

Lactate-Induced HNRNPA1 Lactylation Regulates PKM2 Splicing and Glycolytic Reprogramming in Bladder Cancer

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Abstract

Lactylation represents a newly discovered post-translational modification triggered by lactate that influences tumor metabolic processes. Its importance and the specific proteins it modifies in bladder cancer (BLCA) are still largely unexplored. Immunohistochemical staining was applied to patient-derived tissues. Comprehensive lactylation proteomics was performed using LC-MS/MS. Functional experiments were conducted in cell cultures and animal models. CRISPR/Cas9 gene editing, plasmid-based overexpression, and drug treatments were utilized to investigate the impact of P300-catalyzed lactylation of HNRNPA1 at lysine 350 on the aggressive behavior of BLCA cells. Metabolomics analysis and measurements of glycolytic rates helped evaluate the metabolic effects. Gene expression profiling and splicing pattern assessments provided further molecular validation. Potential HNRNPA1 inhibitors were screened using molecular docking approaches. Overall, protein lactylation was notably higher in BLCA samples than in normal tissues and was strongly associated with unfavorable clinical outcomes. Among the targets, HNRNPA1 stood out as a major lactylated protein. Enhanced glycolysis led to lactate accumulation, which, in turn, drove P300 to add a lactyl group to HNRNPA1 at K350. This change favored the splicing of PKM pre-mRNA into the PKM2 variant, thereby increasing glycolytic metabolism and promoting tumor expansion. Suppressing glycolysis pharmacologically or reducing LDHA expression reduced HNRNPA1 lactylation, decreased PKM2 production, and hindered BLCA cells' proliferation, migration, and invasion. Metabolic profiling confirmed that HNRNPA1-K350 lactylation intensified aerobic glycolysis within these cancer cells. Through molecular docking, a candidate small-molecule inhibitor was found that binds HNRNPA1, reduces PKM2 levels, and limits cell growth. The research identifies a novel lactate-dependent pathway that connects alternative splicing regulation to metabolic shifts in BLCA via HNRNPA1 lactylation. Lactylation at K350 of HNRNPA1 is an important driver of glycolysis-fueled cancer progression. These insights support the development of therapies targeting HNRNPA1 for the treatment of BLCA.

Keywords: HNRNPA1, Lactylation, Alternative splicing, Glycolytic reprogramming, Bladder cancer

Introduction

Bladder cancer (BLCA) ranks as a frequent malignancy affecting the urinary tract and holds the tenth position

globally for cancer occurrence [1]. Surgical removal continues to be the main therapeutic option; however, the disease commonly returns and displays aggressive features that result in disappointing survival rates [2]. Suitable molecular markers for early detection and effective targeted treatments for BLCA remain insufficient, underscoring the urgency of better understanding the biological pathways underlying its onset and progression.

One fundamental characteristic of cancer cells is their altered metabolism, particularly the Warburg effect, in

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which they favor the conversion of glucose to lactate via aerobic glycolysis despite the availability of oxygen [3]. This adaptation helps meet the elevated energy and building-block requirements for rapid cell division [4]. Far from being a simple waste product, accumulated lactate contributes to greater invasiveness, evasion of immune surveillance, and reduced treatment effectiveness [5, 6]. Furthermore, lactate serves as a precursor for specific post-translational modifications (PTMs) that link metabolic status to cellular signaling and gene regulation [7].

Lysine lactylation is a relatively recent PTM in which lactate is attached to lysine. This modification on histones can function as an epigenetic signal that activates transcription [8]. In various cancers, histone lactylation supports pro-tumor gene programs. As an example, higher levels of histone H3 lysine 18 lactylation in ocular melanoma increase expression of the m6A reader protein YTHDF2. This leads to breakdown of protective mRNAs including PER1 and TP53, ultimately speeding up tumor development [9]. Although BLCA tumors frequently exhibit strong glycolytic activity, the contribution of lactylation in this disease has received limited attention. One study reported that circXRN2 can slow BLCA growth by reducing H3K18 lactylation [10]. In addition to histones, lactylation of non-histone proteins is increasingly recognized as a meaningful regulatory mechanism, but its full implications remain under investigation.

Given BLCA's intense reliance on glycolysis and the resulting lactate-rich environment [11], we suspected that lactylation of non-histone proteins could be crucial for disease progression. Lactyl-proteomic screening revealed heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) as one of the most significantly lactylated proteins in BLCA. HNRNPA1 is a common RNA-binding protein involved in managing alternative splicing and other aspects of RNA biology [12, 13]. It participates in diverse processes that encourage cancerous changes [14, 15]. Various PTMs can adjust the metabolic influence of HNRNP proteins. For instance, removal of acetyl groups from HNRNPA1 lysines by SIRT1 and SIRT6 in liver cancer restricts glycolysis [16]. In lung adenocarcinoma, ESCO2-driven acetylation of HNRNPA1 promotes metabolic alterations that benefit tumor growth [17]. The specific function of HNRNPA1 in BLCA-related metabolism had not been clarified before this investigation.

Our work demonstrates that lactylation at lysine 350 (K350) of HNRNPA1 is elevated in BLCA patient samples. Disrupting HNRNPA1 through knockout or introducing the K350R mutation significantly weakened the cancer-promoting traits and aerobic glycolysis of BLCA cells in laboratory and animal studies. P300 was identified as the enzyme responsible for installing this lactyl modification. At the molecular level, this K350 lactylation promotes tumor growth and metabolic rewiring by regulating pyruvate kinase M2 (PKM2) expression.

Overall, the findings reveal an underappreciated contribution of non-histone protein lactylation to BLCA. Breaking the self-reinforcing cycle of glycolysis, HNRNPA1-K350 lactylation, and PKM2 upregulation appears to be a valuable therapeutic target for addressing metabolic dependencies in this cancer type.

Materials and Methods

Clinical samples

Tumor tissues from the bladder and nearby non-cancerous bladder tissues were gathered from individuals who received radical cystectomy or transurethral resection of bladder tumor (TURBT) procedures at our medical center from September 2019 to December 2022. Pathological review by two experienced pathologists confirmed all samples as urothelial carcinoma. Every participant provided informed consent, and the research protocol was reviewed and approved by the Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University. Tissue microarrays were assembled for immunohistochemistry.

Cell lines and culture

The BLCA cell lines 5637, RT4, EJ-1, T24, 253J, and UM-UC-3, along with the immortalized normal urothelial SV-HUC-1 line and HEK-293T cells, were acquired from the Chinese Academy of Sciences in Shanghai, China. BLCA cells and HEK-293T cells were grown in RPMI-1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS; ExCell Biotech, China). SV-HUC-1 cells were cultured in F-12K medium (Gibco) containing 10% FBS. All cell cultures were maintained at 37 °C under 5% CO₂ in a humidified atmosphere. Transfection procedures were initiated once cells achieved 30–50% confluency.

HNRNPA1 knockout and overexpression

HNRNPA1 was knocked out in EJ-1 and UM-UC-3 cell lines using the CRISPR/Cas9 system. A specific single-guide RNA (sgRNA) directed against HNRNPA1 (sequence: 5'-GCGTTCCCCATTGCTCAAAA-3') was incorporated into the lentiCRISPRv2 plasmid (Abm, Canada). Lentiviruses were generated in HEK-293T cells and applied to infect the target BLCA cells, after which puromycin was used for selection. Individual clones were isolated and examined by Western blot to confirm the absence of HNRNPA1 protein. For complementation experiments, the complete human HNRNPA1 coding sequence (NM_031157.4) was inserted into the pcSLenti-EF1-EGFP-P2A-Puro-CMV-MCS lentiviral vector (Obio Technology, China). Site-directed mutagenesis was performed to create the K350R variant (substituting the AAA codon with AGA), and the change was validated through sequencing. Lentiviral particles expressing either wild-type HNRNPA1 or the K350R mutant version were introduced into the knockout cells to generate stable rescue lines. Comparable expression levels across clones were verified by western blotting before proceeding with downstream assays.

Plasmid construction and siRNA transfection

Constructs designed for overexpressing His-tagged PKM2, His-tagged P300, His-tagged CBP, Flag-tagged GCN5, and Flag-tagged PCAF were obtained from Obio Technology and General Bio. YH DURO developed the Flag-tagged HNRNPA1 overexpression vector. Delivery of these plasmids and siRNAs into the various cell lines was achieved with X-tremeGENE HP DNA Transfection Reagent (Roche, Switzerland) as specified by the manufacturer's protocol.

Drug treatment

Global protein lactylation was boosted by incubating cells with sodium lactate (NaLa, catalog 867-56-1, Sigma-Aldrich) for 24 hours. To block glycolytic activity, cells were exposed to oxamate (HY-W013032A, MCE) or 2-deoxyglucose (2-DG, HY-13966, MCE) for 24 hours. P300 was stimulated by treatment with CTB (HY-134964, MCE) for 24 hours, while inhibition of P300 was performed using C646 (HY-13823, MCE) over the same timeframe. The impact of polydatin (catalog 27208-80-6, TargetMol, USA) on cell growth was assessed after 24 hours of exposure. All drug experiments included DMSO as the corresponding vehicle control. After each treatment, cell survival rates and lactylation

status were checked to verify effective dosing without overt toxicity.

CCK-8 and colony formation assays

BLCA cells were initially plated in 6 cm culture dishes, subjected to plasmid transfection or drug exposure for 24 hours, then harvested and counted for use in functional tests. In the CCK-8 viability assay, 1,000 cells were seeded per well in 96-well plates. Cell growth was monitored at 0, 24, 48, 72, 96, and 120 hours using a CCK-8 detection kit (New Cell & Molecular Biotech, China) according to the manufacturer's instructions. Absorbance readings were taken at 450 nm on a plate reader.

For IC₅₀ determination, 8,000 cells per well were placed in 96-well plates and treated with graded concentrations of the compound for 24 hours before CCK-8 analysis. Colony-forming capacity was evaluated by seeding 1,000 cells per well in 6-well plates and culturing for 7–10 days. Colonies were fixed in methanol, stained using crystal violet solution (Beyotime, China), photographed, and counted for statistical evaluation.

EdU assay

Proliferation rates were measured with an EdU incorporation kit (Ribobio, China). SV-HUC-1 and BLCA cells (3,000 cells/well) were seeded into 96-well plates and incubated overnight at 37 °C. SV-HUC-1 cells received treatment with 0.1% DMSO (vehicle control), 10 mM NaLa, or 20 mM NaLa for 24 hours. Following this, EdU labeling reagent was added for 2 hours. Cells were then fixed in methanol for 15 minutes, permeabilized with 0.4% Triton X-100 (Servicebio, China) for 10 minutes, and incubated with 100 µl of Apollo fluorescent dye for 30 minutes. Nuclear staining was performed with Hoechst for 30 minutes. Fluorescent images were obtained via an Olympus inverted microscope. The proliferation rate was expressed as the ratio of EdU-labeled (red) cells to total Hoechst-stained (blue) nuclei.

In vivo tumorigenesis assay

Four-week-old male BALB/c nude mice were purchased from SLAC Laboratory Animal Co. (Shanghai, China) and randomly divided into experimental groups (five animals per group). Each mouse was subcutaneously injected with 1×10^7 cells in 100 µl of serum-free RPMI 1640 medium into the right flank. In lactate supplementation studies, parental EJ-1 cells (1×10^7

cells/mouse) were injected, and from day 4 onward, mice received daily intraperitoneal administration of NaLa (150 mg/kg) or saline control for 14 consecutive days. For polydatin treatment, animals received 25 mg/kg polydatin or DMSO vehicle via intratumoral injection every other day for 2 weeks. Tumor dimensions were recorded with calipers every 3–4 days, and volumes were computed using the standard formula ($\text{length} \times \text{width}^2/2$). Mice were sacrificed 2 weeks post-injection, and the resulting xenograft tumors were excised for further analysis.

Wound healing assay

Roughly 8×10^5 cells were plated into 6-well plates and grown until a complete monolayer formed. A linear scratch was introduced using a sterile 1,000 μl pipette tip. Two PBS washes removed loose cells, and fresh serum-free medium was added. Wound photographs were taken at 0 and 18 hours with an Olympus inverted microscope (Japan). Wound closure was quantified by measuring the remaining open area in ImageJ.

Transwell invasion assay

Invasion potential was tested by placing 5×10^4 cells into the upper chamber of Transwell inserts (Corning, USA) pre-coated with Matrigel when necessary, using serum-free medium. The lower chamber contained medium supplemented with 10% FBS to act as a chemoattractant. After 24 hours at 37°C in 5% CO₂, non-invaded cells on the upper side were wiped off with a cotton swab. Invaded cells on the underside were fixed, stained with crystal violet for 20 minutes, and counted under a microscope.

Cellular apoptosis evaluation via flow cytometry

Cells were exposed to 0.1% DMSO (control), 20 mM oxamate, or 2-DG for 48 hours. After treatment, 5×10^5 cells were harvested, rinsed, and stained with FITC Annexin V combined with propidium iodide (PI) (catalog 40302ES, Yeason, China). Mixtures were gently vortexed and incubated in the dark at room temperature for 20 minutes. Flow cytometry analysis was conducted on a FACS instrument (Beckman Coulter, USA) following the manufacturer's guidelines. Quantitative data were obtained using FlowJo software.

Western blot analysis

Extracted proteins were separated by SDS-PAGE electrophoresis and transferred to PVDF membranes.

Membranes were blocked, then incubated overnight at 4 °C with primary antibodies of interest. After washing, HRP-linked secondary antibodies were applied for 1 hour at room temperature. Blots were washed three times for 10 minutes each in TBST buffer. Protein bands were visualized with an enhanced chemiluminescence (ECL) reagent.

Co-IP assay

HEK-293T cells, EJ-1 cells, UM-UC-3 cells, and fresh BLCA tissue samples were homogenized in 500 μl of IP lysis buffer (New Cell & Molecular Biotech, China) containing PMSF. Cleared lysates were obtained after centrifugation at $12,000 \times g$ for 10 minutes at 4°C. The supernatants were mixed with target-specific antibodies and Protein A/G agarose beads (Santa Cruz, USA), and the mixture was rotated gently overnight at 4 °C. The beads were washed 6 times, and the precipitated proteins were eluted and examined by western blotting as outlined earlier.

Site-specific antibody production

A custom rabbit polyclonal antibody recognizing lactylated HNRNPA1 at lysine 350 was produced by PTM Bio (China). Immunization was performed with a synthetic peptide corresponding to the HNRNPA1 region around K350, bearing a lactyl group. Affinity purification was performed with the lactylated peptide, followed by depletion with the unmodified peptide to ensure high specificity. The antibody's selectivity was confirmed through dot blot and western blot experiments: it reacted only with the lactylated peptide. It detected the correct HNRNPA1 band in wild-type cells but not in K350R mutant cells.

IHC

Immunohistochemical staining was performed as described in a prior report [18]. Hematoxylin (Solarbio, China) was used for nuclear counterstaining. For analysis of tissue microarrays, scores for pan-lactylation and P300 expression were generated by multiplying staining intensity by the percentage of positively stained cells.

qPCR

Cellular RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Reverse transcription to generate cDNA was carried out using a cDNA Synthesis Kit (ACCURATE BIOTECHNOLOGY (HUNAN) CO.,

LTD, China). Real-time quantitative PCR reactions were run with SYBR Green Master Mix (ACCURATE BIOTECHNOLOGY (HUNAN) CO., LTD, China) on an appropriate detection system.

Metabolite measurements

Specific quantification of important glycolytic metabolites, lactate, ATP, ADP, NADPH, and NADP⁺ was carried out through multiple reaction monitoring (MRM) using a triple quadrupole mass spectrometer. Concentrations were calculated based on standard curves prepared from pure metabolite standards. Cellular glucose uptake was evaluated using a commercial colorimetric assay kit (Abcam, UK) according to the manufacturer's instructions. Intracellular lactate content was determined using a Lactate Assay Kit (Abcam, UK). Glucose levels were measured according to the manufacturer's instructions (Boxbio Science & Technology, China). ATP concentration was assessed with an ATP Determination Kit (Beyotime Biotechnology, China). Ratios of NADP⁺/NADPH and GSH/GSSG were analyzed using the corresponding commercial kits from Beyotime Biotechnology, according to the manufacturer's instructions.

Extracellular Acidification Rate (ECAR)

The Seahorse XF Glycolysis Stress Test (Agilent Technologies) was used to determine ECAR. Briefly, 4×10^4 cells were plated into each well of XF24 plates and incubated overnight. On the assay day, cells were washed and equilibrated in Seahorse assay medium. ECAR readings were collected under basal conditions and after successive addition of glucose (10 mM), oligomycin (1 μ M), and 2-DG (50 mM). Basal glycolysis rate and overall glycolytic capacity were derived according to the manufacturer's guidelines.

Single-cell sequencing and Gene Set Enrichment Analysis (GSEA)

Single-cell RNA sequencing data originating from eight BLCA specimens and three normal bladder samples were downloaded from the Genome Sequence Archive (GSA, <https://ngdc.cnbc.ac.cn/gsa/>) with accession code HRA000212 [19]. Data processing, including integration, normalization, and cell clustering, was executed with the Seurat package (v4.0). Tumor and normal urothelial cell populations were distinguished via unsupervised clustering and characteristic marker genes. Lactylation-related genes (LRGs) encompassed enzymes

that add or remove lactyl groups (writers/erasers) as well as proteins involved in lactate generation and transport [20]. GSEA was performed to assess enrichment differences in glycolysis, pyruvate metabolism, and lactic acid metabolism pathways between cell subsets. Relevant gene sets were obtained from Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and WikiPathways resources.

Receptor-based virtual screening for HNRNPA1 inhibitors

A receptor-driven virtual screening was conducted to identify candidate compounds that bind HNRNPA1 and interfere with its RNA-binding function. Roughly 2,300 FDA-approved drugs were retrieved from the DrugBank database and prepared for docking studies by removing salts. The three-dimensional structure of HNRNPA1 (PDB ID: 4YOE) was acquired from the RCSB PDB website (<https://www.rcsb.org/structure/4YOE>). Water molecules were removed, and hydrogens were added before it was designated as the docking receptor. Docking simulations were run using the GPU version of AutoDock4 [21], employing the Lamarckian genetic algorithm and its empirical free-energy scoring function. The RNA-binding site on HNRNPA1 was defined as the key target region for screening [22]. Flexible docking was applied, permitting movement of both the ligand and nearby residues within a 5 Å radius of the pocket center. Each molecule was docked through 50 independent genetic algorithm runs, and the best-scoring pose was kept. Final ranking of compounds was based on their docking scores.

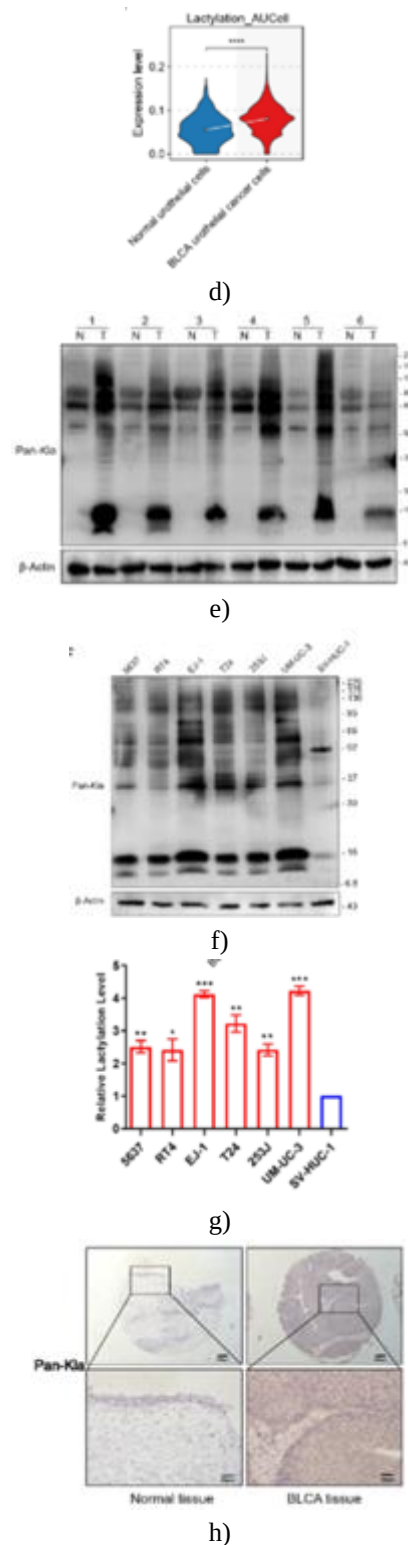
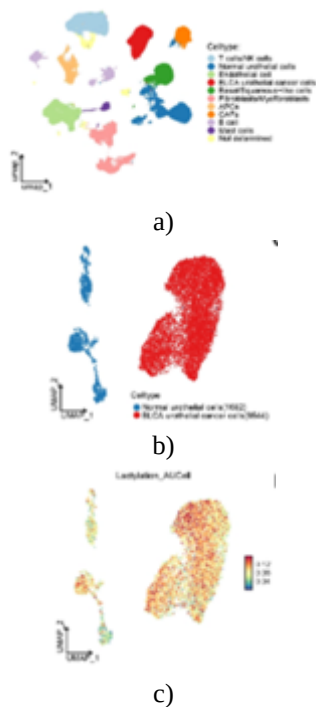
Statistical analysis

Quantitative results are shown as mean $\pm$ standard deviation (mean $\pm$ SD) in all figures. Statistical tests were carried out using GraphPad Prism version 8. Two-tailed Student's t-test analyzed two-group comparisons. For multiple group comparisons, one-way ANOVA was utilized. Relationships between pan-Kla levels and various clinicopathological variables were tested using the Chi-square test or Fisher's exact test, as appropriate. Overall survival was evaluated by Kaplan–Meier curves, and differences were compared using the log-rank test. Statistical significance was defined as $P < 0.05$.

Results and Discussion

Global lactylation is elevated in bladder cancer and correlates with poor prognosis

To investigate the cellular makeup and metabolic features of BLCA, spectral clustering was applied in an unsupervised manner to all BLCA cells (**Figure 1a**). Levels of lactylation-associated genes were clearly upregulated in the urothelial cancer cells of BLCA when compared with normal urothelial cells (**Figures 1b–1d**). Additionally, GSEA indicated strong enrichment of multiple metabolism-related pathways in BLCA cell populations, such as glycolysis, the glycolytic process, pyruvate metabolism, and monocarboxylic acid metabolism. These observations indicate a metabolic shift in BLCA cells toward increased glycolysis and lactate production. Western blot experiments demonstrated markedly higher overall protein lactylation in tumor tissues from BLCA patients than in paired non-cancerous adjacent tissues (**Figure 1e**). In line with this, cultured BLCA cell lines displayed elevated basal lactylation compared to the normal urothelial SV-HUC-1 line (**Figure 1f**), consistent with the characteristic glycolytic preference of cancer cells. Quantitative analysis of band intensities verified that lactylation was significantly increased in every BLCA cell line tested relative to SV-HUC-1 (**Figure 1g**).



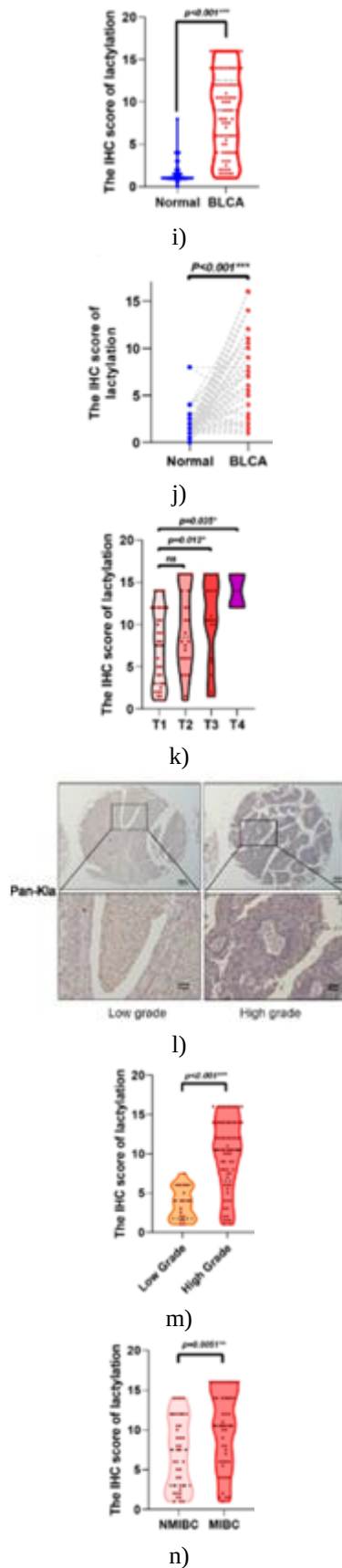


Figure 1. Lactylation level was increased in bladder cancer and associated with poor survival in patients with bladder cancer

UMAP plot from single-cell RNA sequencing data illustrating the classification of different cell types (a). (b, c) UMAP representations displaying the expression distribution of genes connected to lactylation in normal urothelial cells compared to bladder cancer urothelial cells. (d) Quantitative comparison of lactylation-related gene expression levels. (e) Immunoblotting results showing total lactylation abundance in six matched pairs of normal bladder tissue (N) and tumor tissue (T). (f) Immunoblot analysis of baseline lactylation levels in the normal urothelial SV-HUC-1 cell line versus several BLCA cell lines. (g) Bar graph of densitometric quantification of global lactylation (normalized to β -Actin) with statistical comparison between SV-HUC-1 and BLCA cells. (h) Immunohistochemical detection of lactylation in normal and cancerous bladder tissues. Scale bar: 200 μ m (top panels), 50 μ m (bottom panels). (i, j) Quantification and statistical evaluation of lactylation IHC scores in normal versus tumor tissues (unpaired t-test in i; paired t-test in j). (k) Lactylation intensity across AJCC stages T1–T4. (l) IHC images comparing lactylation staining in low-grade and high-grade bladder tumors. Scale bar: 200 μ m (top), 50 μ m (bottom). (m) Statistical comparison of lactylation scores between low- and high-grade tumors (unpaired t-test). (n) Comparison of lactylation levels in non-muscle-invasive versus muscle-invasive bladder cancer specimens (unpaired t-test). (o) Kaplan–Meier plots depicting overall survival differences between patients with low versus high lactylation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

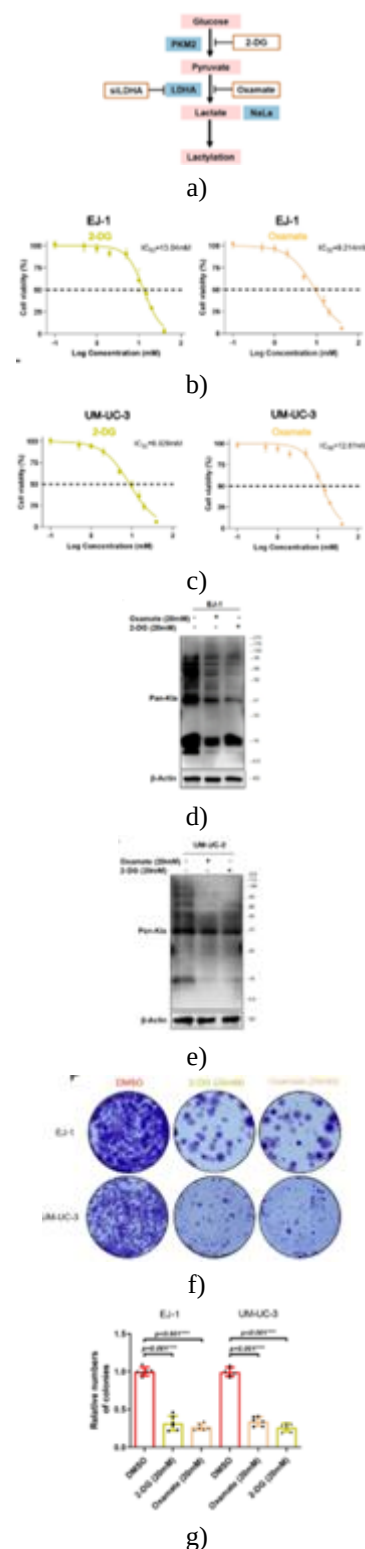
Tissue localization of lactylation was assessed through immunohistochemistry targeting lactylated lysine in clinical bladder cancer samples. Strong nuclear and cytoplasmic staining was prominent in tumor regions, while normal urothelial areas displayed only faint signals

(Figure 1h). Both unpaired and paired statistical tests showed markedly higher IHC scores for lactylation in cancerous tissues compared with adjacent normal tissues (Figures 1i and 1j); ($P < 0.001$). When stratified by disease progression, lactylation increased with advancing tumor stage (Figure 1k), was elevated in high-grade compared to low-grade malignancies (Figures 1l-1m), and was higher in muscle-invasive cases than in non-muscle-invasive cases (Figure 1n). These patterns associate heightened lactylation with more aggressive tumor biology. Survival analysis using the Kaplan–Meier method revealed that individuals with high lactylation had significantly reduced overall survival compared with those with low lactylation (Figure 1o). In summary, bladder cancer exhibits upregulated protein lactylation that correlates with increased tumor grade, later stage, and inferior patient prognosis.

Lactate-driven protein lactylation promotes bladder cancer cell proliferation and tumor growth

We then examined whether the rise in lactylation actively supports tumorigenic properties in BLCA. Pharmacological and genetic methods were used to manipulate lactate production and the consequent protein lactylation (Figure 2a). Glycolysis was first suppressed using inhibitors in EJ-1 and UM-UC-3 cells. Treatment with 2-deoxyglucose (2-DG), which interferes with glucose utilization, and oxamate, which blocks lactate dehydrogenase activity, was performed. Effects on cell viability and IC_{50} values were evaluated by CCK-8 assays (Figures 2b and 2c). Immunoblotting demonstrated that both inhibitors (20 mM, 24 h) caused a pronounced drop in global lactylation (Figures 2d and 2e). Extended exposure to 2-DG or oxamate also strongly suppressed colony formation ability (Figures 2f-2g). Inhibition further increased programmed cell death.

A parallel genetic intervention involved knockdown of lactate dehydrogenase A (LDHA), the enzyme primarily responsible for lactate synthesis from pyruvate. Reducing LDHA expression via siRNA lowered lactylation in EJ-1 and UM-UC-3 cells (Figures 2h and 2i). This reduction led to decreased proliferative capacity as shown by CCK-8 growth curves (Figure 2j). LDHA silencing also impaired cell migration and invasion, reinforcing the link between reduced lactylation and attenuated malignant phenotypes in bladder cancer cells.



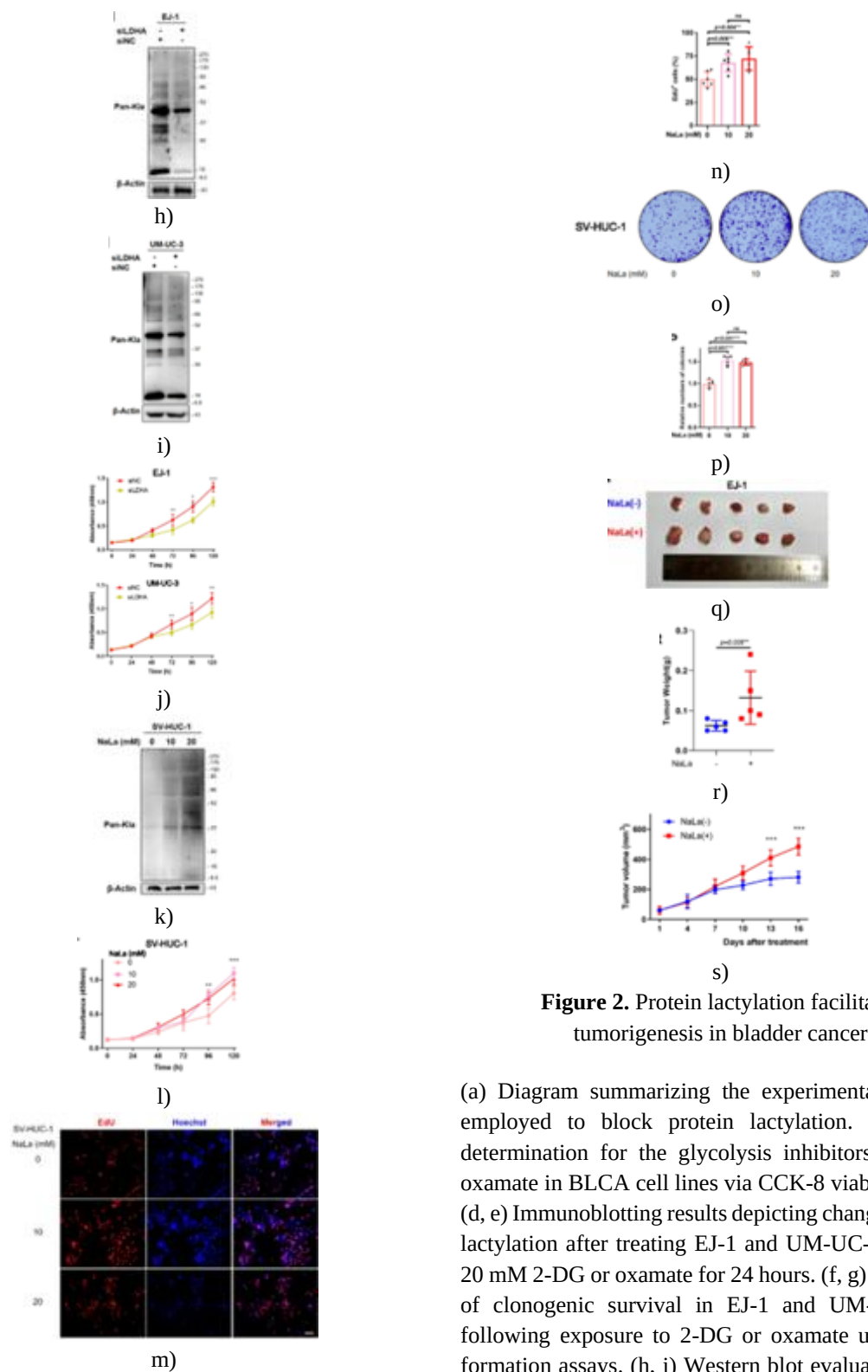


Figure 2. Protein lactylation facilitates tumorigenesis in bladder cancer

(a) Diagram summarizing the experimental strategies employed to block protein lactylation. (b, c) IC₅₀ determination for the glycolysis inhibitors 2-DG and oxamate in BLCA cell lines via CCK-8 viability testing. (d, e) Immunoblotting results depicting changes in global lactylation after treating EJ-1 and UM-UC-3 cells with 20 mM 2-DG or oxamate for 24 hours. (f, g) Assessment of clonogenic survival in EJ-1 and UM-UC-3 cells following exposure to 2-DG or oxamate using colony formation assays. (h, i) Western blot evaluation of total lactylation levels in EJ-1 and UM-UC-3 cells with LDHA knockdown. (j) Growth kinetics of EJ-1 and UM-UC-3 cells following LDHA depletion as measured by CCK-8 assay. (k) Western blot analysis of lactylation

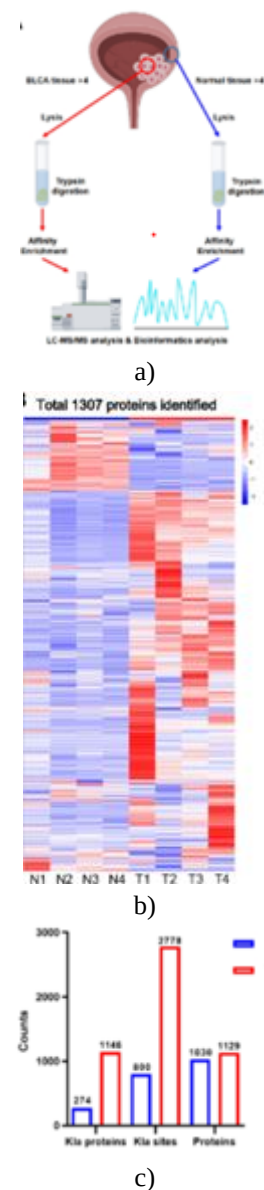
status in SV-HUC-1 cells incubated with or without sodium lactate (NaLa) for 24 hours. (l) Impact of NaLa supplementation on SV-HUC-1 cell proliferation determined by CCK-8 assay. (m-p) Evaluation of proliferative capacity in NaLa-exposed SV-HUC-1 cells through EdU staining and colony formation experiments. (q, r) Subcutaneous EJ-1 cell xenografts in nude mice treated daily with intraperitoneal injections of NaLa (150 mg/kg) or control vehicle for 14 days. Tumors were collected on day 16. Representative images of excised tumors and quantification of tumor weights (unpaired two-tailed Student's t-test, $n = 5$). (s) Longitudinal monitoring of tumor volume changes throughout the study period. Data expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

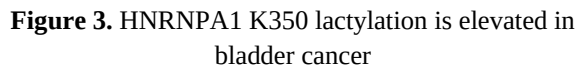
We further investigated whether elevating lactylation could confer proliferative advantages to non-cancerous cells. Normal SV-HUC-1 urothelial cells were supplemented with sodium lactate (NaLa) at 10 mM and 20 mM concentrations. Exogenous NaLa increased overall protein lactylation (**Figure 2k**) and markedly enhanced cell growth rates (**Figure 2l**). Treated cells also produced significantly more colonies and showed greater EdU uptake, demonstrating heightened DNA synthesis activity (**Figures 2m–2p**). These findings indicate that raising lactylation in normal urothelial cells can promote a proliferative phenotype resembling that of tumor cells. In vivo validation was achieved by supplementing lactate in a mouse xenograft model. Animals receiving NaLa developed significantly larger tumors than controls. Endpoint tumor weights were elevated in the lactate-treated cohort (**Figures 2q and 2r**), while growth curves revealed accelerated volume increase upon NaLa administration. Taken together, the data establish that protein lactylation exerts a direct pro-tumorigenic effect in bladder cancer both in cultured cells and in living animals.

HNRNPA1 K350 lactylation is elevated in bladder cancer tissues

With evidence supporting the involvement of lactylation in BLCA progression, attention shifted to identifying the principal modified proteins and their associated mechanisms. Label-free quantitative proteomics was utilized to map lactylated proteins in tumor versus normal bladder tissues. Four matched pairs of BLCA tumors and adjacent non-tumor tissues were processed for LC-MS/MS following selective enrichment of lactyl-lysine peptides (**Figure 3a**). The analysis yielded a total

of 1,307 identified proteins (**Figure 3b**). Normal tissues contained 274 lactylated proteins with 800 modification sites, whereas tumor samples showed a dramatic expansion to 1,146 lactylated proteins and 2,778 sites (**Figure 3c**). A total of 217 proteins (**Figures 3d and 3e**) and 503 sites (**Figure 3f**) were shared between normal and cancerous groups. Bioinformatic enrichment of proteins with differential lactylation revealed prominent links to glucose metabolic processes and monosaccharide breakdown pathways.





(a) Workflow diagram outlining the LC-MS/MS proteomic profiling and subsequent bioinformatics analysis performed on bladder cancer tissues (T) and matched adjacent normal tissues (N) (n = 4). (b) Heatmap displaying the expression of 1,307 detected

proteins across individual biological replicates from N and T groups. (c) Bar chart summarizing the total number of identified proteins, lactylated proteins, and lactylation sites from the mass spectrometry data. (d) Heatmap of the 217 overlapping lactylated proteins present in both N and T samples, shown for each replicate. (e) Venn diagram illustrating the overlap and unique lactylated proteins between normal and tumor groups. (f) Venn diagram depicting shared and group-specific lactylation sites. (g) Identification of 15 significantly altered lactylation sites among the 503 shared sites using thresholds of fold change > 1.5 and $P < 0.05$. These 15 sites are ranked by fold change. (h, i) Immunoprecipitation from two independent BLCA tissue samples using anti-Pan K1a antibody or control IgG, followed by immunoblotting for HNRNPA1. (j, k) Reciprocal immunoprecipitation from two independent BLCA samples using anti-HNRNPA1 antibody or control IgG, followed by detection of Pan K1a. (l) Dot blot validation of the specificity of the anti-HNRNPA1-K350 lactylation antibody. (m, n) Immunoprecipitation with anti-HNRNPA1 antibody from two independent BLCA tissues, followed by detection of K350 lactyl-lysine. (o, p) Immunoprecipitation of HNRNPA1 from EJ-1 and UM-UC-3 cell lysates followed by immunoblotting for HNRNPA1-K350 lactyl lysine.

We specifically searched for lactylation sites showing substantial upregulation in tumors. Applying criteria of > 1.5 -fold increase in modification level (tumor versus normal) and statistical significance ($P < 0.05$), 15 sites were found to be significantly enriched in BLCA (**Figure 3g**). Notably, lysine 350 in HNRNPA1 ranked among the top sites, showing a strong elevation in lactylation in cancerous tissues compared with normal tissues. This positioned HNRNPA1-K350 as a promising functionally important modification in bladder cancer (**Figure 3g**).

To confirm this modification in clinical specimens, immunoprecipitation experiments validated HNRNPA1 lactylation in BLCA tissues, aligning with the proteomics results (**Figures 3h–3k**). A custom site-specific antibody directed against lactylated K350 on HNRNPA1 was generated and validated (**Figure 3l**). This antibody reliably detected K350 lactylation in patient-derived BLCA samples (**Figures 3m and 3n**). Using CRISPR/Cas9, HNRNPA1 was knocked out in EJ-1 and UM-UC-3 cells to create a clean background. These knockout cells were then reconstituted with either wild-type HNRNPA1 (WT) or the K350R mutant, which

is unable to undergo lactylation at this site. As anticipated, sodium lactate (NaLa) treatment did not increase lactylation on the K350R mutant, while glycolysis inhibition with oxamate or 2-DG decreased K350 lactylation on HNRNPA1 (**Figures 3o and 3p**). Moreover, the K350 residue and flanking sequence are highly conserved across species including human, mouse, cow, and rat, underscoring its potential importance and justifying focused investigation.

HNRNPA1 functions as a versatile RNA-binding protein that participates in pre-mRNA splicing, RNA stability control, and translational regulation. Its emergence as a major lactylation target prompted us to explore how this modification might alter its regulatory functions in cancer-related pathways. Subsequent studies therefore centered on determining the impact of HNRNPA1-K350 lactylation on cellular behavior and metabolic reprogramming in BLCA.

HNRNPA1-K350 lactylation promotes BLCA progression

To determine the biological significance of HNRNPA1 K350 lactylation, we generated HNRNPA1 knockout BLCA cell lines using CRISPR-Cas9 technology. Loss of HNRNPA1 led to pronounced decreases in cell viability and colony-forming potential. HNRNPA1 depletion also strongly reduced the migration and invasion abilities of BLCA cells relative to control cells. Immunoblotting with the K350-specific lactylation antibody confirmed strong modification in cells expressing wild-type HNRNPA1. At the same time, no signal was observed in cells expressing the K350R mutant (**Figure 4a**). Both CCK-8 and EdU proliferation assays revealed significantly lower growth rates in K350R-expressing cells compared to WT (**Figures 4b–4d**). Colony formation experiments showed that WT HNRNPA1 cells produced abundant large colonies, whereas K350R cells formed far fewer and smaller ones (**Figure 4e**). Similar patterns emerged in motility assays. Wound-healing experiments demonstrated faster wound closure in cells with WT HNRNPA1 than in those with the K350R mutant, indicating superior migration (**Figure 4f**). Transwell invasion assays through Matrigel further confirmed that WT cells had greater invasive capacity than K350R cells (**Figure 4g**). These results demonstrate that HNRNPA1 drives proliferation, migration, and invasion in BLCA, with these oncogenic activities being dependent on lactylation at lysine 350. In xenograft models, both complete HNRNPA1 knockout

and the K350R mutation resulted in markedly suppressed tumor growth, reflected by reductions in tumor volume and weight (Figures 4h–4j).

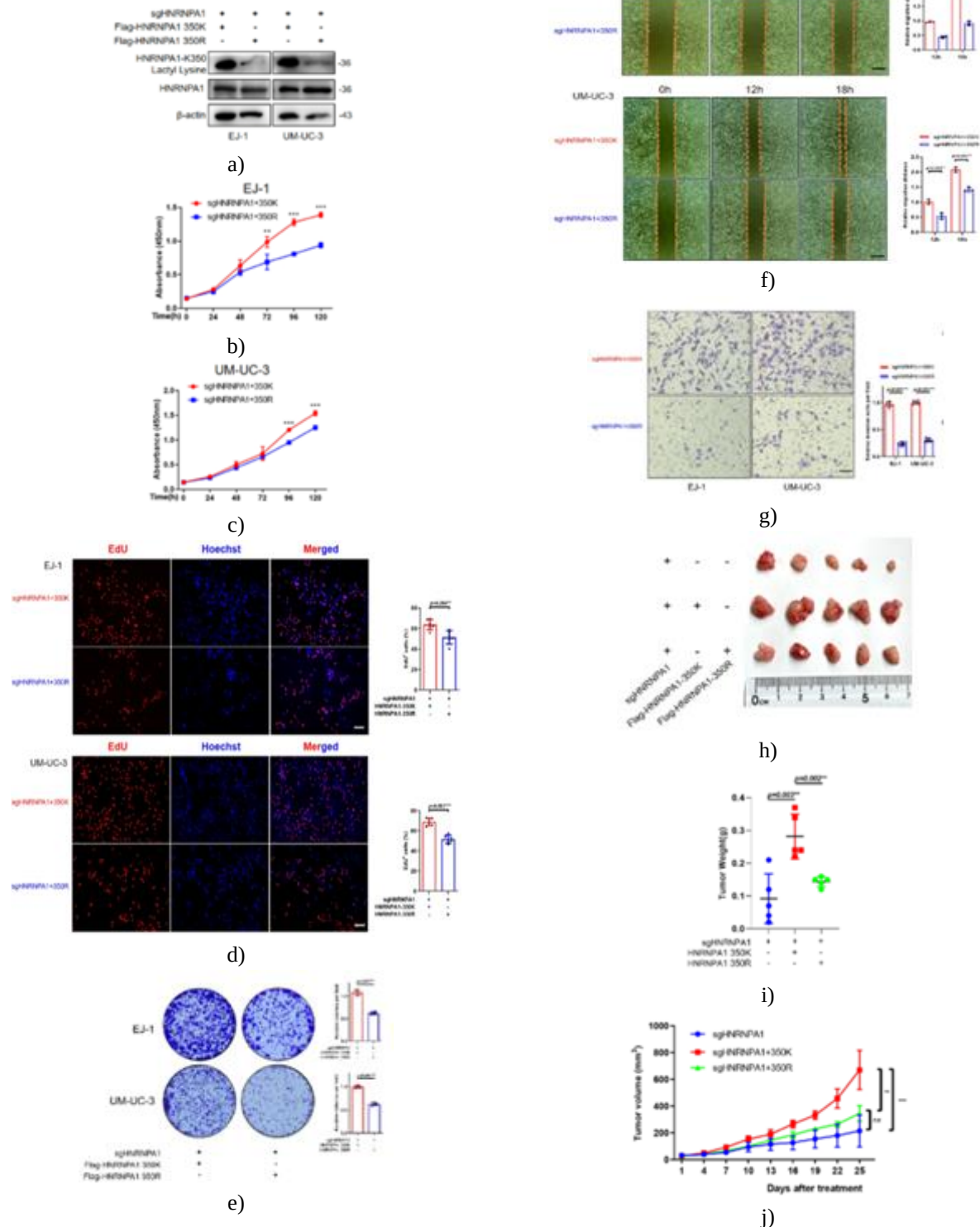


Figure 4. HNRPA1-K350 lactylation promotes bladder cancer progression.:

(a) Western blot detection of HNRNPA1 modified by lactylation at K350 in EJ-1 and UM-UC-3 cell lines. (b-e) Analysis of proliferative activity and growth rates in EJ-1 and UM-UC-3 cells when HNRNPA1-K350 lactylation was blocked, assessed through CCK-8 assays (b, c), EdU labeling (d), and colony formation tests (e). (f) Examination of migratory behavior in EJ-1 and UM-UC-3 cells with suppressed HNRNPA1-K350 lactylation using wound closure assays (scale bar: 200 μ m). (g) Measurement of invasive potential in EJ-1 and UM-UC-3 cells after inhibition of HNRNPA1-K350 lactylation via Transwell chamber assays (scale bar: 20 μ m). (h, i) Subcutaneous implantation of EJ-1 cells into nude mice, with tumors excised on day 25. Representative photographs of harvested tumors and quantification of tumor masses. Data analyzed by one-way ANOVA ($n = 5$). (j) Changes in tumor volume recorded throughout the study. Results expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

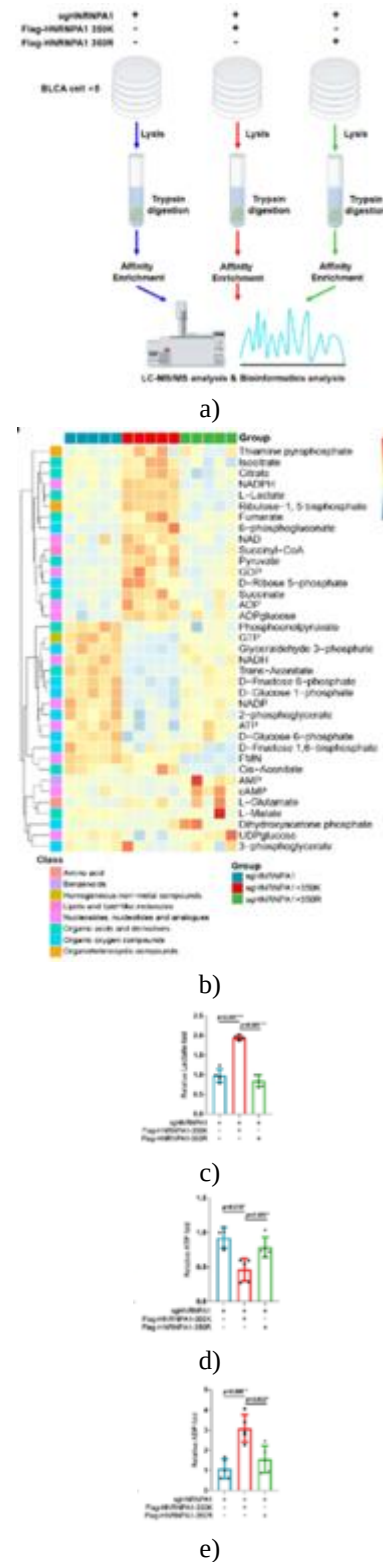
In summary, these experiments demonstrate that the cancer-driving capabilities of HNRNPA1 in bladder cancer are heavily dependent on its lactylation at lysine 350. Elimination of this modification severely impairs the protein's ability to enhance cell proliferation, motility, invasiveness, and overall tumor growth. This identifies HNRNPA1-K350 lactylation as a key factor underlying the aggressive traits of BLCA cells.

HNRNPA1-K350 lactylation enhances aerobic glycolysis in BLCA cells

Given that HNRNPA1 lactylation appeared to drive tumor growth, we next explored the mechanisms underlying this effect. Because HNRNPA1 is known to regulate RNA splicing and gene expression, and given the close relationship between lactate production and this modification, we suspected that lactylation at K350 might rewire cellular energy metabolism, favoring aerobic glycolysis over mitochondrial respiration. We therefore compared metabolic signatures in BLCA cells depending on the lactylation status of HNRNPA1.

Targeted metabolomic profiling was conducted on EJ-1 cells in three conditions: complete HNRNPA1 knockout, rescue with wild-type HNRNPA1, and rescue with the non-lactylatable K350R mutant. All groups were grown under identical conditions. Polar metabolites were extracted and analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Figure 5a). Unsupervised clustering of the resulting metabolite data revealed clear separation among the

groups, with cells expressing wild-type HNRNPA1 displaying a distinct metabolic profile characteristic of increased glycolytic flux (Figure 5b).



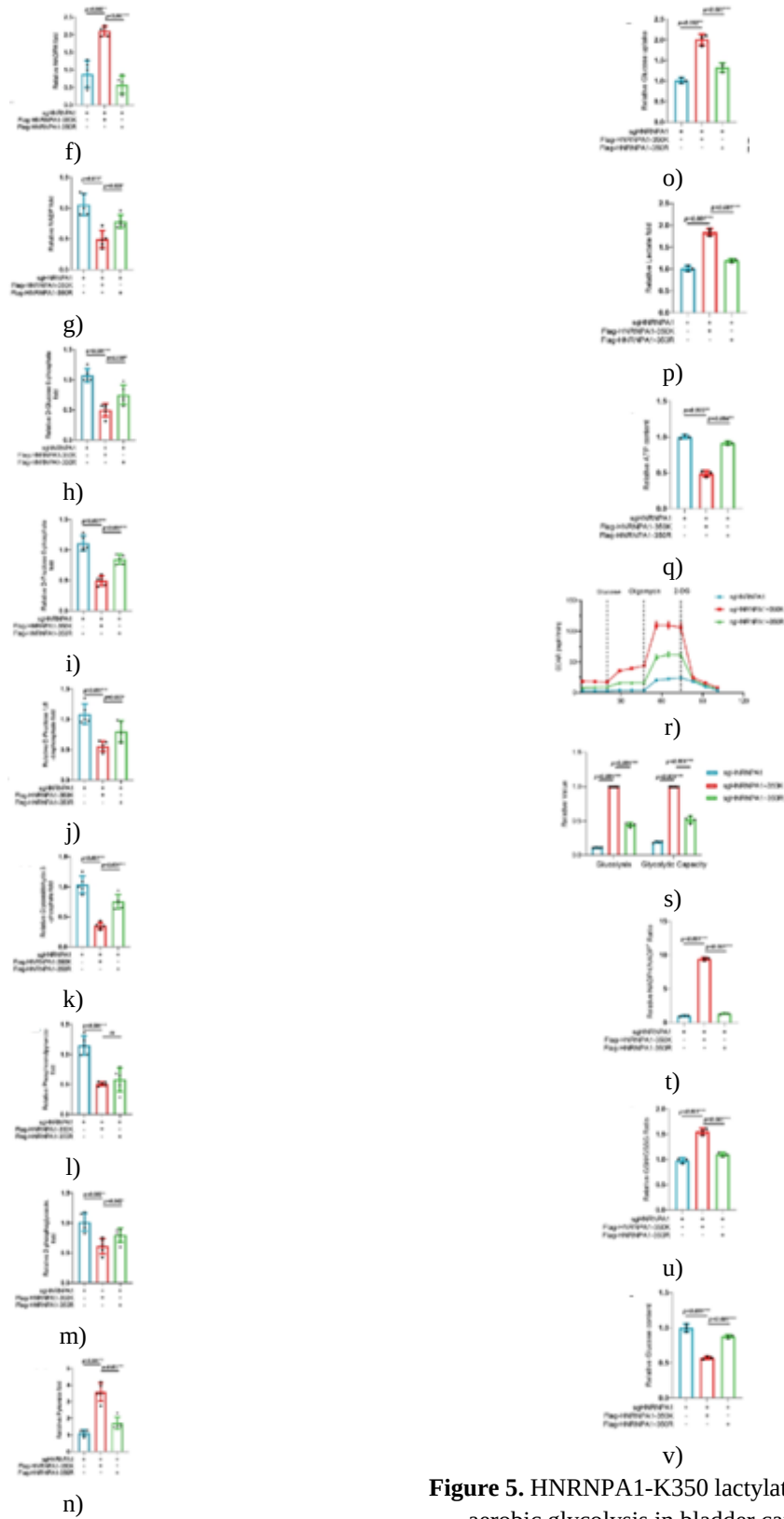


Figure 5. HNRNPA1-K350 lactylation enhances aerobic glycolysis in bladder cancer cells

(a) Schematic overview of the targeted LC–MS/MS metabolomics workflow and bioinformatics processing applied to three groups of BLCA cells (five replicate plates per group). (b) Heatmap displaying variation in metabolite abundance across the different experimental groups. (c–n) Relative abundance of specific metabolites quantified by LC–MS/MS in the three cell groups. (o, p) Measurement of glucose consumption and lactate secretion. (q) Cellular ATP levels determined using a commercial ATP assay kit in EJ-1 cells. (r, s) Analysis of extracellular acidification rate (ECAR) to evaluate basal glycolysis and maximum glycolytic capacity. (t) NADPH/NADP⁺ ratio assay. (u) GSH/GSSG ratio assay. (v) Intracellular glucose content measurement. All quantitative data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Quantitative LC–MS/MS analysis showed that, compared with the knockout group, cells rescued with wild-type HNRNPA1 had higher lactate concentrations (**Figure 5c**), lower ATP levels, and increased ADP (**Figures 5d and 5e**). The WT rescue group also displayed elevated NADPH (**Figure 5f**) and reduced NADP⁺ (**Figure 5g**). Levels of several glycolytic intermediates such as glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, glyceraldehyde-3-phosphate, phosphoenolpyruvate, and 2-phosphoglycerate were decreased (**Figures 5h–5m**), while pyruvate was increased (**Figure 5n**). Importantly, the metabolic profile of the K350R rescue group closely resembled that of the knockout cells, indicating that lactylation specifically at K350 is required for these metabolic shifts.

Functional validation of glycolytic activity was then performed. Glucose uptake assays revealed that HNRNPA1-WT cells consumed substantially more glucose than K350R or knockout cells (**Figure 5o**). Correspondingly, lactate release into the culture medium was significantly higher in WT cells and markedly reduced in the K350R and KO groups (**Figure 5p**). These observations corroborate the metabolomics findings and confirm that HNRNPA1 lactylation augments glycolytic flux.

Cellular ATP measurements indicated higher ATP concentrations in HNRNPA1-KO and K350R cells compared to WT cells (**Figure 5q**). Although this may seem unexpected, it is consistent with the lower ATP yield per glucose molecule in glycolysis-dominant metabolism. The elevated ATP levels in cells lacking K350 lactylation suggest a greater reliance on oxidative

phosphorylation in its absence. Supporting this interpretation, extracellular acidification rate (ECAR) measurements showed significantly higher basal glycolysis and glycolytic capacity in HNRNPA1-WT cells than in K350R or knockout cells (**Figures 5r and 5s**).

Redox balance was further evaluated by measuring the NADPH/NADP⁺ and GSH/GSSG ratios. HNRNPA1-WT cells exhibited elevated NADPH/NADP⁺ and GSH/GSSG ratios compared to K350R and KO cells (**Figures 5t and 5u**). This indicates that K350 lactylation improves the cells' antioxidant defenses and supports biosynthetic needs through increased reducing power. In contrast, cells without this modification showed lower ratios of reduced to oxidized forms, potentially increasing vulnerability to oxidative stress.

Intracellular glucose levels were also quantified as an indirect measure of consumption. HNRNPA1-WT cells maintained the lowest steady-state glucose concentrations, while K350R and knockout cells had significantly higher intracellular glucose (**Figure 5v**). This pattern aligns with more rapid glucose utilization in cells expressing lactylatable HNRNPA1.

Overall, these findings demonstrate that HNRNPA1-K350 lactylation drives metabolic reprogramming toward intensified aerobic glycolysis in bladder cancer cells. This metabolic shift provides a mechanistic explanation for the tumor-promoting effects of the modification.

P300 functions as the acyltransferase for HNRNPA1-K350 Lactylation

Given the central importance of HNRNPA1-K350 lactylation in both tumor progression and metabolic changes in BLCA, we aimed to identify the enzyme that catalyzes this modification. Among several potential lysine acyltransferases, including P300, CBP, GCN5, and PCAF [8, 23–25], P300 was of particular interest due to its previously reported role in histone lactylation. Overexpression experiments revealed that only P300 significantly increased HNRNPA1 lactylation levels. Co-immunoprecipitation assays performed in both BLCA cells and HEK-293T cells confirmed a physical interaction between P300 and HNRNPA1 (**Figure 6a**). Ectopic expression of P300 enhanced HNRNPA1 lactylation, but this increase was not observed with the K350R mutant, confirming site specificity.

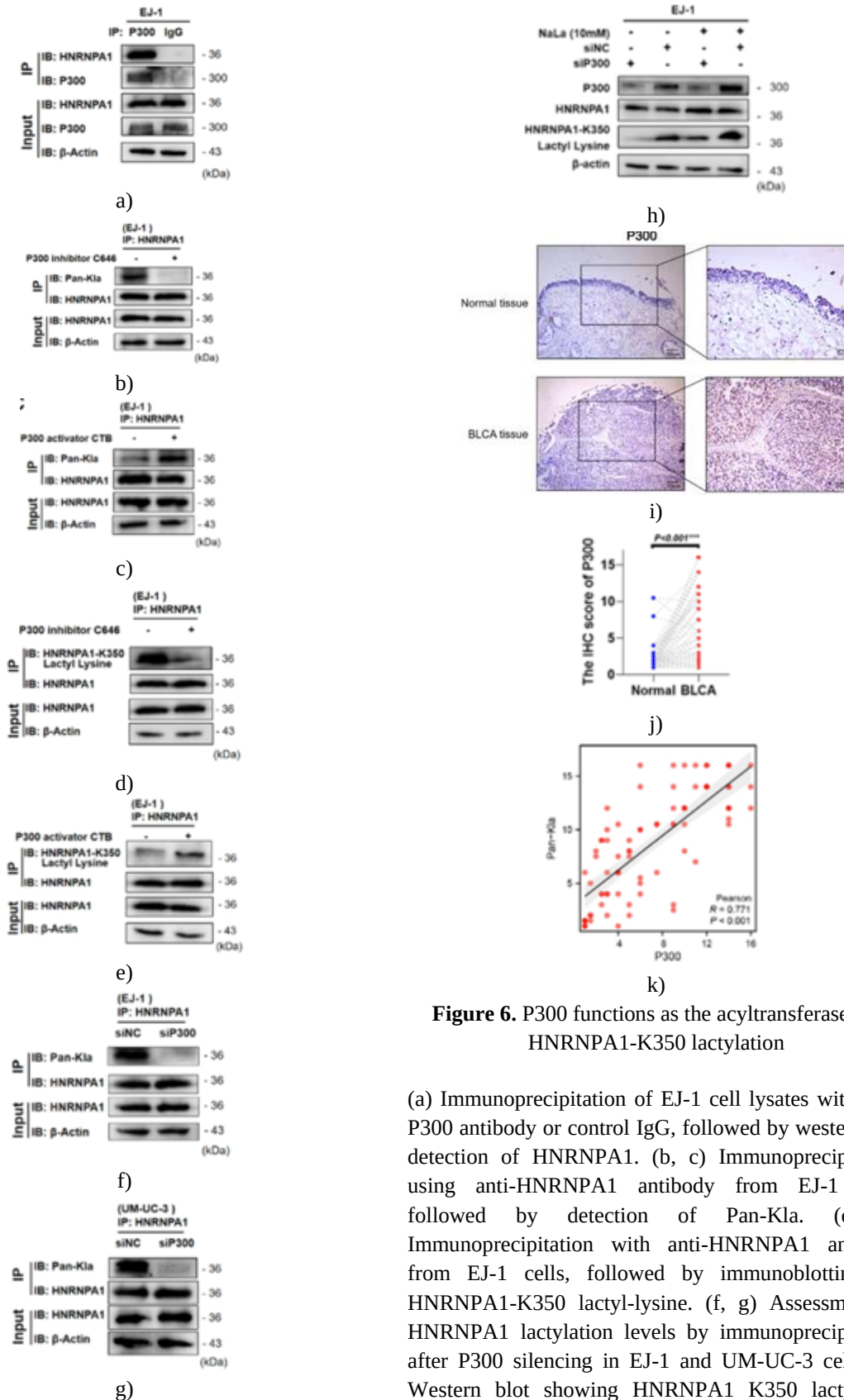


Figure 6. P300 functions as the acyltransferase for HNRNPA1-K350 lactylation

(a) Immunoprecipitation of EJ-1 cell lysates with anti-P300 antibody or control IgG, followed by western blot detection of HNRNPA1. (b, c) Immunoprecipitation using anti-HNRNPA1 antibody from EJ-1 cells, followed by detection of Pan-Kla. (d, e) Immunoprecipitation with anti-HNRNPA1 antibody from EJ-1 cells, followed by immunoblotting for HNRNPA1-K350 lactyl-lysine. (f, g) Assessment of HNRNPA1 lactylation levels by immunoprecipitation after P300 silencing in EJ-1 and UM-UC-3 cells. (h) Western blot showing HNRNPA1 K350 lactylation

under conditions of NaLa treatment combined with P300 knockdown. (i) Immunohistochemical staining of P300 expression in normal and tumor bladder tissues. Scale bar: 100 μ m (left panels), 50 μ m (right panels). (j) Statistical comparison of P300 IHC scores between normal and tumor samples using a paired t-test. (k) Pearson correlation analysis between Pan-Kla levels and P300 expression based on IHC scores. Each point represents an individual sample. *** $P < 0.001$.

We further manipulated P300 activity using pharmacological agents. Treatment of EJ-1 cells with a P300 inhibitor resulted in a clear reduction of HNRNPA1-K350 lactylation, while application of a P300 activator increased this modification (**Figures 6b–6e**). Consistent with these findings, siRNA-mediated depletion of P300 caused a substantial drop in HNRNPA1 lactylation (**Figures 6f and 6g**). Western blotting confirmed that P300 knockdown strongly suppressed HNRNPA1 lactylation, even in the presence of NaLa (**Figure 6h**). These data establish that P300 is an essential mediator of lactate-induced lactylation of HNRNPA1.

If P300-driven HNRNPA1 lactylation contributes to BLCA malignancy, then altering P300 levels should affect cancer cell behaviors. Indeed, knockdown of P300 in EJ-1 and UM-UC-3 cells reduced cell proliferation. Wound healing and Transwell assays also showed impaired migration and invasion upon P300 silencing. These phenotypic changes closely resemble those seen when HNRNPA1-K350 lactylation is prevented, suggesting that many of P300's tumor-promoting effects operate through this modification.

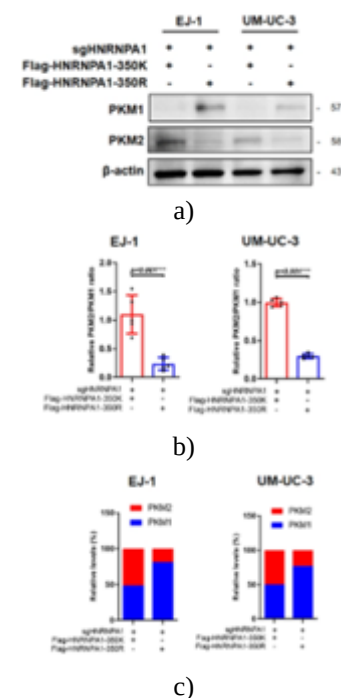
Examination of clinical specimens revealed higher P300 protein levels in bladder cancer tissues compared to adjacent normal tissues by IHC (**Figures 6i–6j**). Moreover, a strong positive correlation existed between Pan-Kla levels and P300 expression ($r = 0.771$, $P < 0.001$), (**Figure 6k**). Collectively, these results identify P300 as the primary “writer” enzyme responsible for installing the lactyl group at K350 on HNRNPA1 in BLCA. Through this activity, P300 likely links metabolic signals to downstream changes in RNA splicing and cellular metabolism.

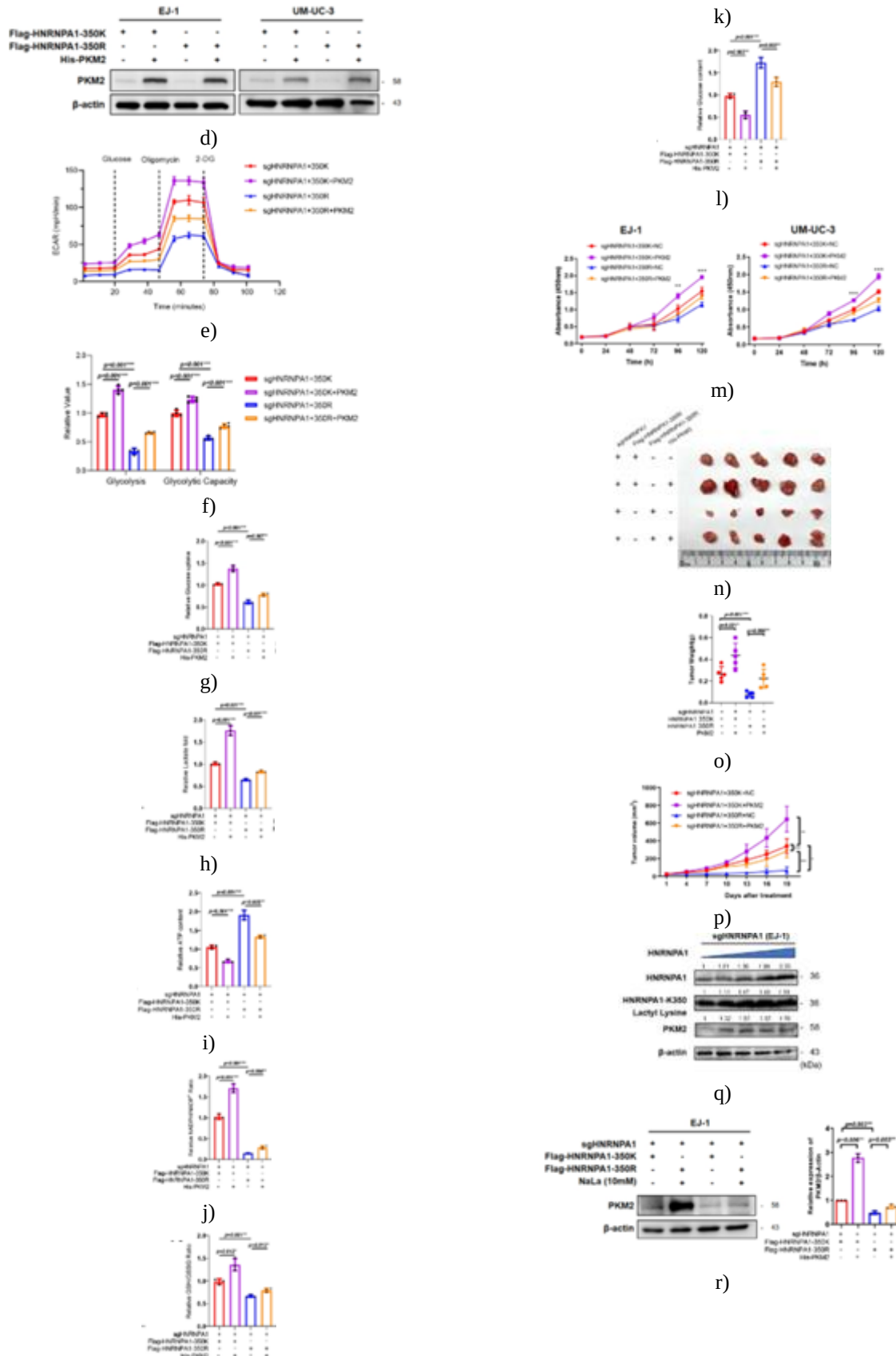
HNRNPA1-K350 lactylation drives PKM2 splicing to promote aerobic glycolysis and tumor aggressiveness

In mammalian cells, the choice of pyruvate kinase isoform is a major determinant of whether metabolism favors aerobic glycolysis or oxidative phosphorylation.

Tumor cells typically upregulate the embryonic isoform PKM2, which supports the Warburg effect, whereas the PKM1 isoform is associated with more efficient oxidative metabolism [26]. The two isoforms are produced through mutually exclusive alternative splicing of the same pre-mRNA, with HNRNP proteins known to promote inclusion of exon 10, thereby favoring PKM2 production [27]. Although increased PKM2 expression has been documented in bladder cancer [28], the specific contribution of HNRNPA1 and its post-translational modifications to this process has not been established. We therefore examined whether HNRNPA1-K350 lactylation influences PKM splicing and thereby contributes to the glycolytic phenotype and aggressiveness of BLCA.

Western blot and qPCR analyses showed that re-expression of wild-type HNRNPA1 increased both PKM2 protein and mRNA levels, while decreasing PKM1 levels. In contrast, the K350R mutant shifted the balance toward higher PKM1 and lower PKM2 expression (**Figures 7a and 7b**). Quantification of the PKM2/PKM1 ratio confirmed that cells expressing lactylatable wild-type HNRNPA1 had a significantly higher proportion of PKM2 (**Figure 7c**). These results indicate that lactylation at K350 on HNRNPA1 promotes alternative splicing that favors the PKM2 isoform.





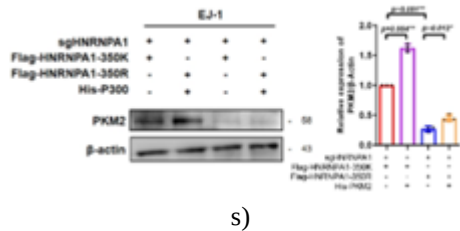


Figure 7. HNRNPA1-K350 lactylation mediates aerobic glycolysis and promotes the proliferation of bladder cancer cells via PKM splicing

(a) Western blot detection of PKM1 and PKM2 protein levels in bladder cancer cells. (b, c) qPCR analysis of the PKM2/PKM1 ratio and individual expression levels of PKM2 and PKM1. (d) Confirmation of PKM2 overexpression efficiency by Western blot in BLCA cells. (e, f) Measurement of basal glycolysis and glycolytic capacity using extracellular acidification rate (ECAR) assays. (g, h) Quantification of glucose uptake and lactate production. (i) Cellular ATP levels measured by ATP assay kit. (j) Assessment of NADPH/NADP⁺ ratio. (k) Evaluation of GSH/GSSG ratio. (l) Determination of intracellular glucose content. (m) Analysis of cell proliferation using CCK-8 assay. (n, o) Subcutaneous injection of EJ-1 cells into nude mice. Tumors were collected on day 19. Representative tumor images and tumor weight quantification. Statistical analysis performed with one-way ANOVA (n = 5). (p) Tumor volume growth curves. (q-s) Western blot detection of PKM2 expression under conditions of varying HNRNPA1 levels (q), NaLa treatment (r), or P300 knockdown (s). All data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

To establish a direct causal link between the splicing alteration and metabolic reprogramming, we tested whether restoring PKM2 levels could reverse the glycolytic defects caused by loss of HNRNPA1 lactylation. PKM2 was overexpressed in HNRNPA1-K350R cells (**Figure 7d**). ECAR analysis revealed that K350R cells exhibited reduced basal glycolysis and glycolytic reserve, but forced PKM2 expression restored these parameters to levels comparable to wild-type cells (**Figures 7e and 7f**). This rescue indicates that the suppression of aerobic glycolysis resulting from blocked HNRNPA1 lactylation is primarily mediated by reduced PKM2.

Likewise, the decreases in glucose uptake and lactate secretion seen in K350R cells were reversed by PKM2 overexpression (**Figures 7g and 7h**). The higher ATP

levels observed in K350R cells (reflecting greater dependence on oxidative phosphorylation) returned to lower levels upon PKM2 restoration (**Figure 7i**). Additionally, the reduced NADPH/NADP⁺ and GSH/GSSG ratios in K350R cells were normalized by PKM2 re-expression (**Figures 7j and 7k**). Intracellular glucose accumulation in K350R cells was also lowered following PKM2 overexpression, consistent with resumed high-rate glycolysis (**Figure 7l**). These metabolic rescue experiments confirm that PKM2 acts as a major downstream mediator of HNRNPA1-K350 lactylation's effects on cellular energy metabolism.

Functional validation was extended to cell behavior and tumor growth. In HNRNPA1-K350R cells, ectopic PKM2 expression significantly enhanced proliferation as measured by a CCK-8 assay (**Figure 7m**) and partially rescued in vivo tumor growth (**Figures 7n–7p**). Wound healing and Matrigel invasion assays further showed that PKM2 overexpression counteracted the migration and invasion defects caused by the K350R mutation. These results connect the oncogenic advantages conferred by HNRNPA1 lactylation to its regulation of PKM2-dependent metabolic reprogramming.

Dose-dependent effects were examined by introducing increasing amounts of HNRNPA1 expression plasmid into HNRNPA1-knockout EJ-1 cells. Higher HNRNPA1 levels led to corresponding increases in K350 lactylation and progressive upregulation of PKM2 protein, demonstrating that both HNRNPA1 abundance and its lactylation status control PKM2 expression (**Figure 7q**). Treatment with NaLa or P300 overexpression elevated PKM2 in cells expressing wild-type HNRNPA1 but not in K350R mutant cells (**Figures 7r and 7s**). Conversely, glycolysis inhibition (using 2-DG or oxamate) or LDHA knockdown reduced PKM2 levels, supporting the idea that lactate produced through glycolysis feeds back to sustain PKM2 expression via HNRNPA1 lactylation. Taken together, these findings reveal that HNRNPA1-K350 lactylation promotes alternative splicing of PKM pre-mRNA in favor of the PKM2 isoform. This shift enhances aerobic glycolysis and drives the aggressive behavior of bladder cancer cells.

Small-molecule drug inhibits the lactylation and biological activity of HNRNPA1 lactylation in bladder cancer

Since HNRNPA1 lactylation promotes PKM2 splicing and accelerates BLCA progression, it represents a potential therapeutic vulnerability. We therefore

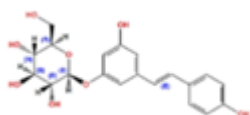
performed structure-based screening to identify compounds that bind the RNA-binding pocket of HNRNPA1. Ten candidates with favorable binding energies were selected (**Figure 8a**), with polydatin showing the strongest predicted affinity. The chemical structure of polydatin is illustrated in **Figure 8b**. Molecular docking indicated that polydatin interacts with key amino acid residues within the active site pocket of HNRNPA1 (**Figure 8c**).

Functional testing demonstrated that polydatin inhibited BLCA cell proliferation in a dose-dependent manner, with an IC_{50} value of 25.06 μ M (**Figure 8d**). Importantly, polydatin treatment blocked HNRNPA1 K350 lactylation. Under conditions of exogenous lactate supplementation, polydatin also suppressed colony formation by EJ-1 cells (**Figure 8f**). In vivo studies showed that polydatin significantly restrained tumor growth (**Figures 8g and 8i**). Immunohistochemical staining for PKM2 further revealed that polydatin prevented the PKM2 upregulation normally induced by HNRNPA1 lactylation (**Figure 8j**), confirming its ability to disrupt this pathway. These results support polydatin as a promising small-molecule inhibitor targeting HNRNPA1 in bladder cancer.

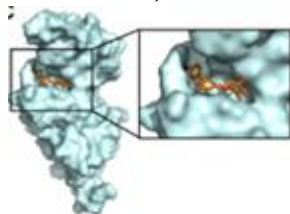
Compound list

ID	Affinity score (kcal/mol)
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DB14879	-15.45
DB09827	-15.44
DB13345	-15.39
DB13947	-15.37
DB00115	-15.26
DB09038	-15.07
DB11630	-15.07
DB01177	-15.06
DB09949	-15.04

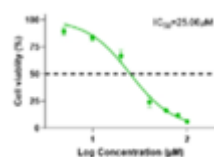
a)



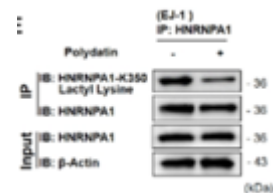
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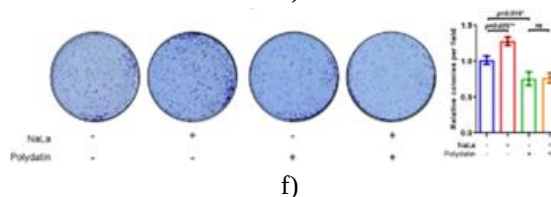
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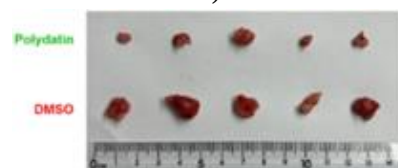
d)



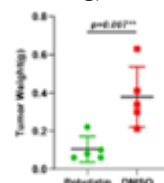
e)



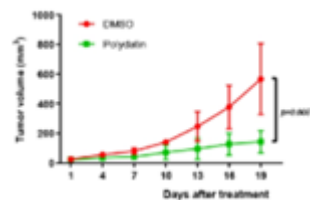
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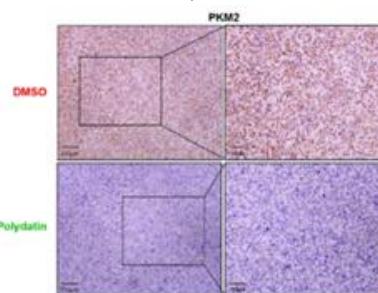
g)



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i)



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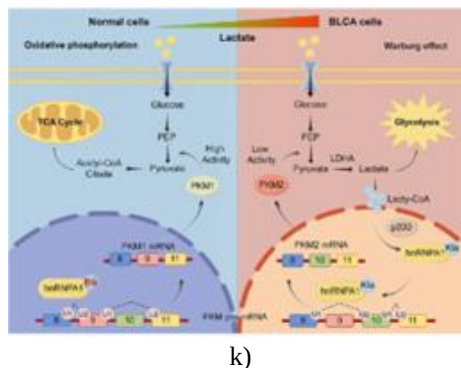


Figure 8. Polydatin inhibits HNRNPA1 lactylation and its biological activity in bladder cancer

(a) Ranking of binding free energies for ten candidate compounds docked into the HNRNPA1 active site pocket. (b) Chemical structure of polydatin. (c) Molecular docking model showing polydatin positioned within the HNRNPA1 binding pocket. (d) Determination of polydatin IC_{50} in EJ-1 cells by CCK-8 assay. (e) Immunoprecipitation of EJ-1 cell lysates with anti-HNRNPA1 antibody followed by detection of K350 lactyl-lysine. (f) Colony formation assay evaluating the proliferative ability of EJ-1 cells after 24-hour treatment with 30 μ M polydatin alone or in combination with 20 mM NaLa. (g, h) Representative images of excised tumors and quantification of tumor weights in nude mice following subcutaneous EJ-1 cell injection. Tumors were collected on day 19. Statistical analysis performed using unpaired two-tailed Student's *t*-test ($n = 5$). (i) Serial tumor volume measurements during the *in vivo* experiment. (j) Immunohistochemical staining of PKM2 expression in xenograft tumor tissues. Scale bars: 100 μ m (left), 50 μ m (right). (k) Diagram summarizing the proposed mechanistic pathway involving HNRNPA1 lactylation. * $P < 0.05$, ** $P < 0.01$.

Metabolic reprogramming is a well-established hallmark of cancer that provides malignant cells with the energy and biosynthetic precursors needed for uncontrolled expansion [29]. Aerobic glycolysis, commonly known as the Warburg effect, is especially prominent in bladder cancer and many other malignancies. This metabolic preference generates large amounts of lactate, which was historically regarded simply as a byproduct. However, recent research has shown that lactate exerts active biological functions, including acidification of the tumor microenvironment that aids invasion and immune suppression, as well as serving as a substrate for post-translational modifications [30]. The present study

extends this concept by demonstrating that lactate promotes protein lactylation, which, in turn, reprograms gene regulation and supports malignant progression in BLCA.

Elevated protein lactylation in bladder cancer tissues correlated strongly with adverse clinical features such as higher histological grade, more advanced stage, and poorer patient survival, indicating that lactylation may function as a marker of disease aggressiveness. This observation is consistent with findings in other tumor types where increased lactylation is linked to worse outcomes [31]. Importantly, our functional experiments demonstrate that lactylation is not just a passive marker but an active driver of tumorigenesis in BLCA. Suppressing lactate production or lactylation markedly impaired cancer cell growth and invasiveness, whereas elevating lactylation accelerated proliferation — even in non-transformed cells. Treatment of SV-HUC-1 cells with either 10 mM or 20 mM NaLa produced similar increases in global lactylation and comparable proliferative stimulation. This suggests that while lactate-induced lactylation promotes cell division, its effects reach a plateau in non-malignant cells, possibly due to saturation of lactylation signaling, transporter capacity, or negative feedback mechanisms within cellular metabolism.

Unbiased lactyl-proteomics identified HNRNPA1 as a major lactylated target in BLCA. HNRNPA1 is an extensively studied RNA-binding protein that regulates alternative splicing and mRNA metabolism. Our data reveal that the tumor-promoting functions of HNRNPA1 in bladder cancer depend not only on its abundance but critically on its modification by lactylation at lysine 350. This finding illustrates how metabolic conditions (as reflected in lactate abundance) can directly modulate HNRNPA1 splicing activity.

A crucial splicing event in cancer metabolism concerns the mutually exclusive production of PKM2 or PKM1 isoforms from the PKM transcript [32]. PKM2 predominates in proliferating and embryonic cells and supports aerobic glycolysis, while PKM1 is expressed in differentiated tissues and favors oxidative phosphorylation. HNRNP family members are known to repress exon 9 inclusion, thereby promoting PKM2 expression [27]. Our work extends this model by demonstrating that lactylation at K350 enhances HNRNPA1's ability to drive PKM2 splicing in BLCA. When this lactylation site is mutated, splicing shifts toward PKM1, reducing glycolytic activity and slowing

tumor growth. This mechanism creates a positive feedback circuit in which high lactate levels in the tumor microenvironment reinforce the Warburg effect by increasing PKM2, which, in turn, generates more lactate (**Figure 8k**).

P300 is a highly versatile acyltransferase implicated in numerous physiological and disease processes. It serves as a writer for various acyl modifications including acetylation, succinylation, and β -hydroxybutyrylation [24, 33, 34]. Our results indicate that P300 can also utilize lactyl-CoA to attach lactyl groups to non-histone targets such as HNRNPA1 at K350. Pharmacologic and genetic modulation of P300 confirmed its essential role in this process. While previous efforts to target P300 in cancer have mainly focused on its histone acetyltransferase activity, our study provides additional justification for P300 inhibition, as it would also disrupt lactylation-dependent oncogenic pathways.

Although lactylation was first described on histones in immune cells [8], it is now evident that this modification affects many non-histone proteins and influences diverse cellular functions. Examples include regulation of inflammatory responses in sepsis [35] and modulation of cardiac fibrosis [36]. In oncology, lactylation of metabolic enzymes, chromatin regulators, and oncoproteins has been reported [37, 38]. Our discovery that HNRNPA1 — an RNA-binding protein — undergoes lactylation adds a new layer, showing that this PTM can directly control RNA splicing and thereby link metabolism to transcriptomic reprogramming.

From a translational standpoint, virtual screening of approved drugs against the HNRNPA1 RNA-binding pocket identified polydatin as the top candidate with the strongest predicted binding. Polydatin, a natural stilbenoid glucoside from *Polygonum cuspidatum* and a precursor to resveratrol, possesses known anti-inflammatory, antioxidant, and anti-cancer activities [39, 40]. In this study, polydatin effectively inhibited HNRNPA1 K350 lactylation, reduced cell proliferation and colony formation, and suppressed tumor growth in vivo. It also prevented the downstream upregulation of PKM2, supporting its potential as a targeted agent against the HNRNPA1 lactylation axis. Further mechanistic studies are warranted to fully elucidate its mode of action.

Conclusion

Although our investigation primarily addressed cell-autonomous effects of lactylation within cancer cells, particularly the HNRNPA1–PKM2 pathway, this modification likely also influences the broader tumor microenvironment. Lactate-driven lactylation has been shown to promote M2 macrophage polarization, impair cytotoxic T-cell function, and alter cytokine profiles, thereby aiding immune escape [30, 41, 42]. Because our in vivo models used immunodeficient nude mice, the impact on tumor-immune interactions could not be fully assessed. Future work employing syngeneic or humanized models will be important to clarify how targeting lactylation affects the interplay between cancer cells and the immune system. In conclusion, this study provides important new insights into metabolic and epigenetic regulation in bladder cancer. We show that protein lactylation levels correlate with tumor aggressiveness and poor clinical outcomes, positioning it as a potential prognostic indicator. Mechanistically, lactate in the tumor milieu drives HNRNPA1-K350 lactylation, which alters PKM splicing to favor the oncogenic PKM2 isoform and sustains aerobic glycolysis. A small-molecule compound (polydatin) binds HNRNPA1, blocks its lactylation, reduces PKM2 expression, and inhibits tumor progression. These discoveries reveal a self-reinforcing glycolysis–HNRNPA1-K350 lactylation–PKM2 axis that fuels BLCA development and identify this pathway as an attractive target for novel therapeutic interventions.

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