis.

## LC-MS/MS Analysis

We dissolved the tryptic peptides in 0.1% formic acid (solvent A) and directly loaded them onto a home-made reversed-phase analytical column (15-cm length, 75  $\mu$ m i.d.). The gradient consisted of an increase from 6% to 23% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, followed by an increase to 35% in 8 min and climbing to 80% in 3 min, then holding at 80% for the last 3 min, all at a constant flow rate of 400 nL/minute on an EASY-nLC 1000 UPLC system. The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive Plus (Thermo) coupled online with the UPLC. The electrospray voltage applied was 2.0 kV. For full scan, the m/z scan range was 350 to 1800, and intact peptides were detected in Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28, and the fragments were detected in the Orbitrap at a resolution of 17,500. We used a data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0 s dynamic exclusion. Automatic gain control (AGC) was set at 5E4, and the fixed first mass was set as 100 m/z.

## Database Search

The obtained MS/MS data were processed using Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against the human UniProt database (<https://www.uniprot.org/>) concatenated with a reverse decoy database. Trypsin/P was specified as the cleavage enzyme allowing up to 4 missing cleavages. The mass tolerance for precursor ions was set at 20 ppm in the first search and 5 ppm in the main search, while the mass tolerance for fragment ions was set at 0.02 Da.

Carbamidomethyl on Cys was specified as fixed modification and acetylation modification and oxidation on Met were specified as variable modifications. FDR was adjusted to <1% and minimum score for modified peptides was set >40.

## Bioinformatics Analysis

Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database (<http://www.ebi.ac.uk/GOA/>). Firstly, we converted identified protein IDs to UniProt IDs and mapped them to GO IDs using protein IDs. If some identified proteins were not annotated by the UniProt-GOA database, we used the InterProScan software to annotate the protein's GO function based on the protein sequence alignment method. We then classified proteins by Gene Ontology annotation based on three categories: biological process, cellular component, and molecular function. For each category, we employed a two-tailed Fisher's exact test to test the enrichment of differentially modified proteins against all identified proteins. The GO with a corrected p-value < 0.05 was considered significant. To predict subcellular localization, we used "WoLF PSORT", a subcellular localization prediction software that is an updated version of PSORT/PSORT II for the prediction of eukaryotic sequences (<https://wolfsort.hgc.jp/>). For prokaryotic species, we used the subcellular localization prediction software CELLO (<http://cello.life.nctu.edu.tw/>).

Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to annotate related protein pathways. Firstly, we used the KEGG online service tool KAAS to annotate the KEGG database description (<https://www.genome.jp/kegg/kaas/>). We then depicted the mapping of the annotation result on the KEGG pathway database using the KEGG online service tool KEGG mapper (<https://www.genome.jp/kegg/mapper/>). To test the enrichment of differentially modified proteins against all identified proteins, we employed a two-tailed Fisher's exact test. The pathway with a corrected p-value < 0.05 was considered significant. For each category of proteins, we retrieved data from the InterPro database (<https://www.ebi.ac.uk/interpro/>), which provides functional analysis of protein sequences by classifying them into families and predicting the presence of domains and important sites. A two-tailed Fisher's exact test was used to test the enrichment of differentially modified proteins against all identified proteins. Protein domains with a corrected p-value < 0.05 were considered significant.

The Soft MoMo (motif-x algorithm) was employed to analyze a model of sequences constituted with amino acids at specific positions of modify-21-mers (10 amino acids upstream and downstream of the site, but phosphorylation with modify-13-mers that 6 amino acids upstream and downstream of the site) in all protein sequences. We set all the database protein sequences as background database parameter and set the minimum number of occurrences to 20. We ticked "emulate original motif-x" and set other parameters to default. All differentially expressed modified protein database accessions or sequences were searched against the Search Tool for the Retrieval of Interacting Genes (STRING) database (<http://www.string-db.org/>) for protein-protein interactions. We only selected interactions between the proteins belonging to the searched data sets, thereby excluding external candidates. STRING defines a metric called "confidence score" to define interaction confidence. We fetched all interactions that had a confidence score  $\geq 0.7$  (high confidence). The interaction network from STRING was visualized using Cytoscape software (version 3.6.0).

## Lentiviruses and Transfection

Knockdown of HSP90AB1 in CAL27 cells was conducted using a lentiviral vector (Genechem, Shanghai, China) carrying a short-hairpin RNA (shRNA) specific for HSP90AB1, according to the manufacturer's instructions. The sequence of the HSP90AB1 shRNA and its transfection operations can be found in our previous reports.<sup>29</sup>

## Plasmids and Transfection

The human HSP90AB1 expression plasmid GV657 vector with a Flag tag-HSP90AB1 (WT) and the corresponding mutant plasmid M265 (K265R, K and R denote lysine and arginine, respectively) were constructed by KeyGEN Biotech (Nanjing, China). For transfection assays, the plasmids were transfected into cells using Lipofectamine 8000 reagents (Beyotime, Shanghai, China) following the manufacturer's instructions.

## Coimmunoprecipitation and Immunoblotting

After appropriate treatments, cells were lysed using 20 mM PIPES (pH 7.0), 100 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.1% Nonidet P-40, 20 mM Na<sub>2</sub>MoO<sub>4</sub>, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 30 mM NaF, and protease inhibitors. The lysates were then immunoprecipitated for 1 hr at 4°C with anti-HSP90AB1 antibody (2 µg; Abcam, ab203085, Cambridge, UK) and incubated with sepharose G beads overnight. The beads were washed with PBS and eluted with SDS-PAGE sample buffer.

The subsequent immunoblotting was carried out essentially as we reported before.<sup>23</sup> Briefly, protein samples were separated by SDS-PAGE gel electrophoresis using a certain percentage of gel depending on the molecular weight of the protein of interest, and then transferred onto a PVDF membrane. The membrane was blocked with 5% non-fat milk in TBS-T buffer and incubated with primary antibodies overnight at 4°C. Then, the membrane was washed with a TBS-T buffer and incubated with secondary antibodies conjugated with horseradish peroxidase for 1.5 hr at room temperature. Finally, the protein bands were visualized using an enhanced chemiluminescence detection system (Tanon 4600, Tanon, Shanghai).

The antibodies used for immunoblotting were as follows: anti-pan-crotonyllysine antibody (1:1000; PTM Biolab, Hangzhou, China), anti-HSP90AB1 antibody (1:5000; Abcam, ab203085, Cambridge, UK), anti-HIF-1α (1:2000; Proteintech, Wuhan, China), anti-β-actin (1:5000; Proteintech, Wuhan, China), and goat anti-rabbit IgG (H+L) HRP (1:5000, SA00001-2, Proteintech, Wuhan, China).

## Detection of Glycolysis Products

The detection of pyruvate, ATP and lactic acid were carried out as we reported before.<sup>29</sup> Pyruvate assay kit (A081-1-1), ATP assay kit (A095-2-1) and Lactic Acid assay kit (A019-2-1) were used (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

## Statistical Analyses

All data are expressed as mean ± SD. Statistical differences were measured using an unpaired two-sided Student's *t*-test or one-way ANOVA for multiple comparisons when appropriate. All statistical analyses were performed using SPSS 22.0 statistical software package (SPSS Inc, Chicago, IL, USA). Statistical significance was set at an alpha value of *P*<0.05. All the graphs were drawn using GraphPad Prism 9 (GraphPad Software Inc).

## Results

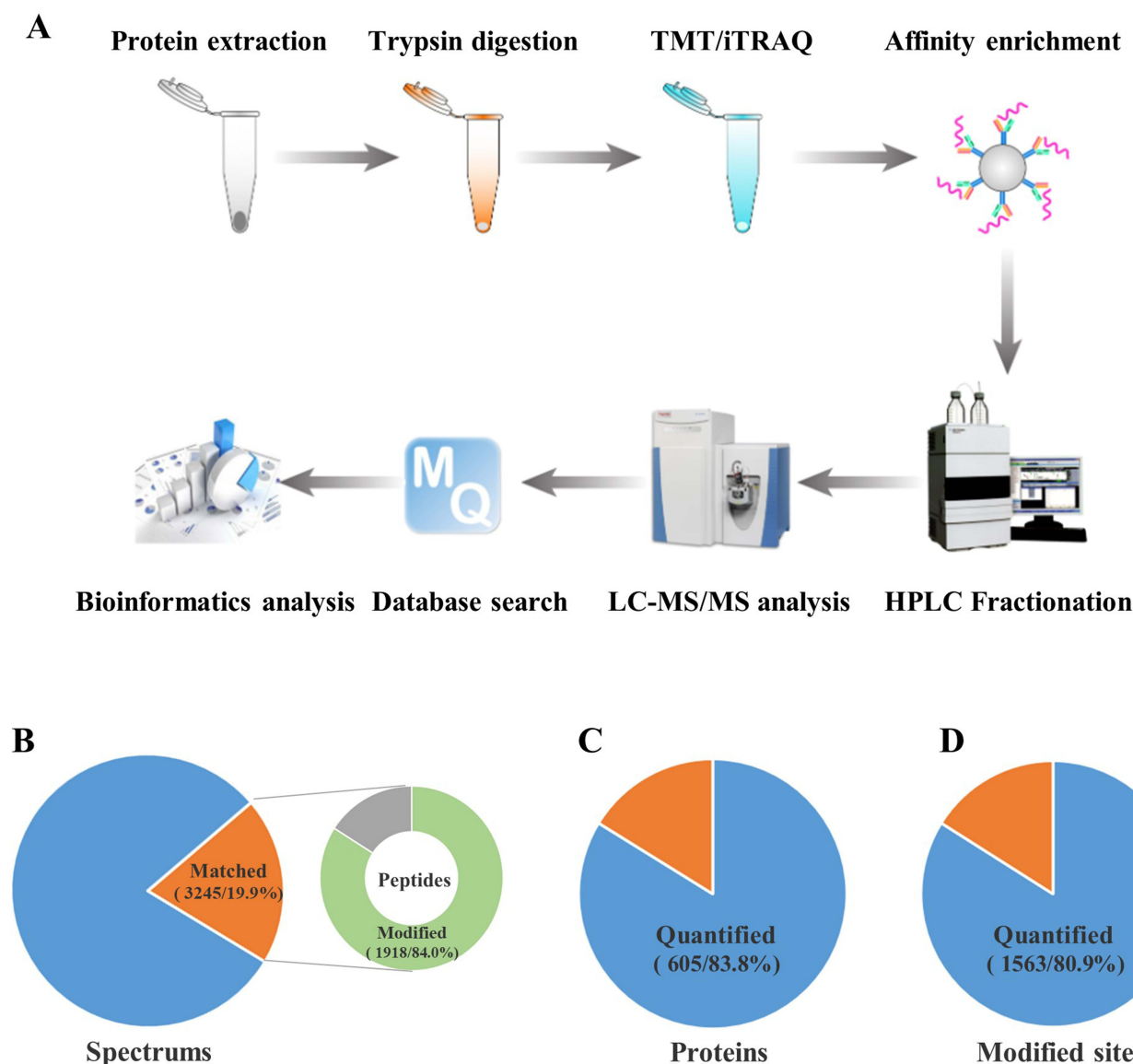
### Large-Scale Analysis of Lysine Crotonylated Proteins in Hypoxia Condition

To investigate the impact of hypoxic microenvironments on protein crotonylation in OSCC, we implemented a multi-step approach combining liquid-chromatography fractionation, affinity enrichment of crotonylated peptides, and high-resolution mass spectrometry. The detailed process is depicted in Figure 1A. Initially, proteins were extracted and digested using trypsin to generate peptides. Subsequently, crotonylated peptides were enriched using either a pan anti-lysine crotonylation antibody or basic reversed-phase high-performance liquid chromatography (HPLC) followed by antibody affinity enrichment from each fraction. The eluted peptides were then subjected to LC-MS/MS analysis employing high-resolution mass spectrometry. In total, we obtained 16,330 secondary spectrograms from the mass spectrometry analysis. After comparison with the protein database, we identified 3245 available and effective spectrograms, resulting in a spectral graph utilization rate of 19.9%. Through these analyses, we successfully identified 2282 peptides, including 1918 crotonylated modified peptides (Figure 1B). Ultimately, we discovered 1933 crotonylated modification sites on 722 proteins, among which 1563 sites on 605 proteins had quantitative information (Figure 1C and D).

### Analysis of Crotonylation Site Motif

To gain insights into the characteristics of lysine crotonylated peptides, we conducted an analysis of the motifs present in all identified crotonylated peptides. Our analysis revealed the presence of 16 conserved motifs, including AxKK, AxxxKK, KK, and others (Figure 2A). Notably, the AxKK motif exhibited the highest score and largest fold change among the identified motifs (Table 1). Additionally, we investigated the enrichment of specific amino acid residues





**Figure 1** Large-scale identification of crotonylation sites in oral squamous cell carcinoma (OSCC) following hypoxic treatment.

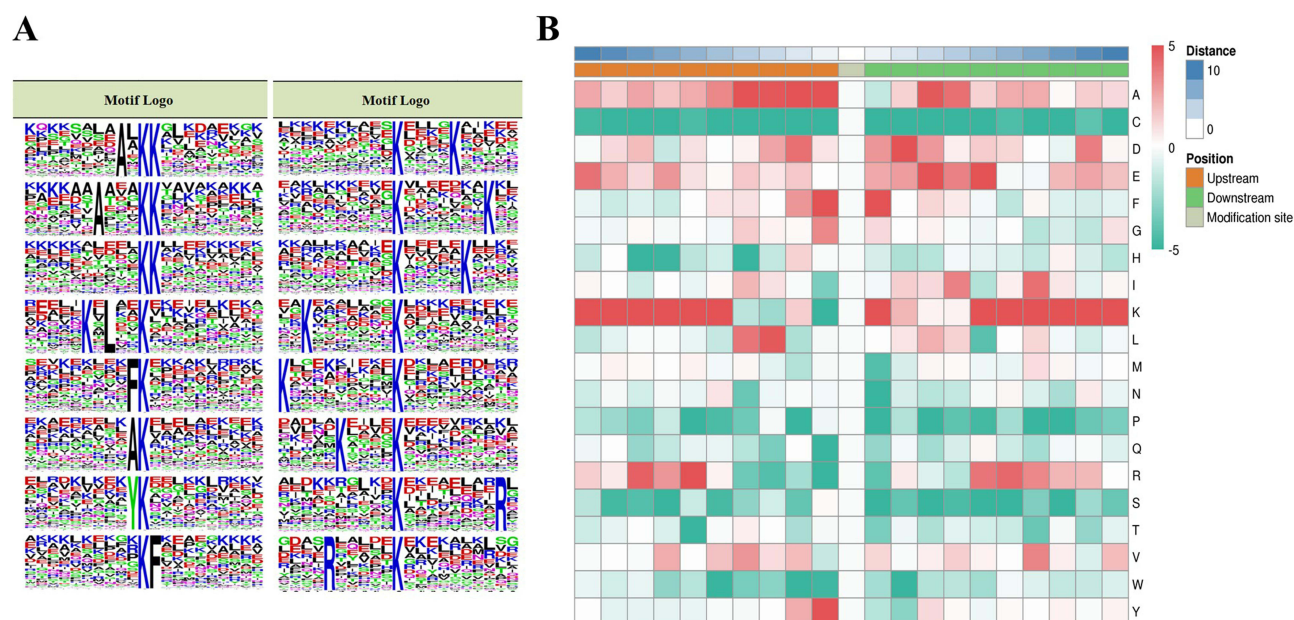
**Notes:** (A) Schematic illustration detailing the workflow for the analysis of crotonylated proteins in CAL27 cells after 24 hr of normoxia and hypoxia culture. The process involved TMT-labeled LC-MS/MS analysis. (B) Proportional diagram displaying the number of available effective spectrograms and identified crotonylated modified peptides obtained through mass spectrometry analysis. (C) Proportional diagram illustrating the quantified crotonylated proteins. (D) Proportional diagram depicting the quantified crotonylated modification sites.

**Abbreviations:** OSCC, oral squamous cell carcinoma; TMT, Tandem Mass Tags; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography with tandem mass spectrometry.

surrounding crotonylated modification sites within each motif. Our analysis demonstrated that residues A, E, K, and D were more likely to be found in proximity to the crotonylated site, while residues C, S, P, and W exhibited significant decreases in their occurrence near the modified site (Figure 2B).

## The Up-Regulated Crotonylated Proteins Were Enriched in Non-Histones and the Down-Regulated Were Enriched in Histones

Among the 1563 crotonylated modification sites on 605 proteins, we set a threshold fold change of more than 1.5 to identify significantly altered sites (Figure 3A). As depicted in Figure 3B, there were 62 up-regulated sites on 41 proteins and 49 down-regulated sites on 33 proteins. Notably, the level of crotonylated modification on histones exhibited



**Figure 2** Analysis of crotonylation site motifs in oral squamous cell carcinoma (OSCC).

**Notes:** (A) Sequence logos representing significantly enriched crotonylation site motifs surrounding the differential crotonylation sites in OSCC. Each logo visually depicts the consensus sequence and the degree of conservation at each position. (B) Heatmap illustrating the amino acid preference on both sides of the crotonylated lysine sites, displaying the frequency of different amino acids adjacent to the modified residue in OSCC. Red marks indicate enrichment, while green marks indicate depletion.

**Abbreviation:** OSCC, oral squamous cell carcinoma.

a consistent decrease ([Supplementary Table 1](#)). Subsequently, subcellular localization analysis revealed that the majority of proteins exhibiting altered crotonylation levels were localized in the cytoplasm (44.59%), with this proportion reaching 63.41% among the up-regulated proteins ([Figure 3C and D](#)). Conversely, most of the down-regulated proteins were located in the nucleus (33.36%), which may be attributed to the involvement of histones ([Figure 3E](#)).

## Functional Enrichment of Crotonylated Proteins by Bioinformatics

To explore the potential functions of crotonylated proteins, we conducted a Gene Ontology (GO) analysis. For biological processes, up-regulated crotonylated proteins were predominantly associated with cellular processes, single-organism processes, and metabolic processes. On the other hand, the down-regulated crotonylated proteins showed enrichment in cellular processes, biological regulation, and single-organism processes. In terms of cellular components, both up- and down-regulated crotonylated proteins were mainly related to cells or organelles. Regarding molecular function, both up- and down-regulated crotonylated proteins were associated with binding and catalytic activity ([Figure 4A and B](#)).

Furthermore, KEGG pathway enrichment analysis unveiled that the up-regulated crotonylated proteins were primarily associated with metabolic pathways, carbon metabolism, and glycolysis/gluconeogenesis. Conversely, the down-regulated crotonylated proteins were more related to alcoholism and systemic lupus erythematosus ([Figure 4C and D](#), [Supplementary Figure 1](#)). Domain analysis of crotonylated proteins revealed that up-regulated proteins exhibited a higher prevalence of EF-hand domains, while down-regulated proteins were enriched in histone-related domains, including histone H2A/H2B/H3, histone-fold and linker histone H1/H5, and domain H15. This finding aligns with the observation that down-regulated proteins are primarily located in the nucleus ([Figure 4E and F](#)).

## Functional Relationships Between Crotonylated Proteins

To explore potential relationships between crotonylated proteins, we constructed a protein–protein interaction (PPI) network ([Figure 5A](#)). Within the PPI network, key rate-limiting enzymes in glycolysis, such as phosphoglycerate kinase 1 (PGK1), fructose-bisphosphate aldolase A (ALDOA), alpha-enolase (ENO1), and ATP-dependent 6-phosphofructokinase (PFK), occupied prominent positions among the up-regulated crotonylated proteins, even among all crotonylated proteins. Notably,

**Table 1** Characteristic Sequence and Enrichment Statistical Information of Crotonylation Sites in OSCC After Hypoxic Treatment

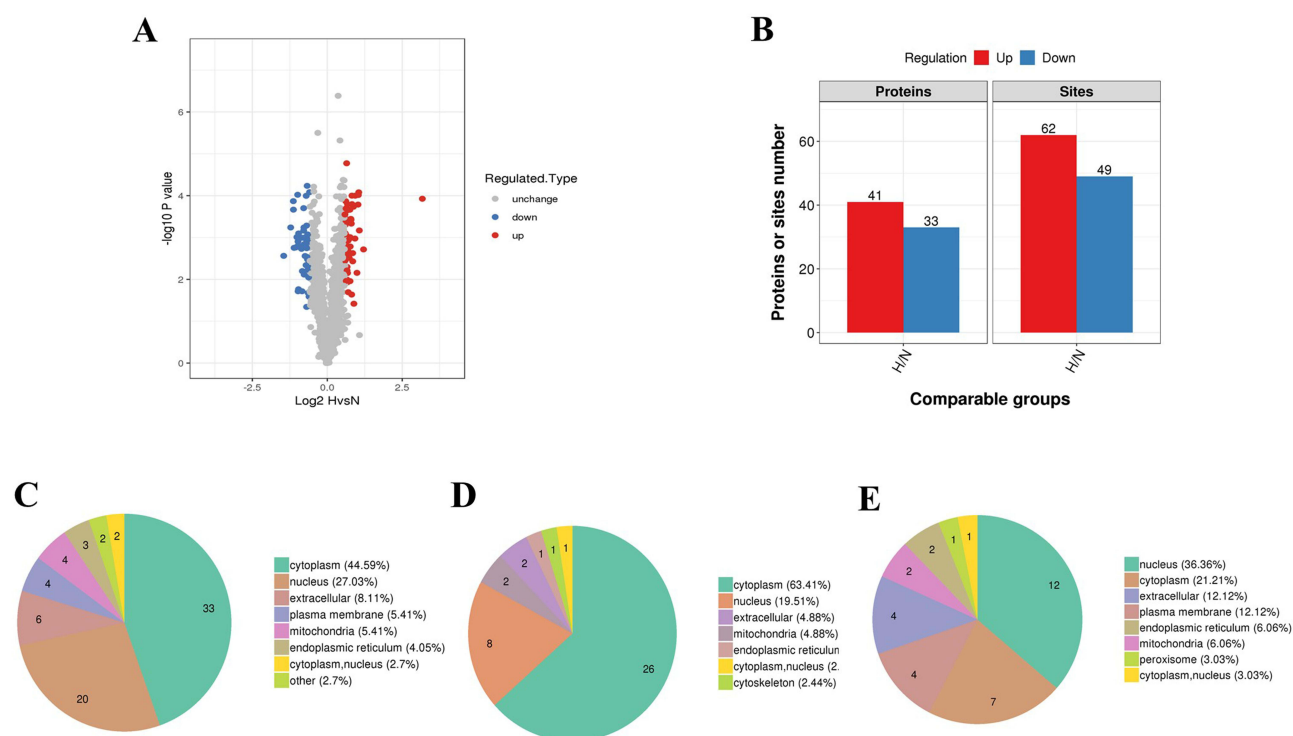
Motif	Motif Score	Foreground Matches	Foreground Size	Background Matches	Background Size	Fold Increase
xxxxxxxxAx_K_Kxxxxxxxx	24.72	58	1933	2952	604,147	6.1
xxxxxxAxxx_K_Kxxxxxxxx	22.33	45	1875	2633	601,195	5.5
xxxxxKxLxx_K_Kxxxxxxxx	22.46	49	1531	4147	556,159	4.3
xxxxxxxxY_K_Kxxxxxxxx	16.00	98	1191	15,098	502,852	2.7
xxxxxxxxF_K_Kxxxxxxxx	16.00	122	1482	17,216	552,012	2.6
xxxxxxxx_K_K_Fxxxxxxxx	13.85	88	1093	15,692	487,754	2.5
xxxxxxxx_K_K_Kxxxxxxxx	16.00	299	1830	42,403	598,562	2.3
xxxxxxxx_K_K_xxxxxxxRx	6.73	52	425	17,194	304,352	2.2
xxxxRxxxx_K_K_Kxxxxxxxx	6.67	49	373	17,067	287,158	2.2
xxxxxxxxA_K_K_Kxxxxxxxx	16.00	169	1360	31,944	534,796	2.1
xxxxxxxx_K_K_xxxKxxxx	12.98	147	1005	36,434	472,062	1.9
xxxxxxxx_K_K_xxxxxKxx	9.74	114	858	31,076	435,628	1.9
xxKxxxxxx_K_K_Kxxxxxxxx	8.02	86	646	26,208	375,408	1.9
Kxxxxxxxx_K_K_Kxxxxxxxx	7.23	74	560	23,887	349,200	1.9
xxxxxKxxxx_K_K_Kxxxxxxxx	6.19	61	486	20,961	325,313	1.9
xxxxxxxx_K_K_xxxxKxxxx	8.10	98	744	29,144	404,552	1.8

PGK1 exhibited multiple crotonylation sites, while ALDOA, ENO1, PFKL, and PFKP had only one site each ([Supplementary Table 1](#)). It is worth highlighting that HSP90AB1 occupied a central position within the PPI network along with the key glycolytic enzymes. Furthermore, HSP90AB1 displayed the most significant up-regulation, reaching up to a 9-fold change ([Table 1](#), [Figure 5B](#), [Supplementary File 2](#)).

## Crotonylation of HSP90AB1 Affects Its Glycolysis Regulation Ability

In our previous study, we have demonstrated the involvement of HSP90AB1 in regulating glycolysis in OSCC.<sup>29</sup> To investigate whether crotonylation impacts the regulatory ability of HSP90AB1 in glycolysis, we generated both mutant and wild-type plasmids for HSP90AB1. Specifically, lysine 265 of HSP90AB1 was mutated to arginine to prevent crotonylation ([Figure 6A](#)). To enhance the expression of exogenous HSP90AB1, we utilized lentivirus-mediated knock-down of endogenous HSP90AB1 in CAL27 cells ([Supplementary Figure 2](#), [Supplementary File 2](#)). Subsequently, we transfected either the wild-type or mutant plasmids into the HSP90AB1 knockdown cell lines for subsequent experiments. As a result, the crotonylation level of HSP90AB1 in CAL27 cells transfected with mutant plasmids was significantly reduced compared to that in the wild-type and control groups ([Figure 6B](#), [Supplementary File 2](#)). In the subsequent assessment of glycolytic products, we observed significant reductions in pyruvic acid, ATP, and lactic acid levels in the mutant group compared to the wild-type group ([Figure 6C-E](#)). These findings suggest that crotonylation of HSP90AB1 under hypoxic conditions may enhance its regulation of glycolysis.





**Figure 3** Analysis of differential crotonylation sites and corresponding proteins in OSCC under hypoxia conditions.

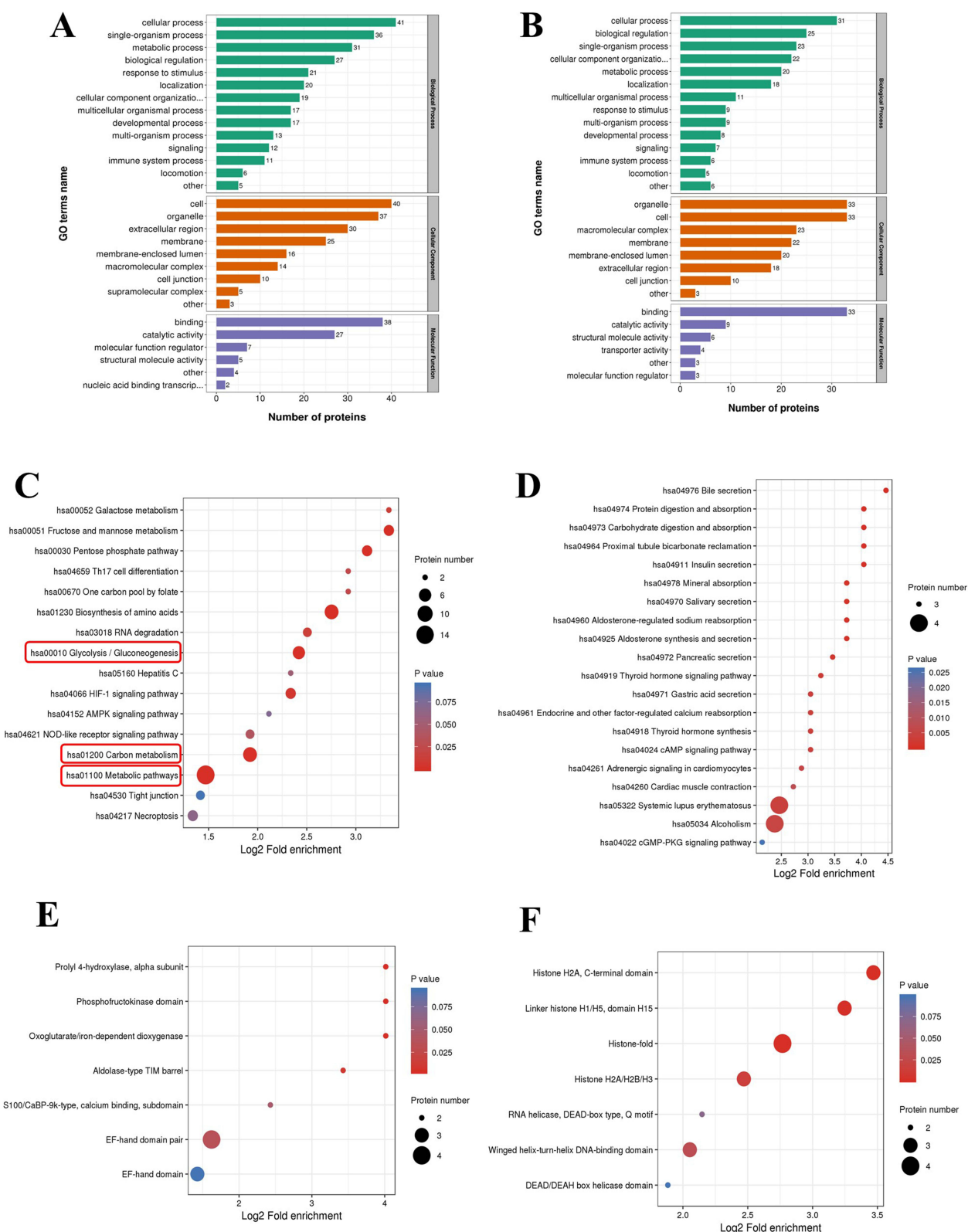
**Notes:** (A) Volcano plot depicting the differentially expressed crotonylation sites in OSCC under hypoxic conditions. Crotonylation sites with a fold change  $> 1.5$  and  $P < 0.05$  are indicated by red dots, representing upregulated sites. Crotonylation sites with a fold change  $< -1.5$  and  $P < 0.05$  are indicated by blue dots, representing downregulated sites. (B) Histogram illustrating the distribution of crotonylated proteins and associated sites with a fold change greater than 1.5. (C) Subcellular localization analysis of the crotonylated proteins. (D) Subcellular localization analysis specifically focusing on the up-regulated crotonylated proteins. (E) Subcellular localization analysis specifically focusing on the down-regulated crotonylated proteins.

**Abbreviations:** OSCC, oral squamous cell carcinoma; H, hypoxia; N, normoxia.

## Discussion

Emerging evidence suggests that crotonylation can modulate protein function in human tumor tissues.<sup>30,31</sup> Global crotonylation levels have been found to be significantly increased in various carcinoma types, including thyroid, esophagus, colon, pancreas, and lung, while reduced in liver, stomach, and kidney carcinomas.<sup>32</sup> These observations indicate that crotonylation may play diverse roles in cancer progression by influencing distinct pathways. In our study, we investigated the differential crotonylation levels in OSCC under hypoxic conditions using liquid-chromatography fractionation, affinity enrichment of crotonylated peptides, and high-resolution mass spectrometry. Using these methods, we identified a total of 1933 crotonylated modification sites on 722 proteins, with quantitative information available for 1563 sites on 605 proteins. Consistent with previous studies, our findings demonstrate that crotonylation occurs not only on histones but also on numerous non-histone proteins.<sup>2,14,33</sup>

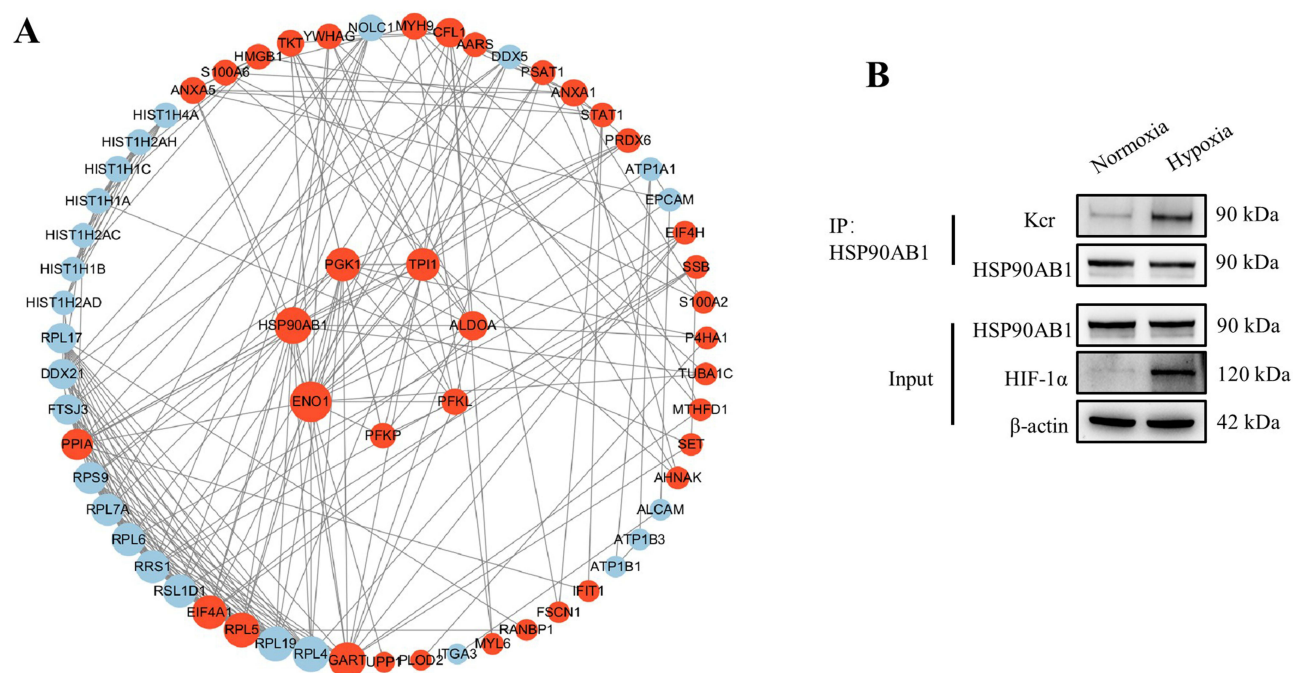
Tumor hypoxia, characterized by low oxygen levels in the tumor microenvironment, has been implicated in the regulation of protein crotonylation.<sup>34</sup> A study suggests that the YEATS2 protein, a selective histone crotonylation reader, is regulated by hypoxia-inducible factor 1- $\alpha$  (HIF-1 $\alpha$ ). HIF-1 $\alpha$  increases the expression of YEATS2 through binding to its hypoxia response element (HRE), thereby promoting the promotion of proliferation and migration of pancreatic cancer cells under hypoxic conditions.<sup>35</sup> Previous studies have demonstrated that hypoxia can significantly enhance protein crotonylation in liver cancer cells.<sup>32</sup> In our study, we observed a decrease in global crotonylation in OSCC under a hypoxic microenvironment. Preliminary analysis revealed a down-regulation of histone crotonylation levels, while crotonylation levels of non-histone proteins were up-regulated. Interestingly, a majority of the up-regulated crotonylated proteins (up to 63.41%) were localized in the cytoplasm. This intriguing differential distribution suggests spatial changes in the regulatory mechanisms within the cell.



**Figure 4** Functional enrichment of proteins corresponding to differential crotonylation sites in OSCC.

**Notes:** (A) GO enrichment analysis of proteins associated with up-regulated crotonylation sites, including cellular component, molecular function, and biological process. (B) GO enrichment analysis of proteins associated with down-regulated crotonylation sites, encompassing cellular component, molecular function, and biological process. (C) KEGG pathway enrichment analysis of proteins linked to up-regulated crotonylation sites. The red border highlights cellular metabolic-related signaling pathways. (D) KEGG pathway enrichment analysis of proteins linked to down-regulated crotonylation sites. (E) Protein domain analysis of proteins associated with up-regulated crotonylation sites. (F) Protein domain analysis of proteins associated with down-regulated crotonylation sites.

**Abbreviations:** GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.



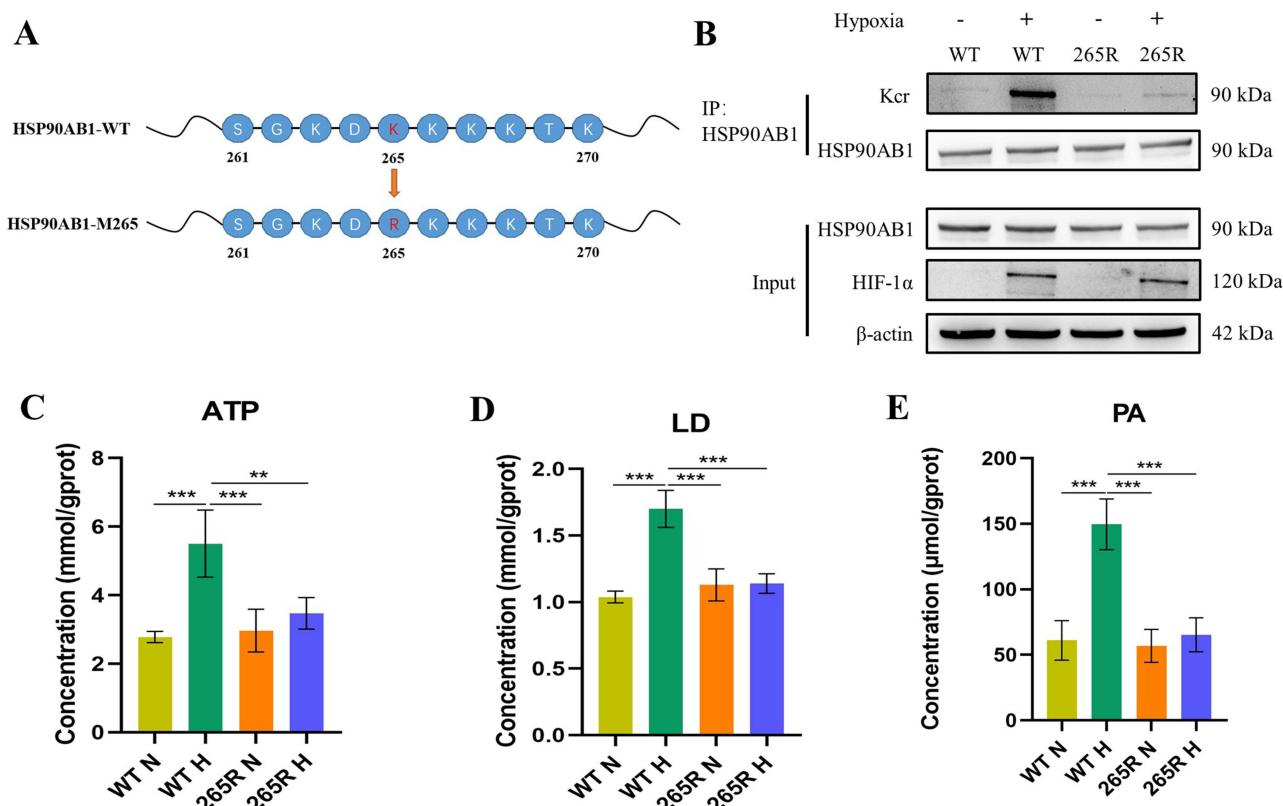
**Figure 5** Functional relationships between proteins corresponding to differential crotonylation sites in OSCC.

**Notes:** (A) Protein–protein interaction (PPI) network of proteins associated with differential crotonylation sites. Each protein is represented by a circle, with the size of the circle indicating the number of interacting proteins. Connecting lines represent the relationships between proteins. Different colors denote up-regulated (red) or down-regulated (blue) proteins. (B) Immunoprecipitation and Western blot assay performed to detect the crotonylation modification level of HSP90AB1 in CAL27 cells following 24 hr of hypoxic culture.

**Abbreviation:** PPI, protein–protein interaction.

Lysine crotonylation may occur either enzymatically or nonenzymatically. Intracellular levels of crotonyl-CoA have been proposed to influence the overall level of crotonylation.<sup>36,37</sup> The regulation of crotonylation involves a dynamic balance between writer and eraser proteins, known as crotonyltransferases (KCT) and decrotonylases (KDCR), respectively. Three major families have been identified: p300/CBP (p300/CREB-binding protein),<sup>36</sup> MYST,<sup>38</sup> and GNAT (GCN5-related N-acetyltransferase).<sup>39</sup> Currently, p300 and hMOF in MYST family and PCAF in GNAT family were recognized to be non-histone crotonyltransferase.<sup>12</sup> For KDCRs, both class I and III HDACs (Histone deacetylases) were reported as histone decrotonylases.<sup>40,41</sup> This suggests that hypoxia might alter the distribution of crotonyltransferase or decrotonylase between cytoplasm and nucleus, which sheds light on our follow-up study. Therefore, exploring the distribution of KCTs/KDCRs or crotonyl-CoA in cytoplasm and nucleus is an effective way to uncover the crotonylated proteome of OSCC under hypoxia condition.

Crotonylation of non-histone proteins has been shown to impact their original biological functions. For instance, Wei W et al demonstrated that the decrotonylase activity of crotonylated HDAC1 was lower compared to its unmodified form.<sup>42</sup> Additionally, Dan Zhang et al reported that crotonylation of lamin A at the K265/270 site maintained its subcellular localization and promoted liver cancer cell proliferation.<sup>30</sup> In our study, protein–protein interaction (PPI) analyses revealed that many of the identified non-histone crotonylated proteins in OSCC were closely associated with glycolysis, including PGK1, ALDOA, ENO1, and PFK. Notably, the crotonylation level of HSP90AB1, a critical upstream regulator of glycolysis, was reported to be up-regulated by up to a 9-fold change at the K265 site.<sup>29</sup> Hence, we speculate that the crotonylation level of HSP90AB1 could influence its regulatory function in glycolysis, and preliminary experimental results have provided support for this hypothesis.



**Figure 6** Crotonylation of HSP90AB1 affects its regulation of glycolysis in OSCC.

**Notes:** (A) Schematic diagram illustrating the plasmid mutation strategy for HSP90AB1. Lysine 265 of HSP90AB1 was mutated to arginine to prevent crotonylation. (B) Immunoprecipitation and Western blot analysis performed to detect the crotonylation modification level of HSP90AB1 in CAL27 cells under different treatments. The HSP90AB1low CAL27 cells were transfected with either wild-type (WT) or mutant plasmids and cultured under normoxic or hypoxic conditions for 24 hr. (C) Quantification of ATP levels in CAL27 cells transfected with WT or mutant HSP90AB1 plasmids and subjected to normoxia or hypoxia culture. (D) Measurement of lactic acid levels in CAL27 cells after transfection with WT or mutant HSP90AB1 plasmids and exposure to normoxic or hypoxic conditions. (E) Analysis of pyruvic acid levels in CAL27 cells transfected with WT or mutant HSP90AB1 plasmids and treated with normoxia or hypoxia. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to control.

**Abbreviations:** WT, wild type; ATP, adenosine triphosphate.

HSP90AB1 has emerged as a significant player in cancer development and progression, functioning as a molecular chaperone that stabilizes and activates client proteins involved in cell growth and survival. Notably, HSP90AB1 has been demonstrated to stabilize extracellular fibronectin in breast cancer cells, thereby indicating its involvement in the tumor microenvironment.<sup>43</sup> Moreover, in gastric cancer, HSP90AB1 has been found to stabilize low-density lipoprotein receptor-related protein 5 (LRP5), leading to the activation of AKT and Wnt/ $\beta$ -catenin signaling pathways, which in turn promotes epithelial–mesenchymal transition and cancer progression.<sup>28</sup> Furthermore, an interaction between HSP90AB1 and EEF1A2 has been observed, enhancing TGF- $\beta$ /SMAD signaling and facilitating lung adenocarcinoma metastasis.<sup>27</sup> These findings align with our previous study, revealing that HSP90AB1 promotes cell proliferation, migration, and glycolysis.<sup>29</sup> More importantly, HSP90AB1 has also been linked to drug resistance, as its expression has been associated with resistance to HSP90 inhibitors in mammary and colorectal cancer.<sup>44</sup> In ovarian cancer, targeting HSP90AB1 has emerged as a viable strategy, as its inhibition can reverse cisplatin resistance and impede tumor growth.<sup>45</sup> Collectively, these investigations underscore the multifaceted role of HSP90AB1 in cancer biology, exerting influence on diverse cellular processes and pathways that contribute to tumor development and progression. Further exploration of HSP90AB1 and its interactions with client proteins holds promise for the development of targeted therapies in cancer treatment.

Aligned with the findings from our KEGG pathway enrichment analysis, multiple studies have provided evidence for the involvement of crotonylation in the regulation of various metabolic pathways, including glycolysis, carbon metabolism, and amino acid metabolism.<sup>14,46,47</sup> In our study, the large-scale analysis also revealed elevated levels of crotonylation in key enzymes of glycolysis, such as PGK1, ALDOA, ENO1, and PFK. Thus, it is plausible that the crotonylation

modification of these enzymes may impact their activity. Notably, a recent study demonstrated that crotonylation of lysine 420 in ENO1 significantly enhances its catalytic activity, thereby promoting the proliferation, migration, and invasion of colorectal cancer cells.<sup>48</sup> Furthermore, a co-occurrence of crotonylation and 2-hydroxyisobutyrylation was observed in proteins involved in glycolysis in end-stage renal disease, with ENO1 and PGK1 being the most abundant dual-modified proteins in the glycolysis or gluconeogenesis pathway.<sup>49</sup> Therefore, in our study, changes in enzyme activity resulting from crotonylation modifications of these rate-limiting enzymes might also contribute to enhanced glycolytic function under hypoxic conditions. However, further research is necessary to fully elucidate the underlying mechanisms through which crotonylation regulates glycolysis and its implications in the context of OSCC.

There are several limitations in our study that warrant mention. Firstly, it is important to highlight that our research relies on bioinformatic analysis to uncover the global crotonylome in hypoxic conditions of OSCC. The underlying regulatory mechanism of this modification difference and the effect of crotonylation on protein function still require further investigation. Additionally, although we have verified the expression pattern of HSP90AB1 in hypoxic OSCC and its association with glycolysis, it is crucial to confirm the precise biological roles of HSP90AB1 in OSCC through additional molecular and animal experiments. These investigations will provide deeper insights into the functional significance of HSP90AB1 in OSCC.

## Conclusion

Our study revealed significant dysregulation of global crotonylation in OSCC under a hypoxic microenvironment. Most of histone crotonylation levels were down-regulated, while crotonylation levels of glycolysis-related proteins were up-regulated. Notably, HSP90AB1 exhibited the most significant up-regulation, and further experiments confirmed that its crotonylation under hypoxic conditions might promote glycolysis. These findings offer new insights into the regulatory mechanism of crotonylation in OSCC under hypoxia and suggest that targeting crotonylation of glycolysis-related proteins could be a potential therapeutic strategy for OSCC treatment.

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## Disclosure

The authors report no conflicts of interest in this work.

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