Identification of lysine-lactylated substrates in gastric cancer cells

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Highlights
2375 Kla sites in 1014 proteins were identified in gastric cancer AGS cells
Lysine lactylation could be a prognostic marker in gastric cancer
Lactylated proteins were significantly enriched in spliceosome
Lactate could influence RNA splicing in gastric cancer AGS cells
Identification of lysine-lactylated substrates in gastric cancer cells

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SUMMARY
Cancer cells tend to utilize aerobic glycolysis to generate energy and metabolites; the end product of aerobic glycolysis is lactate, which promotes lysine lactylation (Kla). Kla is a newly discovered histone post-translational modification (PTM) that plays important roles in regulating gene expression. However, Kla in non-histone mammalian proteins is unclear. Here, a comprehensive analysis of lactylated proteins in gastric cancer AGS cells was conducted. There were 2375 Kla sites found in 1014 proteins. Interestingly, KEGG pathway analysis showed that these proteins were significantly enriched in spliceosome function. In addition, Kla was more abundant in gastric tumors than in adjacent tissues, and high levels of Kla in gastric tumors were associated with poor prognosis. These results suggest that Kla could be a prognostic marker in gastric cancer. This lysine lactylome analysis in gastric cancer cells, the first of its kind, provides a valuable foundation for further studies of Kla.

INTRODUCTION
Despite progress in available treatments, gastric carcinoma remains the third most common cause of cancer-related death worldwide (Joshi and Badgwell, 2021; Sung et al., 2021). Deeper understanding of the biological nature of this disease is therefore needed. The Warburg effect is one of the most common characteristics of cancers, including gastric carcinoma (Liu et al., 2019); this effect involves enhanced glycolysis and metabolism of pyruvate to lactate rather than oxidative phosphorylation under aerobic conditions. Lactate dehydrogenase A (LDHA), the key enzyme in lactogenesis, is a predictor of poor prognosis in patients with gastric cancer (Kolev et al., 2008). In addition, levels of lactate in gastric juice are correlated with tumor size and the degree of invasion in gastric cancer (Armstrong et al., 1984). Although lactate was recognized as a metabolic byproduct or energy source in previous studies, its non-metabolic functions in gastric cancer remain unclear.

In 2019, Zhao et al. reported lactate-derived histone lysine lactylation (Kla) as a novel post-translational modification (PTM) that stimulated gene transcription in macrophages (Zhang et al., 2019a). The functions of histone Kla were then explored in mouse embryo fibroblasts (MEFs) (Li et al., 2020a), ocular melanoma cells (Yu et al., 2021), and non-small cell lung cancer (NSCLC) cells (Jiang et al., 2021); these studies indicated that Kla has important effects in regulating pluripotency and oncogenesis. Few studies have focused on Kla of non-histone proteins, although such studies have been conducted in Botrytis cinerea and Oryza sativa (Gao et al., 2020; Meng et al., 2021). Yang et al. reported lactylation of HMGB1 in macrophages, but the specific lactylation sites were not studied (Yang et al., 2021). Thus, identification of lactylated substrates and specific lactylation sites in mammals should be conducted to help comprehend the non-metabolic functions of lactate.

Although basal lactate production averages ~0.8 mmol/kg body weight/h, the concentration can reach 12.9 mmol/kg body weight/h in glycolytic tumors (Vinasco et al., 2019; Walenta et al., 1997, 2000). Concentrations of lactate in gastric juice were shown to be higher in patients with gastric carcinoma than in those from control groups (Armstrong et al., 1984), suggesting that the concentration of lactate is higher in gastric tumors than in normal gastric tissues. In this study, we compared Kla abundance in gastric cancer and para-cancerous tissues from 76 patients by immunohistochemical staining (IHC). Levels of Kla were higher in human gastric tumors than in adjacent gastric tissues and high levels of Kla in gastric tumors indicated poor prognosis in patients. We also performed proteomic analysis of gastric cancer cells and identified 2375 Kla sites in 1014 proteins, including a lactylation site in HMGB1. These lactylated proteins were distributed in multiple cellular compartments, with more than half localized to the nucleus. In
addition, we identified 63 lactylated spliceosomal proteins, indicating that this PTM was abundant in the spliceosome and may affect RNA splicing.

Together, the results of this study validate the prognostic value of Kla in gastric cancer, expand the known profile of protein substrates and pathways affected by Kla, and provide a valuable foundation for further studies on functions of Kla in human diseases.

RESULTS
Systematic profiling of the Kla proteome in AGS cells

Although Kla can occur on non-histone proteins, proteome-wide identification of Kla substrates had not previously been conducted in mammalian cells. To identify proteins bearing Kla, we conducted a proteomic screen using affinity-directed mass spectrometry.

First, we confirmed Kla modifications in gastric cancer cells via Western blot. Lactate increased the abundance of Kla in AGS cells (Figure 1A). Multiple protein bands with different molecular weights were observed, indicating that many non-histone proteins were lactylated in gastric cancer cells (Figure 1A).

We then conducted affinity-directed mass spectrometry. Briefly, proteins were extracted from cells and digested with trypsin, and then the lactylated peptides were enriched with anti-Kla antibodies. Peptides were identified by liquid chromatography/tandem mass spectroscopy (LC-MS/MS) analysis and database searching (Figure 1B). To improve the reliability of identified sites, we filtered out peptides with MaxQuant scores below 40 and selected those with localization probabilities >0.75 as previously described (Huang et al., 2021). This led to the identification of 2375 high-confidence Kla sites in 1014 proteins (Table S1).

Among the 1014 proteins, about 52.7% contained single Kla sites, whereas 3.6% contained more than eight Kla sites, which corresponded to about 19.7% of all identified sites (Figure 1C). In addition, we identified 27 core histone Kla sites, including eight previously unknown sites (Table S2). We also identified the lactylated site in HMGB1; lactylation of this protein is associated with mortality due to sepsis (Yang et al., 2021). Representative MS/MS spectra of lactylated HMGB1 and H2AX are shown in Figure 1D. As shown in the upper panel in Figure 1D, 11 ions (y1 to y8 and b2 to b4) were detected, and ion b2 consisted of lysine and threonine. The mass to charge ratio (m/z) of b2 would be around 230.149 if the amino acids were not modified, but the actual m/z value of b2 was 302.17. This corresponds to a mass shift of 72.021, consistent with the shift caused by addition of a lactyl group (Zhang et al., 2019a). Ions b3 and b4 also had mass shifts of 72.021 while ions y1 to y8 did not. These results provided evidence that the first lysine of the KTSATVGPK sequence was lactylated.

Properties of Kla peptides in AGS cells

To define the possible Kla motifs in lactylated proteins, we compared the amino acid sequences surrounding confirmed Kla sites and identified 10 conserved motifs; xxxxxxxxxx_K_xxGxxxxxxx (present in 80 of the 1014 peptides), xxxxxxxxxx_K_xGxxxxxxx (100 peptides), xxxxxxxxxx_Px_K_xxxxxxxx (87 peptides), xxxxxxxxxx_K_xGxxxxxxxKx (132 peptides), xxxxxxxxxx_K_xxxxxxKxxx (152 peptides), xxxxxxxxxx_K_xxxxxRxxxx (149 peptides), xxxxxxxxxx_K_xxxxxKxxxx (202 peptides), xxxxxxxxxx_K_xxxxxKxxxx (240 peptides), xxxxxxxxxx_K_xxxxxxxx (325 peptides), and xxxxxxxxxx_K_xxxxxxKxxx (274 peptides) (Figure 2A). These motifs differ from those identified in O. sativa and B. cinerea (Gao et al., 2020; Meng et al., 2021). Heatmaps of the amino acid sequences surrounding lactylation sites demonstrated that K residues were enriched in the regions from −10 to −3 and from 3 to 10, and that A residues were enriched in the regions from −8 to −1 and from 1 to 6 (Figure 2B).

Structure analysis was performed to investigate the correlation between Kla and protein structures in gastric cancer cells. Among the 2375 Kla sites, 2269 were located in regions with ordered secondary structures, 31% were located in alpha-helices, and 64% were located in coils (Figure 2C). In addition, lactylated sites in the protein surface were more likely to be exposed than other lysine residues (Figure 2D).

Characterization of the Kla proteome in AGS cells

To better characterize lactylated proteins in gastric cancer cells, we analyzed the subcellular localization of the lactylated substrates. More than half of the proteins (56.6%) were localized to the nucleus, whereas
29.3% and 5.2% were distributed in the cytoplasm and mitochondria, respectively (Figure 3A). We then conducted biochemical pathway analysis using annotations from the Kyoto Encyclopedia of Genes and Genomes (KEGG). This analysis showed that proteins related to the spliceosome were more likely to be lactylated (Figure 3B), suggesting that Kla may affect RNA splicing. This finding was further supported by analysis of Gene Ontology (GO) functional annotations; analysis of the biological processes associated with lactylated proteins showed enrichment proteins that participate in RNA processing or splicing (Figure 3C); enrichment analysis of molecular functions also showed that proteins involved in RNA binding were more likely to be lactylated (Figure 3D). Meanwhile, immunoprecipitation was used to confirm...
Lactylation of hnRNPA1 and SF3B1 (Figure S1), which are key proteins that regulate alternative splicing (Howard et al., 2018; Liu et al., 2020).

The results support that Kla is abundant in spliceosomal proteins in these gastric cancer cells. A summary of lactylated proteins related to the spliceosome is shown in Figure 3E.

Lactate changed RNA splicing events in AGS cells

Because Kla proteins were enriched in spliceosome functions, we predicted that lactate may affect RNA splicing in gastric cancer cells. We therefore performed an RNA-seq analysis using the program rMATS (Hwang et al., 2020). The presence of lactate altered RNA splicing events (Figure 4A), including skipped exon (SE), alternative 5′ splice site (A5SS), alternative 3′ splice site (A3SS), mutually exclusive exons (MXE), and retained intron (RI) events in AGS cells (Table S3). RNA splicing events are visualized as volcano plots in Figures 4B–4F.

Pan-kla was more abundant in gastric tumors than that in adjacent tissues

Kla substrates were identified in our proteomic screening of gastric tumor cells. However, whether this type of PTM is correlated with gastric tumors has not yet been determined. We therefore examined Kla levels by

Figure 2. Properties of Kla peptides

(A) Sequence motif logo showing a representative sequence for all Kla sites. The central K represents the lactylated lysine. The number of instances of each conserved motif in the samples from the present study is shown below.

(B) Heatmap of the amino acid composition around Kla sites. Green indicates low frequency of an amino acid and red indicates high frequency.

(C) Estimated probabilities of Kla sites in of coil, beta-strand, and alpha-helix structures.

(D) Estimated surface accessibility of Kla sites in substrates. p values were calculated with Fisher’s exact test.

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IHC in a tissue microarray, which included 76 gastric tumor sections and corresponding para-cancerous sections. The results showed that among the 76 samples, Kla levels were significantly higher in 51 tumor tissues than their corresponding para-cancerous tissues (Figures 5A and 5B). Furthermore, three gastric cancer cell lines showed higher Kla levels and sensitivity to lactate dehydrogenase (LDH) inhibition than gastric epithelial cells (Figure S2). We also studied the relationships between Kla levels in tumor tissues and patient age/level of tumor differentiation. Kla was more abundant in poorly differentiated tumors than in the moderately differentiated tumors (Figure 5C). Pearson correlation analysis suggested that age was not correlated with Kla abundance (Figure 5D, p = 0.2874).

Pan-Kla abundance predicted prognosis in gastric tumor patients

The above results showed that Kla was more abundant in tumor tissues than in adjacent tissues, we therefore then examined whether Kla levels were correlated with patient prognosis. Using TNM staging, those with Stage IIIA-IIIB (TNM staging) gastric carcinoma had higher Kla levels than those with Stage I-II carcinoma (Figure 5E). Tumors from patients with lymph node metastasis were also more lactylated than those from patients without lymph node metastasis (Figure 5F). Kaplan-Meier analysis was conducted, which confirmed that high Kla levels were correlated with poor overall survival in patients with gastric cancer (Figure 5G). The hazard ratios of Kla levels and differentiated levels against overall survival were 2.77 and 0.56, respectively, in a multivariate Cox regression proportional hazards model (Figure 5H).

DISCUSSION

As the primary byproduct of the Warburg effect, lactate has long been associated with tumors. The tumor-promoting actions of lactate include regulating immune surveillance (Brand et al., 2016; Colegio et al., 2014; Peng et al., 2016; Zhang et al., 2019b), accelerating tumor angiogenesis (Giatromanolaki et al., 2006; Kolev et al., 2008; Shi et al., 2001; Vegran et al., 2011), promoting metastasis (Cantelmo et al., 2016; Tasdogan et al., 2020), and even affecting fibroblasts (Formby and Stern, 2003; Stern et al., 2002). The effects of lactate on PTM that may mediate these processes were not discovered until 2019 (Zhang et al., 2019a). Lactate was found in 2014 to promote the transcription of Arg1 and Vegfa in macrophages (Colegio et al., 2014), but the mechanism remained unclear. In 2019, Zhao et al. found that histone lactylation controlled the transcription of Arg1 and Vegfa (Zhang et al., 2019a), and further elucidated the effects of lactate on macrophage polarization. Kla was also found to occur in non-histone proteins. Gao et al. identified 273 kla sites in 166 proteins in B. cinerea (Gao et al., 2020), and Meng et al. identified 638 kla sites in 342 proteins in rice grains, suggesting that Kla could be associated with fungal pathogenicity and carbon metabolism. Moreover, Yang et al. reported lactylation of HMGB1 in macrophages, but the specific lactylation sites in HMGB1 were not studied (Yang et al., 2021). Thus, Kla substrate sites in non-histone proteins have remained unknown in the context of human diseases. In this study, we identified 2375 Kla sites in 1014 proteins in AGS cells, including eight previously unknown histone Kla sites and a lactylated site in HMGB1.

The maturation of precursor mRNA (pre-mRNA) relies on RNA splicing, which removes introns and exons from pre-mRNA (Kitamura and Nimura, 2021). Alternative splicing increases the diversity of proteins by affecting the complexity of mRNA, and is known to regulate multiple cellular processes (Zhang et al., 2021). It was previously reported that RNA splicing could affect lactate metabolism (Klipfel et al., 2021; Leyva-Carrillo et al., 2019; Li et al., 2020b), however, whether lactate could affect RNA splicing was unknown. We have identified 193 Kla sites in 70 spliceosome-related proteins. KEGG pathway analysis showed that lactylated proteins were enriched in spliceosome functions, suggesting a global influence of lactate on RNA splicing. Moreover, as shown in the RNA-seq analysis, treatment with lactate caused differential splicing events (namely SE, A5SS, A3SS, MXE, and RI) to occur in AGS cells.
Finally, we compared pan-Kla levels in gastric tumors and adjacent tissues. Kla levels were significantly higher in 51 tumor tissues than in the corresponding para-cancerous tissues. In addition, higher levels of Kla were correlated with more poorly differentiated tumors, lymph node metastasis, and poorer overall patient survival rates. In summary, this study not only suggests a prognostic value of Kla in gastric tumors but also identified Kla substrates via LC-MS/MS. As the first lysine lactylome analysis in mammalian cells, our findings provide a valuable foundation for further studies related to the function of Kla in human diseases.

Figure 4. Lactate treatment caused differential alternate splicing (AS) events in AGS cells
AGS cells were treated with 20 mM lactate or vehicle for 24 h.
(A) Numbers of AS events altered by lactate treatment.
(B–F) Volcano plots show how lactate treatment altered different types of AS events, namely (B) Retained intron (RI), (C) Mutually exclusive exon (MXE), (D) Alternative 5′ splice site (A5SS), (E) Alternative 3′ splice site (A3SS), and (F) Skipped exon (SE) events.

Finally, we compared pan-Kla levels in gastric tumors and adjacent tissues. Kla levels were significantly higher in 51 tumor tissues than in the corresponding para-cancerous tissues. In addition, higher levels of Kla were correlated with more poorly differentiated tumors, lymph node metastasis, and poorer overall patient survival rates. In summary, this study not only suggests a prognostic value of Kla in gastric tumors but also identified Kla substrates via LC-MS/MS. As the first lysine lactylome analysis in mammalian cells, our findings provide a valuable foundation for further studies related to the function of Kla in human diseases.
A

Tumor

Adjacent

B

C

D

Pearson correlation

R = 0.1236
P = 0.2874

E

F

G

Overlay survival

K-lactylation

high (n = 38)

low (n = 38)

H

| Prognostic factor          | HR    | P value | 95% CI  |
|---------------------------|-------|---------|---------|
| Lysine-Lactylation (High) | 2.77  | 0.001   | 1.47 to 5.26 |
| Genders (Male)            | 1.73  | 0.091   | 0.92 to 3.26 |
| Age                       | 0.98  | 0.128   | 0.95 to 1.01  |
| Differentiated Level      | 0.56  | 0.004   | 0.38 to 0.83  |
Limitations of the study
The underlying mechanism of how Kla affects gastric cancer progression was not investigated here. Future studies, which should include genetic manipulation and mutagenesis experiments, are needed to verify the effects of Kla on protein functions and tumor progression.

DATA AVAILABILITY
All data generated or analyzed during this study are included in this article.

ETHICS STATEMENT
Tissue arrays were provided by the Nanjing Pukou Central Hospital and approved by Ethics Committee of the Nanjing Pukou Central Hospital.

STAR+METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104630.

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Figure 5. Kla abundance in gastric tumors and its relationship with the prognosis
(A and B) Representative images and quantification of immunohistochemistry (IHC) staining of Kla in gastric tumors (n = 76) or adjacent tissues (n = 76). Relative intensities of IHC staining were determined with Image Pro Plus v6.0.
(C) Relative Kla levels in types of gastric tumors: poorly differentiated (n = 27), moderately/poorly differentiated (n = 26), and moderately/highly differentiated (n = 11).
(D) Relative Kla levels in gastric tumors from patients of varying ages (n = 76).
(E) Relative Kla levels in gastric tumors from patients with TNM stages I-II (n = 39) and stages IIIA-IIIB (n = 37).
(F) Relative Kla levels in gastric tumors from patients with lymphatic metastasis (N1, N2, and N3; n = 46) or without lymphatic metastasis (N0; n = 29).
(G) Survival rates for patients with different Kla levels using Kaplan-Meier analysis.
(H) A multivariate Cox regression proportional hazards model was used to analyze the hazard factors in gastric cancer. Students’ T test was employed in Figures 1B and 1D–1F to compare the differences between groups; One-way ANOVA was used in Figure 1C to compare the differences between groups; Significance values are *p < 0.05, **p < 0.01 and ***p < 0.001.
AUTHOR CONTRIBUTIONS

Dawei Yang: Conceptualization, Methodology, Investigation, Writing – original draft. Jie Yin: Methodology, Software. Liuqun Shan: Writing – review & editing. Xingling Yi: Methodology, Writing – review & editing, Funding acquisition. Yongbing ding: Conceptualization, Writing – review & editing, Resources, Supervision, Funding acquisition.

DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest. Each listed author on the manuscript is aware of and agrees to the contents of the manuscript, including the authorship.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-Kla antibody   | Micron Biotechnology Company | Cat# WM101; RRID: Not applicable |
| Anti-hnRNPA1 antibody | ABclonal | Cat# A11564; RRID:AB_2861599 |
| Anti-SF3B1 antibody | ABclonal | Cat# A15801; RRID:AB_2763223 |
| Anti-ß-Actin        | ABclonal | Cat# AC026; RRID:AB_2768234 |
| HRP Goat Anti-Rabbit IgG secondary antibody | ABclonal | Cat# AS014; RRID:AB_2769854 |
| Rabbit Control IgG | ABclonal | Cat# AC005; RRID:AB_2771930 |
| **Biological samples** |        |            |
| Tissue arrays       | Nanjing Pukou Central Hospital | Not applicable |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Sodium lactate      | Aladdin Biochemical Technology | Cat# S108838 |
| ECL substrate       | Yeasen | Cat# 36222ES60 |
| Urea                | Sigma | Cat# US378 |
| NP40                | Sigma | Cat# I3021 |
| Sodium deoxycholate | Sigma | Cat# 30970 |
| EDTA                | Sigma | Cat# E6758 |
| Dithiothreitol      | Solarbio | Cat# D8220 |
| Glycine             | Solarbio | Cat# G8200 |
| Tris                | Solarbio | Cat# T8060 |
| SDS                 | Solarbio | Cat# S8010 |
| Triethylammonium bicarbonate | Sigma | Cat# T7408 |
| Iodoacetamide       | Sigma | Cat# I1149 |
| Formic acid         | Sigma | Cat# 56302 |
| Trifluoroacetic Acid | Sigma | Cat# 302031 |
| Trypsin             | Promega | Cat# VS280 |
| Acetonitrile        | Fisher | Cat# A998-4 |
| TRIzol              | Thermo | Cat# 15596018 |
| **Critical commercial assays** |        |            |
| Protein A Magnetic beads | MCE | Cat# HY-K0203 |
| Cell Counting Kit-8 (CCK-8) | APExBIO | Cat# K1018 |
| C18 Easy Tip        | Micron Biotechnology Company | Cat# ETC185096 |
| Anti-K-la antibody conjugated agarose beads | Micron Biotechnology Company | Cat# WM102 |
| C18 SPE column      | Phenomenex | Cat# 8B-S123-EBJ |
| **Deposited data**  |        |            |
| Mass spectrometry proteomics data | This paper | [PRIDE]: PXD033991 |
| RNA-seq data        | This paper | [SRA data]: PRJNA841340 |
| **Experimental models: cell lines** |        |            |
| AGS cells           | Cell Bank of Type Culture Collection of Chinese Academy of Sciences | TCHu232 |
| HGC-27 cells        | Cell Bank of Type Culture Collection of Chinese Academy of Sciences | TCHu22 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yongbin Ding (njdyb@njmu.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium via the PRIDE partner repository, RNAseq data has been deposited at Sequence Read Archive (SRA, NCBI). The accession numbers are listed in the Key resources table. They are available upon request if access is granted.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the Lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
Gastric cancer AGS and HGC-27 cell lines were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Gastric epithelial GES-1 cells and gastric cancer MGC-803 cells were purchased from iCell Bioscience Inc (Shanghai, China). AGS cells were cultured in Dulbecco’s modified Eagle medium (DMEM) (Gibco, Grand Island, NY). GES-1, MGC-803, and HGC-27 cells were cultured in RPMI 1640 medium (Gibco) in a humidified atmosphere containing 5% CO2 at 37 °C. Both media were supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin.

METHOD DETAILS

Western blot
Gastric cancer cells were treated with sodium lactate (Aladdin Biochemical Technology, Shanghai, China) or vehicle at the indicated concentrations for 24 h, then washed with phosphate-buffered saline (PBS) and lysed with radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA, USA). Samples (10 μg each) were separated with SDS-PAGE electrophoresis. The membranes were blocked with 3% skim milk at room temperature for 1 h, then incubated overnight at 4 °C with primary
antibodies: anti-Kla (Micron Biotechnology Company, Hangzhou, China), anti-hnRNPA1 (ABclonal, Wuhan, China), anti-SF3B1 (ABclonal), and anti-β-Actin (ABclonal). Membranes were then incubated with HRP Goat Anti-Rabbit IgG secondary antibody (ABclonal) for 1 h at room temperature. The blot was developed with ECL substrate (Yeasen, Shanghai, China) and photographed with a Molecular Imager (Bio-RAD, Hercules, CA, USA) or X-ray film exposure.

RNA-seq
AGS cells were treated with sodium lactate (20 mM) or vehicle for 24 h, then washed with PBS and collected. There were three biological replicates for each group. Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific) following the manufacturer’s instructions. Transcriptome sequencing was conducted on an Illumina platform by GENEWIZ (Suzhou, China). rMATS (http://rnaseq-mats.sourceforge.net/) was used to analyze the RNA-seq data and identify differential alternate splicing events, including skipped exon (SE), alternative 5' splice site (A5SS), alternative 3' splice site (A3SS), mutually exclusive exon (MXE), and retained intron (RI) events. rMATS was used to calculate the p value and false discovery rate (FDR) for differences in the isoform ratio of each gene. Events with |IncLevelDifference| ≥ 0.02, p value ≤ 0.001, and FDR ≤ 0.05 were classified as differential alternative splicing events.

Immunoprecipitation (IP)
Protein A magnetic beads (MedChem Express, Shanghai, China) were pre-incubated for 2 h at room temperature with primary antibodies: IgG control (AB), anti-hnRNPA1 (ABclonal), or anti-SF3B1 (ABclonal). AGS cells were pretreated with sodium lactate or vehicle for 24 h, then washed with PBS and lysed with cell lysis buffer to perform western blot and IP (Beyotime, Shanghai, China). Samples were sonicated and centrifuged at 13,000 x g for 15 min at 4°C. The supernatants were collected to obtain the protein samples. These samples were then incubated with the magnetic beads and rotated at 4°C overnight. The magnetic beads were washed with PBS + Tween (PBST) five times before adding 5X SDS loading buffer and boiling the samples for 10 min. The prepared samples were then used for western blot.

Immunohistochemistry (IHC) staining
Pre-prepared tissue microarrays were deparaffinized and hydrated, then washed with PBS. Microwave antigen retrieval was performed in citric acid buffer. Endogenous peroxidase was then blocked with 3% H2O2 for 30 min, and microarrays were incubated with 5% serum containing 0.5% bovine serum albumin (BSA). Microarrays were incubated with primary antibodies at 4°C overnight. After washing with PBS three times, microarrays were incubated with secondary antibody for 1 h at room temperature. Finally, microarrays were stained with 3, 3’-diaminobenzidine (DAB) and counterstained with hematoxylin.

An IHC kit was obtained from ORIGENE (Beijing, China). The microarrays were photographed with an Axio Scope A1 (Zeiss, Germany). The intensities of IHC staining were determined with Image Pro plus v6.0, then divided by the whole area of the tissue slice in each image to calculate relative Kla levels. Tissue arrays were provided by the Nanjing Pukou Central Hospital and approved by the Ethics Committee of the Nanjing Pukou Central Hospital.

Sample preparation for LC-MS/MS
Three independent cultured replicates of AGS cells were harvested and mixed. The cells were sonicated with lysis buffer (a cocktail of 8 M urea, 50 mM Tris 8.0, 1% NP40, 1% NaDOC, 10 mM EDTA, and 1% protease inhibitor). Lysates were centrifuged at 20,000 x g at 4°C for 10 min, and the supernatant was collected as the raw protein sample. Dithiothreitol (DTT) was added to a final concentration of 10 mM to the protein samples, which were then incubated at 37°C for 60 min. Samples were cooled to room temperature before adding 30 mM iodoacetamide (IAM), then incubated at room temperature in the dark for 45 min. Proteins were precipitated with 5X volume cold acetone, then washed with 80% cold acetone three times. The supernatants were discarded and extracted proteins were incubated at room temperature for 1 h.

Peptide fractionation and affinity enrichment
Proteins were dissolved in 0.1 M triethylammonium bicarbonate (TEAB) solution and sonicated on ice. Trypsin was then added at a ratio of 1:50 trypsin:protein (w/w) and samples were incubated at 37°C overnight. Trifluoroacetic Acid (TFA) was then added to a final concentration of 1% to terminate the reaction. Samples were desalted with a C18 SPE column and eluted peptides were dried with a vacuum concentrator.
Peptides were then redissolved and enriched with anti-L-lactyllysine antibody-conjugated agarose beads (Micron Biotechnology Company). Finally, the bonded lactated peptides were eluted, dried with a vacuum concentrator, redissolved, desalted with a C18 EasyTip, and dried with a vacuum concentrator.

**LC-MS/MS analysis**

Peptides were dissolved in 100% H₂O plus 0.1% Formic acid (FA) then centrifuged at 20,000 x g. Supernatants were tested with LC-MS on the Ultimate RSLC nano 3000 coupled with the Thermo Scientific Q Exactive HFX (Thermo Fisher Scientific). Enriched peptides were separated using the Thermo Acclaim PepMap RSLC C18 column (75 um × 500 mm, 2 μm, Thermo Fisher Scientific) at 40°C. The mobile phase was composed of 100% H₂O plus 0.1% FA (A) and 80% acetonitrile (ACN) plus 0.1% FA (B). The gradient program was set as follows: 0–6 min, 2–10% solution B; 6–51 min, 10–20% solution B; 51–58 min, 20–80% solution B; 58–62 min, 80% solution B; 62–63 min, 80–2% solution B; 63–70 min, 2% solution B. The flow rate was 250 μL/min.

The Orbitrap was applied to detect whole peptides and ion fragments at resolutions of 60,000 and 30,000, respectively. The electrospray voltage was set to 2.0 kV. Automatic gain control (AGC) was used to prevent overfilling of the iontrap. The m/z range was from 350–1800 for MS scans. The MS fixed first mass was set at 110 m/z. The affinity enrichment and LC-MS/MS analyses were conducted by the Micron Biotechnology Company.

**Database searching**

The raw MS/MS data were analyzed with MaxQuant (v.1.5.2.8) (http://www.maxquant.org/). The tandem mass spectra were collected and compared against the Homo sapiens database from UniProt. Mass errors of fragment ions and precursors were set to 0.02 Da and 5 ppm. Trypsin/P was specified as the cleavage enzyme with the following limitations: four missing cleavages, five modifications per peptide, and five charges. Carbamidomethylation was set as a fixed modification for cysteine and lactylation on lysine was set as a variable modification. The minimal peptide length was set as seven. The FDR thresholds for modification sites, peptides, and proteins were set at 1%. Kla sites with localization probability < 0.75 and MaxQuant scores < 40 were excluded.

**Bioinformatics analysis**

WoLF PSORT was used to predict the subcellular localization of each protein. Using GO annotations (www.ebi.ac.uk/GOA/), proteins were classified using three categories: biological processes, cellular components, and molecular functions. The KEGG database was used to identify biochemical pathways of proteins with Kla. NetsurfP-1.0 was used to analyze the secondary structures and surface accessibilities of peptides. Enrichment in biological pathways and functional annotations were tested with a two-tailed Fisher’s exact test; categories with a corrected p value < 0.05 were considered statistically significant.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Sample numbers used for statistical analysis are depicted in the figures. GraphPad Prism 8 was used to make statistical comparisons with Student’s t test, one-or two-way ANOVA and Fisher’s exact test (depicted in figure legends). Data are presented as the means +/- SD. Significance values are *p < 0.05, **p < 0.01 and ***p < 0.001.