

Lactylation of RHOA at Oncogenic Hotspots Enhances Oncogenic Activity and Stabilizes the Protein

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Abstract

Uncontrolled RHOA signaling promotes cancer progression, but the mechanisms governing its activation beyond direct gene alterations remain poorly understood. Lactylation, a metabolic modification derived from lactate, connects altered cellular metabolism to cancer development. However, whether this modification can imitate the effects of cancer-causing mutations has not been examined. The present research explores lactylation of RHOA at key mutation-prone sites and its function as a reversible “epi-mutation” mechanism. RHOA lactylation sites were detected through mass spectrometry using a pan-anti-lactyllysine (Kla) antibody. Site-specific lactylation was introduced via an orthogonal Mb-Pyl Kla-RS/Pyl-tRNA system to add lactyl-lysine at positions K118 and K162 in recombinant RHOA; incorporation was confirmed by immunoblotting and fluorescence methods. Molecular dynamics simulations (using AlphaFold 3 and BIOVIA DS) evaluated impacts on GTPase function and hydrogen-bonding patterns. ROCK2-RBD pull-down assays and GTPase activity tests measured RHOA activation status. Protein ubiquitination and stability were analyzed with cycloheximide chase experiments and K48/K63-ubiquitin variants. In vitro lactylation and delactylation reactions with PCAF and HDAC3 established enzyme preferences. Functional effects were assessed in cell migration/invasion assays and in xenograft tumor studies. Clinical significance was assessed in breast cancer specimens and patient survival datasets. Lactylation of RHOA occurs at oncogenic mutation hotspots K118 and K162, controlled by the lactate-PCAF/HDAC3 regulatory axis. Mechanistically, lactylation at K118 leads to persistent RHOA activation by reducing its intrinsic GTPase activity, while modification at K162 enhances RHOA protein stability by blocking ubiquitination; USP9X further supports this stability by deubiquitinating RHOA. These modifications drive increased tumor cell motility, invasiveness, and metastatic spread. In clinical samples, RHOA lactylation levels are higher in breast tumors compared with nearby normal tissue. Combined inhibition of lactate generation (using the LDHA inhibitor sodium oxamate) and RHOA signaling (with the ROCK inhibitor Y-27632) effectively restrained tumor growth. Through a site-specific lactylation approach, we demonstrate that this modification replicates the functional consequences of oncogenic mutations by boosting both RHOA activity and longevity, thereby defining lactylation at mutation-sensitive residues as a dynamic, reversible “epi-mutation” pathway that mirrors the outcomes of genetic mutations. Lactylation of RHOA at K118 (promoting activation) and K162 (promoting stabilization), orchestrated by the PCAF/HDAC3 enzyme pair, sustains continuous oncogenic signaling and accelerates tumor development. Importantly, this work establishes that lactylation at mutation hotspots operates as a reversible “epi-mutation” system in which metabolic modification functionally reproduces the effects of genetic mutations. This concept blurs traditional distinctions between genetic and epigenetic drivers in cancer and highlights combined inhibition of lactylation and standard RHOA pathways as a promising therapeutic avenue.

Keywords: RHOA, Lactylation, USP9X, PCAF, HDAC3

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Introduction

The Rho family GTPase RHOA functions as a molecular switch cycling between an active GTP-bound form and an inactive GDP-bound conformation [1, 2]. This cycling is controlled by five highly conserved G-box motifs (G1–G5) that bind nucleotides [3]. Upon stimulation, RHOA engages downstream effector proteins such as ROCK,

MLC, LIMK, and cofilin, triggering pathways that reorganize the actin cytoskeleton and support cell movement, migration, and metastatic dissemination across multiple cancer types [1, 4].

Common oncogenic mutations in RHOA (e.g., at residues G17, Y42, R5, L57, C16, and E40), often observed in lymphomas and gastric adenocarcinomas, diminish its intrinsic GTPase activity and cause persistent downstream signaling [1, 2, 5]. These alterations maintain RHOA in the GTP-bound state, thereby driving tumor progression [1, 2, 4]. Nevertheless, clinical observations indicate frequent hyperactivation of wild-type RHOA (WT-RHOA) in many human malignancies [4, 6, 7], especially at the leading edges of invasive tumors and in metastatic lesions [8]. This pattern implies the existence of non-mutational mechanisms that enhance RHOA activity.

The Warburg effect, marked by elevated aerobic glycolysis and lactate buildup in tumors, provides a metabolic foundation for protein lactylation [9]. Lactylation has been recognized as an important modulator in conditions including neurodegenerative disorders, inflammatory states, and cancer [9-14]. In this investigation, we show that lactylation of RHOA at oncogenic mutation hotspots (K118 and K162), governed by PCAF-dependent addition and HDAC3-dependent removal, replicates the biological consequences of genetic mutations by causing constitutive RHOA activation and increased protein stability. Moreover, pharmacological suppression of lactate synthesis sensitizes tumors that depend on lactylated RHOA to therapies targeting mutation-mimicking pathways. These discoveries position RHOA lactylation as a reversible “epi-mutation” system and support strategies that target lactylation alongside canonical RHOA signaling to improve precision cancer treatment.

Materials and Methods

Plasmids and antibodies

The human RHOA coding sequence was PCR-amplified from SUM159 cell cDNA and inserted into the pLVX-puro vector. Human KCTD13 and LDHA sequences were similarly amplified from MDA-MB-231 cDNA and cloned into pLVX-Puro. Plasmids pCMV-3×FLAG-USP9X(human)-Neo (P56773) and pCMV-USP33(human)-3×Myc-Neo (P71806) were acquired from MiaoLingBio, China. DNA fragments encoding the Mb-Pyl KlacR-RS/Pyl-tRNA pair were synthesized by

Beijing Tsingke Biotech Co., Ltd. Antibodies targeting RHOA and MLC2 were obtained from Proteintech (cat. nos. 10749-1-AP and 10906-1-AP). Anti-FLAG antibody was from Sigma-Aldrich (cat. no. F3165). Antibodies against β -actin, Myc-tag, USP9X, and phospho-MLC2 were supplied by ABclonal (cat. nos. AC038, A9782, AE070, and AP1503). Anti-HA antibody was purchased from Abcam (cat. no. ab236632). Pan anti-Kla antibody (PTM-1401RM) was from PTM Bio Inc. Goat anti-mouse IgG H&L (Alexa Fluor 488) was from Abcam (cat. no. ab150113). Antibodies against PCAF and SIRT2 were from Abways (cat. nos. CY8354 and CY5545). Custom antibodies specific for lactylation at K118 and K162 were generated by immunizing rabbits at Hangzhou Kelian Rabbit Industry.

Cell culture

Cell lines (MDA-MB-231, SUM159, HEK293T) were sourced from the American Type Culture Collection (ATCC, Manassas, VA) and authenticated by STR profiling. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS according to ATCC guidelines. Stable cell lines overexpressing wild-type or mutant RHOA were generated by selection with 300 ng/mL puromycin for four weeks.

Intracellular expression of site-specific fully lactylated proteins

An optimized eukaryotic orthogonal system incorporating chimeric archaeal ribozymes and multiple tRNA repeats was used to improve lactyl-lysine incorporation efficiency. Cells were co-transfected with a pcDNA plasmid expressing RHOA (with TAG codons introduced at K118 or K162) and the chimeric ribozyme plasmid at a 3:1 ratio. Twelve hours post-transfection, medium was replaced and supplemented with 2 mM lactyl-lysine. After 48 hours, site-specifically lactylated RHOA was verified using custom site-specific antibodies and subjected to further analyses.

3D mass spectrometry targeting RHOA K1a

HEK293T cells stably expressing Flag-tagged RHOA were analyzed for lactylation by Shanghai Applied Protein Technology Co., Ltd. Cells were washed with ice-cold PBS and snap-frozen in liquid nitrogen. Proteins were extracted in RIPA buffer containing protease and de-lactylation inhibitors (20 mM nicotinamide). After BCA quantification, Flag-tagged RHOA was

immunoprecipitated, eluted, reduced, alkylated, and trypsin-digested. Lactylated peptides were enriched with KLa-Pan antibody beads, desalted, and analyzed by LC-MS/MS. Data were processed with MaxQuant (v2.4.9.0) and pFind Studio (v3.1.6) against the UniProt human database, applying appropriate mass tolerances, variable modifications (including +72.02113 Da for lactylation), and FDR < 1%. Sites were manually validated.

Molecular dynamics simulations

Structures of RHOA and mutants (K118Q, K118R, K118N) were generated with AlphaFold 3. Simulations were conducted in BIOVIA DS using a standard cascade protocol: minimization, heating/cooling, equilibration, and production runs [15]. RMSD and RMSF values for C α atoms and GTP-binding pocket residues were calculated, and hydrogen-bond occupancies between GTP and key residues (including K118 and K162) were quantified. Trajectories were visualized and analyzed in UCSF Chimera and DS Visualizer.

GTPase assay

Intrinsic GTP-hydrolyzing activity of both wild-type RHOA and its variants was determined using the Phosphate Assay Kit from Sigma (catalog #MAK113), following the supplier's standard procedure to track ongoing inorganic phosphate liberation. In short, 25 μ L of a 1:10 diluted sample was combined with 50 μ L of the assay reagent. After incubation for 30 minutes in the dark, the optical density was measured at 630 nm on a spectrophotometer. Standard curves for phosphate quantification were included on every 96-well plate.

RHOA activity assay

Cells were maintained until reaching 50–70% confluence across three days, rinsed with cold PBS, and disrupted in Mg²⁺-containing lysis/wash buffer (Sigma-Aldrich, #20–168). Five hundred micrograms of clarified protein lysate were diluted to 500 μ L with MLB buffer. Active GTP-bound RHOA was isolated through immunoprecipitation with Flag-M2 agarose beads, after which binding to the ROCK2-derived RBD probe was visualized via gel electrophoresis and immunoblot analysis.

In vitro lactylation assay

Purified recombinant GST-PCAF fragment (K311–381BG, Sino Biological) was mixed with Flag-tagged RHOA isolated from HEK293T cells inside a buffer system (50 mM HEPES pH 7.5, 30 mM KCl, 0.25 mM

EDTA, 5 mM MgCl₂, 2 mM DTT) that included 20 mM lactyl-CoA (MedChemExpress). The enzymatic reaction was performed at 37 °C for 1 hour and halted by adding 5 $\times$ SDS sample buffer, then heated to 100 °C for 5 minutes. The resulting samples were separated by SDS-PAGE and immunoblotted with relevant antibodies to detect lactylation.

In vitro delactylation assay

Recombinant GST-HDAC3 (H85–30G, Sino Biological) was combined with purified Flag-tagged lactylated RHOA (K118la/K162la from HEK293T cells) in a reaction mixture (50 mM HEPES pH 7.5, 1 mM EDTA, 5 mM MgCl₂, 2 mM DTT) and incubated at 37 °C for 1 hour. The reaction was stopped with 5 $\times$ SDS loading buffer and boiled at 100 °C for 5 minutes. SDS-PAGE separated proteins, blotted onto PVDF membranes, and probed using the designated antibodies.

GTP-sepharose binding assay

HEK-293T cells overexpressing wild-type, lactylated, or mutated RHOA constructs were harvested and lysed on ice in buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) plus protease inhibitors (Roche) with rotation for 1 hour at 4 °C. After high-speed centrifugation to clear the lysates (21,000 $\times$ g, 10 min, 4 °C), normalized protein quantities were incubated with 30 μ L of GTP-agarose beads (Sigma-Aldrich, G9768) for 4 hours at 4 °C under gentle agitation. Beads were rinsed three times, and captured proteins were released in 4 $\times$ SDS buffer for immunoblot detection of GTP-associated RHOA.

Immunoprecipitation and western blotting

Lysis was performed in 0.3% Nonidet P-40 buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) supplemented with a protease/phosphatase/deacetylase inhibitor mix (1 μ g/mL aprotinin, 1 mM PMSF, 1 μ g/mL pepstatin, 1 μ g/mL leupeptin, 1 mM Na₃VO₄, 30 μ M TSA, 1 mM NaF, 15 mM NAM). Insoluble material was pelleted at 13,000 rpm for 15 min at 4 °C. Supernatants were incubated with antibody-coupled agarose for 3 hours at 4 °C. Precipitates were washed three times before boiling and SDS-PAGE analysis. Protein levels were quantified by Bradford assay (Fude Biological Technology). Normalized samples were run on 12% SDS-polyacrylamide gels, transferred to PVDF membranes, blocked in 5% non-fat milk for 1 hour at room temperature, and incubated overnight at 4 °C with

primary antibodies. Following secondary antibody incubation, bands were developed with ECL reagent.

Colony formation assay

The capacity for anchorage-independent proliferation was tested using soft agar colony formation as described earlier [16]. In 24-well plates, a base layer of 0.7% agar in full medium was overlaid with a top layer of 0.35% agar containing 5×10^3 cells per well. Cultures were grown at 37 °C with 5% CO₂ for 21–28 days, receiving fresh medium twice weekly. Colonies exceeding 50 µm were examined at 40× magnification on a Nikon Eclipse Ti microscope and counted with ImageJ (NIH). Assays were conducted in triplicate and repeated three times independently. For visualization, colonies were stained with 0.005% crystal violet for 1 hour. Efficiency was computed as (colonies formed / cells plated) × 100%.

Migration and invasion assays

Transwell Boyden chamber assays (Corning, 8 µm pores) were used to measure migratory and invasive properties, as described previously [17]. Migration assays involved seeding 5×10^4 cells in serum-free medium into the upper compartment, with 10% FBS-containing medium in the lower compartment serving as attractant. For invasion, Matrigel (BD Biosciences, 1:8) was coated onto the membrane and allowed to gel for 4 hours at 37 °C. After 24 hours of incubation, cells remaining on the upper side were wiped away. Those that had traversed the membrane were fixed in 4% paraformaldehyde (15 min), stained with 0.1% crystal violet (20 min), and counted in five randomly selected fields at 100× magnification (Nikon Eclipse Ti). Each condition was run in triplicate and repeated at least 3 times independently. Statistical evaluation was performed using GraphPad Prism 9.0 with two-tailed t-tests ($p < 0.05$), with data normalized to controls.

Lung metastasis model

In vivo experiments complied with protocols approved by the Zhejiang University Institutional Animal Care and Use Committee. Female NOD-SCID mice (5–6 weeks) were randomly divided into groups ($n = 6$ each). They received tail-vein injections of 1×10^6 luciferase-tagged cancer cells (expressing either wild-type or modified RHOA) in 100 µL PBS. Weekly bioluminescence imaging was performed for 4 weeks on an IVIS Lumina III system (PerkinElmer) after a 150 mg/kg D-luciferin injection. At termination, animals were sacrificed and

lungs fixed by perfusion with 4% paraformaldehyde. Tissues were processed for paraffin embedding and H&E staining to enumerate metastatic foci or homogenized for biochemical examination. Two independent blinded pathologists scored sections using a Nikon Eclipse 80i microscope. The number of nodules and luminescence intensity were used to assess metastatic load. Data were analyzed by two-tailed t-test ($P < 0.05$) in GraphPad Prism 9 and reported as mean ± SEM across three separate experiments.

Statistical analysis

Quantitative results are shown as mean ± standard deviation or standard error of the mean, as noted. Inter-group differences were evaluated by one-way ANOVA or two-tailed Student's t-test. Associations were tested using Pearson or Spearman correlation coefficients. Patient survival was analyzed via Kaplan–Meier curves and log-rank tests. Statistical significance was defined as $P < 0.05$ throughout.

Results and Discussion

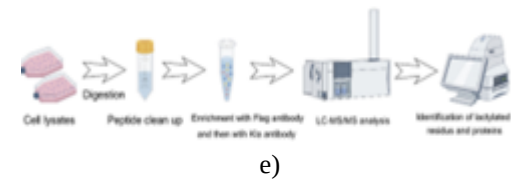
RHOA experiences lactylation at mutation hotspot residues K118 and K162

Excessive RHOA activation is linked to cancer advancement [1, 4]. Our examination revealed a clear association between abnormally high RHOA levels and reduced patient survival. Extensive literature connects lactate-mediated lactylation with malignancy [18]. We examined whether RHOA itself undergoes this modification by exposing various cell lines to increasing concentrations of glucose or lactate, which produced concentration-dependent elevations in RHOA lactylation (**Figures 1a-1d**).

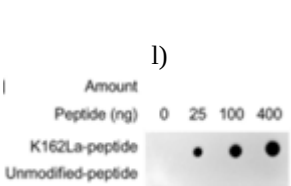
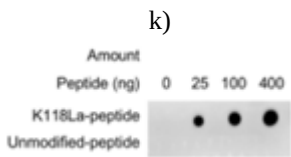
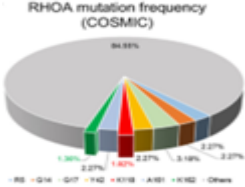
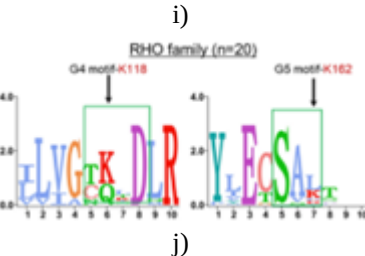
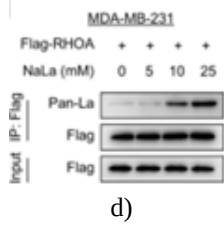
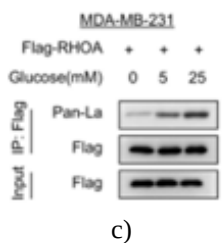
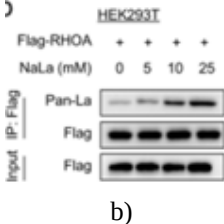
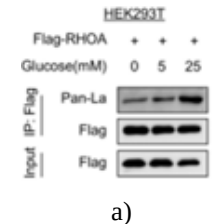
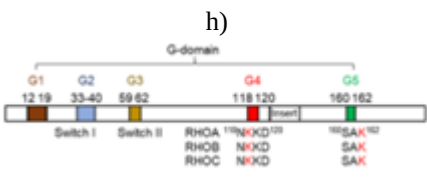
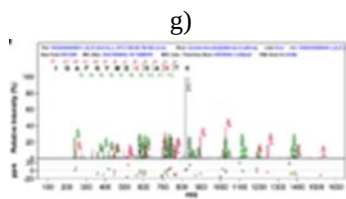
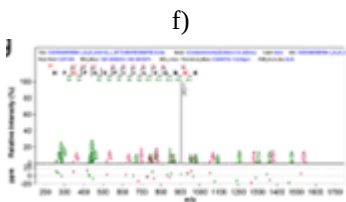
Mass spectrometry screening with a pan-Kla antibody on Flag-RHOA immunoprecipitates from HEK293T cells identified two lactylated peptides corresponding to lysines K118 and K162 (**Figures 1e-1h**). These sites lie within the functionally important G4 and G5 nucleotide-binding boxes of RHOA (**Figure 1i**). Sequence alignments confirmed strong evolutionary conservation of K118 and K162 across RHOA orthologs and the wider RAS GTPase superfamily (**Figure 1j**). Database mining via COSMIC further highlighted K118 and K162 as recurrent sites of somatic missense mutations in human cancers (**Figure 1k**).

To enable precise detection, custom antibodies were raised against the lactylated forms at each residue

(K118la and K162la). Specificity was validated by dot blot analysis, which showed strong recognition of lactylated peptides. Still, negligible binding was observed to unmodified counterparts (**Figure 1m**). Replacing either lysine with arginine substantially lowered overall lactylation signals (**Figures 1n-1q**). These data demonstrate that RHOA is lactylated at two prominent oncogenic mutation hotspots. For definitive functional studies, we applied a genetic-code-expansion strategy using an orthogonal aminoacyl-tRNA synthetase/tRNA pair to site-specifically install lactyl-lysine into RHOA at the desired positions, yielding homogeneously lactylated recombinant protein (**Figure 1r**). System validation involved transient transfection of HEK293T cells with amber-mutant RHOA constructs (K118TAG or K162TAG) plus the PylRS/PylT components, followed by culture with or without exogenous lactyl-lysine. Immunoreactive bands and EGFP fluorescence appeared only in the presence of the modified amino acid, confirming accurate and site-selective incorporation (**Figure 1s**).



Gene Names	Proteins	Lactylated sequence	Position	Score
RHOA	P61586	HFCPNVPILVGNK*K	K118	17.596849
RHOA	P61586	IGAFGYMECSAK*TK	K162	17.279511



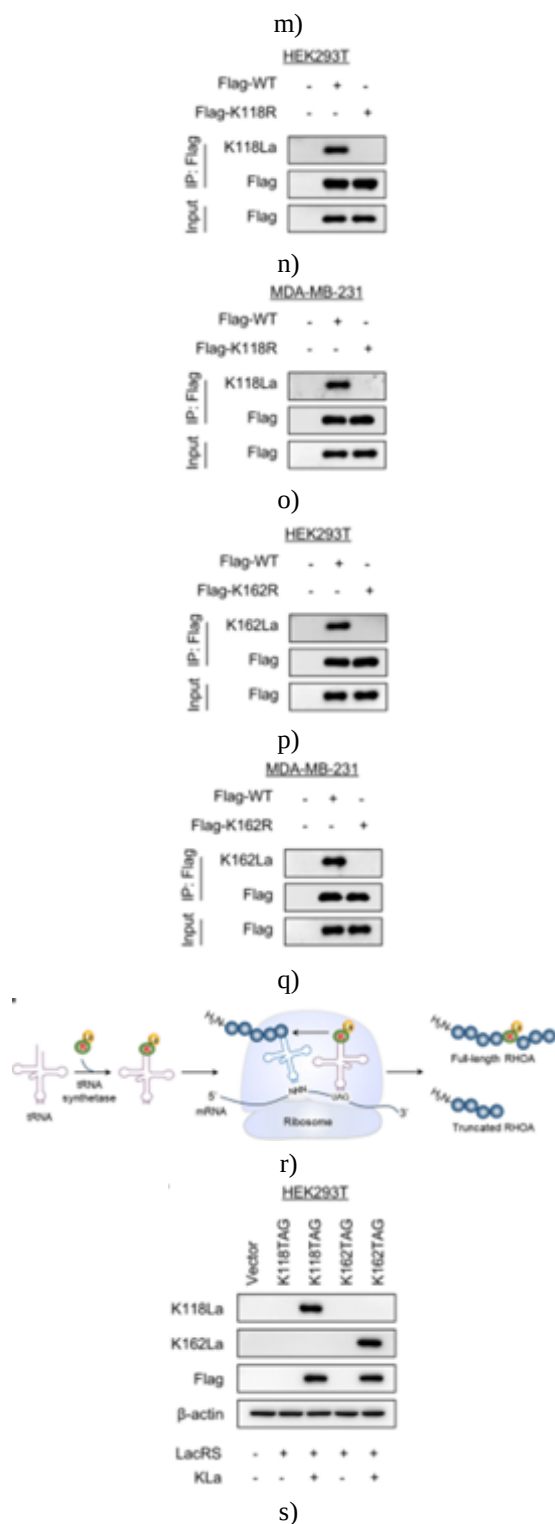


Figure 1. RHOA undergoes lactylation specifically at lysine sites K118 and K162, both of which represent common hotspots for oncogenic mutations.

Panels a–d: HEK293T cells (a, b) along with MDA-MB-231 cells (c, d) were transfected with a Flag-RHOA construct. These cells were then maintained for 24 hours under varying glucose or sodium lactate concentrations. After immunoprecipitation, KLa modification on the isolated protein was evaluated through Western blotting. Panel e illustrates the overall procedure for the 3D mass spectrometry study. Lysates from HEK293T cells stably expressing RHOA underwent processing steps as described for the 3D-MS analysis. Panel f contains a table summarizing the lactylated peptides detected via 3D mass spectrometry. Panels g and h show the MS/MS spectra of RHOA peptides modified by lactylation at K118 (g) and K162 (h).

Panel i includes a diagram of the RHOA G domain together with an alignment of the G4 and G5 motifs from various RHO family members, highlighting the conserved lysine residues in red. Panel j features an amino acid sequence logo depicting the conserved G4 and G5 motifs across the RHO family. Panel k reports the mutation frequencies observed for RHOA in human cancers, drawn from the Catalog of Somatic Mutations in Cancer (COSMIC) database; red bars correspond to K118 and green bars to K162. Panels l and m tested the specificity of antibodies directed against lactylation at K118 (l) and K162 (m) using dot blot analysis. In panels n–q, either wild-type RHOA or the K118R/K162R point mutants were introduced into HEK293T or MDA-MB-231 cells, after which lactylation status at K118 (n, o) and K162 (p, q) was determined post-immunoprecipitation. Panel r outlines the method used to produce recombinant RHOA carrying site-specific lactylation through a lactyl-lysine tRNA-based approach. Panel s confirms selective KLa incorporation at RHOA-K118TAG or K162TAG sites in HEK293T cells employing the Mb-Pyl KLa-RS/Pyl-tRNA system, with protein expression verified by Western blot.

Lactylation at K118 boosts RHOA activity via suppression of its intrinsic GTPase function

The residues K118 and K162 within RHOA exhibit strong evolutionary conservation extending from zebrafish through to humans (**Figure 2a**). To evaluate how lactylation or mutations at these positions affect RHOA function, the RHOA-binding domain (RBD) of human ROCK2 (residues 979–1047), which selectively interacts with the GTP-bound form of RHOA, was fused to both HA and GST tags. Binding capacity under different conditions was subsequently examined using

co-immunoprecipitation and pull-down methods. Results indicated that site-specific lactylation introduced at K118—but not at K162—substantially strengthened the interaction between RHOA and the RBD (**Figure 2b**). Parallel findings showed that various mutants at position K118 (but none at K162) likewise enhanced RHOA-RBD binding (**Figure 2c**).

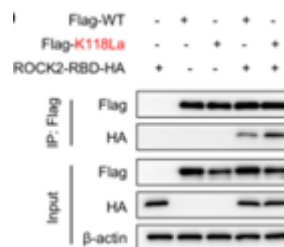
RHOA exhibits intrinsic GTPase activity, and its inactivation triggers downstream pathway activation. The influence of K118 lactylation and cancer-linked mutations on this GTP hydrolysis was quantified by measuring released phosphate using a dedicated assay kit. Data revealed that the GTP-hydrolyzing ability of K118-lactylated RHOA closely resembled that of the K118Q mutant and was markedly diminished relative to wild-type RHOA, K162-lactylated RHOA, or the K162Q version (**Figure 2d**). Additional experiments assessed impacts on downstream signaling cascades in both HEK293T cells and breast cancer lines. In particular, K118 lactylation and corresponding mutations led to substantially higher MLC2 phosphorylation levels than those seen with wild-type RHOA or K162 lactylation (**Figures 2e–2i**).

RHOA activation drives assembly of actin stress fibers, structures essential for cellular morphology and substrate adhesion [19]. To explore cytoskeletal changes linked to ROCK pathway stimulation by RHOA variants, actin stress fibers were visualized by staining. Cells harboring the K118Q mutant displayed a clear increase in stress fiber formation compared with those expressing wild-type RHOA (**Figure 2j**).

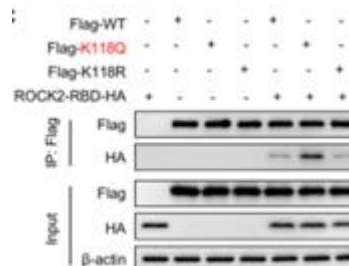
Structurally, the terminal portions of the side chains are as follows: lysine acetylation features a carbonyl ($-C=O$) attached to a nonpolar methyl ($-CH_3$); lysine lactylation features a carbonyl ($-C=O$) attached to a polar hydroxyethyl ($-CH_3CHOH$); and glutamine features a carbonyl ($-C=O$) attached to a polar amino group ($-NH_2$). Because of the shared polarity profile between lactylated lysine and glutamine (distinct from acetylation), the K-to-Q substitution better recapitulates the effects of lysine lactylation. Consistent with this, the biological consequences of Q replacement at these lysines mirrored those obtained with genuine site-specifically lactylated RHOA proteins (**Figures 2b–2j**). Given that the genetic code-expansion system for lactylation supports only short-term expression, the glutamine substitution strategy was adopted to emulate persistent lactylation in subsequent experiments requiring extended time frames.



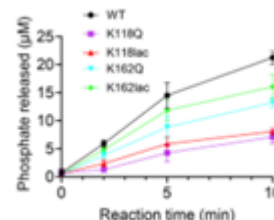
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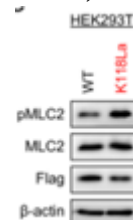
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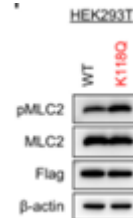
c)



d)



e)



f)

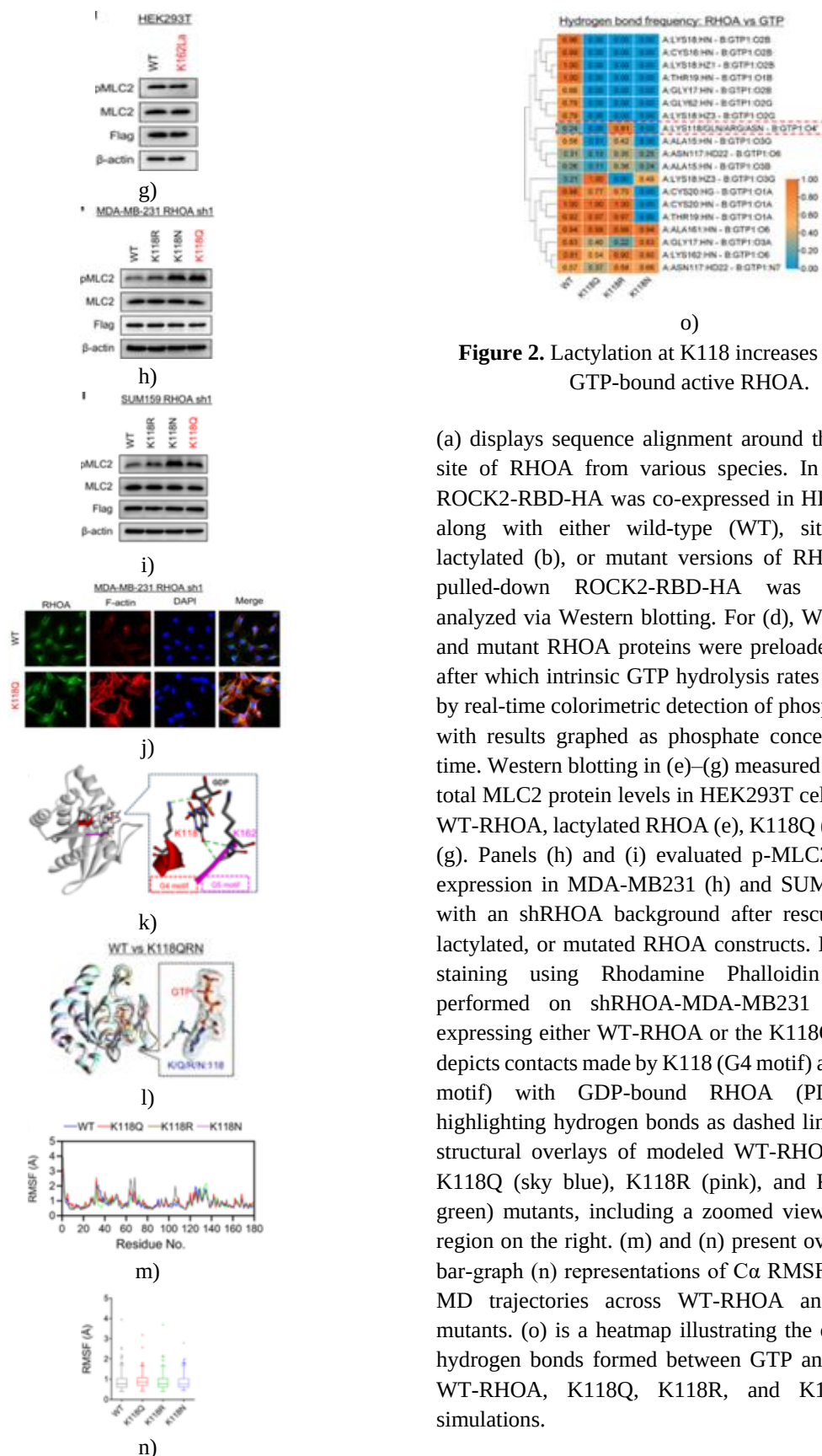


Figure 2. Lactylation at K118 increases the pool of GTP-bound active RHOA.

(a) displays sequence alignment around the lactylation site of RHOA from various species. In (b) and (c), ROCK2-RBD-HA was co-expressed in HEK293T cells along with either wild-type (WT), site-specifically lactylated (b), or mutant versions of RHOA (c). The pulled-down ROCK2-RBD-HA was subsequently analyzed via Western blotting. For (d), WT, lactylated, and mutant RHOA proteins were preloaded with GTP, after which intrinsic GTP hydrolysis rates were tracked by real-time colorimetric detection of phosphate release, with results graphed as phosphate concentration over time. Western blotting in (e)–(g) measured p-MLC2 and total MLC2 protein levels in HEK293T cells expressing WT-RHOA, lactylated RHOA (e), K118Q (f), or K162Q (g). Panels (h) and (i) evaluated p-MLC2 and MLC2 expression in MDA-MB231 (h) and SUM159 (i) cells with an shRHOA background after rescue with WT, lactylated, or mutated RHOA constructs. In (j), F-actin staining using Rhodamine Phalloidin (red) was performed on shRHOA-MDA-MB231 cells stably expressing either WT-RHOA or the K118Q variant. (k) depicts contacts made by K118 (G4 motif) and K162 (G5 motif) with GDP-bound RHOA (PDB: 1FTN), highlighting hydrogen bonds as dashed lines. (l) shows structural overlays of modeled WT-RHOA (tan) with K118Q (sky blue), K118R (pink), and K118N (light green) mutants, including a zoomed view of the GTP region on the right. (m) and (n) present overlaid (m) or bar-graph (n) representations of $C\alpha$ RMSF values from MD trajectories across WT-RHOA and the K118 mutants. (o) is a heatmap illustrating the occupancy of hydrogen bonds formed between GTP and residues in WT-RHOA, K118Q, K118R, and K118N during simulations.

Structural examination indicated that the amino group on the K118 side chain in RHOA creates a hydrogen bond with the ribose portion of the bound guanine nucleotide (**Figure 2k**). Models of RHOA and its K118Q, K118R, and K118N mutants were generated using AlphaFold 3. The predicted WT-RHOA structure matched the crystal structure (PDB: 1FTN) with very high accuracy, validating the modeling approach. Analysis of RHOA structures revealed nearly identical folds among WT-RHOA, the lactylation mimic K118Q, and the cancer-related K118N/K118R mutants, especially around the GTP-binding site (**Figure 2l**).

Molecular dynamics (MD) simulations were run to probe how K118 lactylation or mutation might enhance RHOA function by altering protein dynamics. RMSF calculations assess local flexibility of each residue. RMSF profiles for the K118 mutants were overall comparable to WT-RHOA (**Figures 2m and 2n**). In agreement, separate GTP-binding experiments in vitro showed no major differences in GTP affinity for lactylated RHOA (K118La) or K118Q relative to the wild-type protein. These observations indicate that modifications at K118 have minimal impact on structural flexibility or GTP association.

Hydrogen bonding is essential for precise ligand recognition by proteins. Within RHOA, GTP interacts via hydrogen bonds with C16, G17, K18, T19, and C20 (P-loop/G1), G62 (G3), N117 and K118 (G4), as well as A161 and K162 (G5) [3, 20]. Unmodified K118 is positively charged; its lactylation removes this charge and disrupts favorable contacts with GTP. Thus, K118 lactylation likely perturbs electrostatic interactions. As predicted, both K118Q (lactylation mimic) and K118N (tumor mutant) fully eliminated hydrogen bonding to GTP compared with WT-RHOA, whereas K118R (positively charged) displayed higher bond frequency (**Figure 2o**). Moreover, these K118 changes also reduced or eliminated hydrogen bonds between GTP and nearby residues including C16, G17, K18, T19, and G62—residues important for RHOA's intrinsic GTPase function (**Figure 2o**). Overall, these findings show that K118 lactylation or mutation weakens intrinsic GTPase activity, resulting in greater RHOA activation.

Lactylation at K162 enhances RHOA protein stability through competition with ubiquitination

Mass spectrometry identified RHOA as a potential ubiquitination target, with K118 and K162 appearing as candidate sites (**Figures 3a and 3b**). Supporting this,

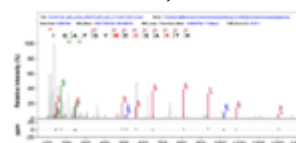
exposure to the proteasome inhibitor MG132 caused clear accumulation of RHOA protein, pointing to regulation via the ubiquitin-proteasome pathway. CHX chase experiments confirmed the relatively short half-life of RHOA (**Figures 3c–3f**). Notably, only K162 lactylation or the K162Q mutation (and not K118 lactylation or K118Q) markedly improved RHOA protein stability (**Figures 3c–3f**).

KCTD13, a BTB/POZ domain protein, serves as a specific adaptor for the BCR (BTB-CUL3-RBX1) E3 ligase complex and interacts with RHOA [21]. Higher KCTD13 levels were associated with less aggressive cancer subtypes, greater drug responsiveness, and better patient outcomes. Co-immunoprecipitation showed strong binding between WT-RHOA and KCTD13, which was substantially reduced by K162La or K162R (**Figure 3g**). K48-linked ubiquitin chains mark proteins for degradation by the proteasome. In contrast, other linkages, such as K63-linked chains, mainly control non-degradative functions, such as activity and localization [22, 23]. Use of ubiquitin mutants revealed that K48R (but not K63R) strongly decreased RHOA ubiquitination (**Figure 3h**), confirming that K162 ubiquitination primarily mediates proteasomal turnover.

Ubiquitination assays were conducted to investigate the relationship between K162 modifications and RHOA stability. Relative to WT-RHOA, both K162 lactylation and the mutants K162Q, K162N, and K162R displayed clearly lower ubiquitination (**Figures 3i–3l**). This indicates that lactylation and mutations at K162 block ubiquitination at the same site in a competitive manner, thereby stabilizing the protein.

Gene Names	Proteins	Ubiquitinated sequence	Position	Raw Score
RHOA	P61586	HFCFNVVPIILVGNK ⁺ K	K118	14.949515
RHOA	P61586	IGAFGYMECSAK ⁺ TK	K162	11.208966

a)



b)



c)

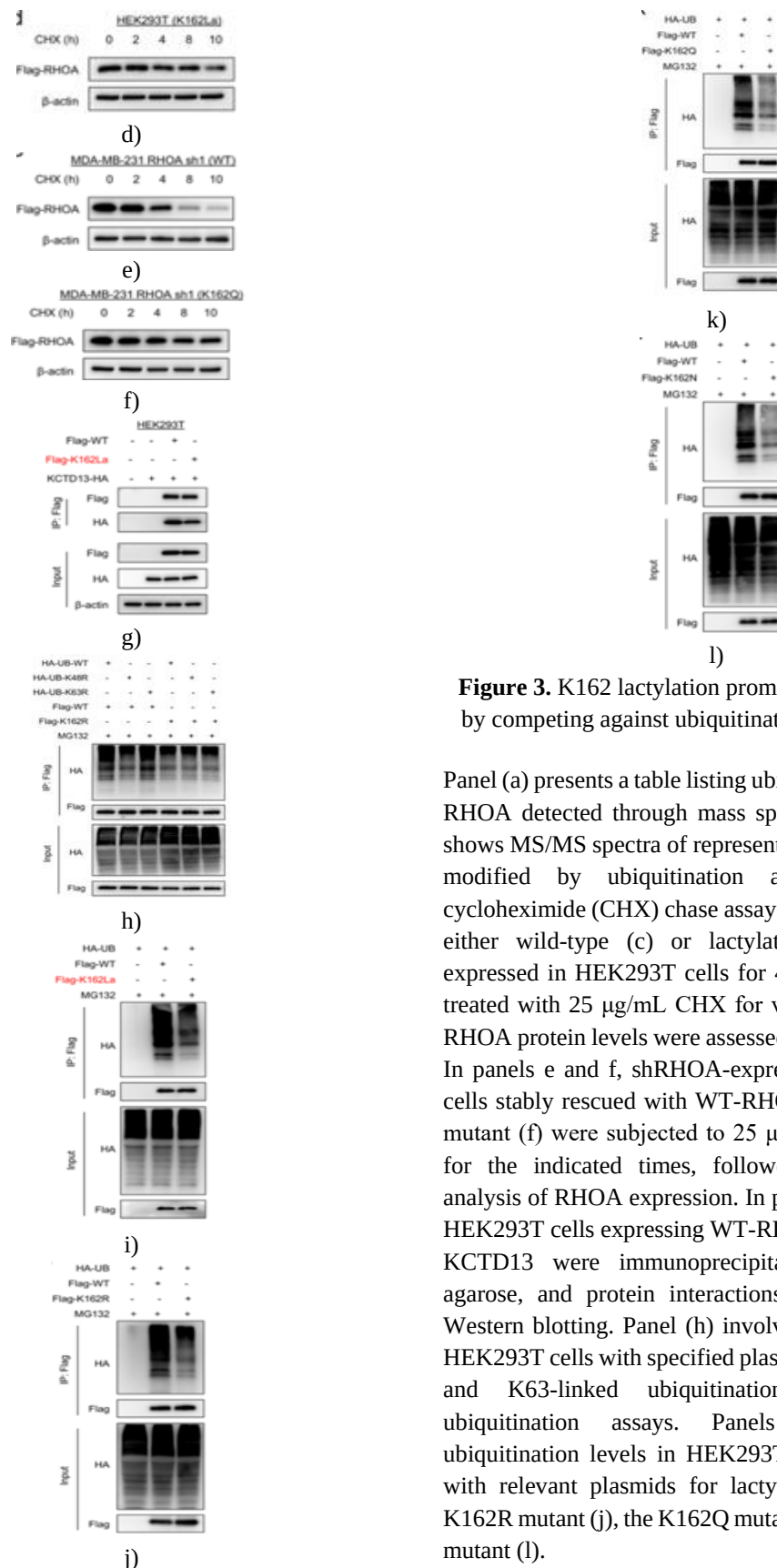
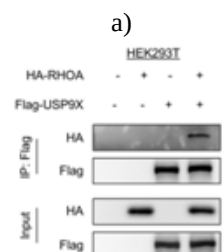
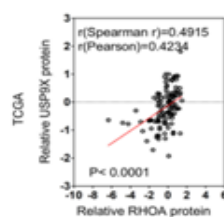


Figure 3. K162 lactylation promotes RHOA stability by competing against ubiquitination at the same site.

Panel (a) presents a table listing ubiquitinated peptides of RHOA detected through mass spectrometry. Panel (b) shows MS/MS spectra of representative RHOA peptides modified by ubiquitination at K162. For the cycloheximide (CHX) chase assays in panels (c) and (d), either wild-type (c) or lactylated RHOA (d) was expressed in HEK293T cells for 48 h. Cells were then treated with 25 μ g/mL CHX for various durations, and RHOA protein levels were assessed by Western blotting. In panels e and f, shRHOA-expressing MDA-MB-231 cells stably rescued with WT-RHOA (e) or the K162Q mutant (f) were subjected to 25 μ g/mL CHX treatment for the indicated times, followed by Western blot analysis of RHOA expression. In panel (g), lysates from HEK293T cells expressing WT-RHOA, K162La, and/or KCTD13 were immunoprecipitated using Flag-M2 agarose, and protein interactions were examined by Western blotting. Panel (h) involved co-transfection of HEK293T cells with specified plasmids to evaluate K48- and K63-linked ubiquitination of RHOA via ubiquitination assays. Panels (i)–(l) measured ubiquitination levels in HEK293T cells co-transfected with relevant plasmids for lactylated RHOA (i), the K162R mutant (j), the K162Q mutant (k), and the K162N mutant (l).

USP9X functions as a deubiquitinase for RHOA

Ubiquitin carboxyl-terminal hydrolase 9X (USP9X) is a deubiquitinase that participates in both ubiquitin precursor processing and removal of ubiquitin from modified proteins [24]. Examination of protein–protein interaction databases identified USP9X as a candidate binding partner and deubiquitinase for RHOA. Clinical dataset analysis revealed that elevated USP9X expression strongly correlates with higher tumor grade and reduced patient survival. Additionally, USP9X levels showed a positive association with RHOA protein abundance (**Figure 4a**). To test whether USP9X acts on RHOA, immunoprecipitation experiments confirmed a direct interaction between USP9X and RHOA. In contrast, USP33 showed no such binding (**Figure 4b**). Knocking down USP9X expression resulted in a marked reduction of RHOA protein levels (**Figures 4c and 4d**). Consistent with this, CHX chase assays demonstrated decreased RHOA protein stability upon USP9X depletion (**Figures 4e–4h**). In contrast, restoring USP9X expression partially reversed the increase in RHOA ubiquitination (**Figure 4i**). Functional studies in cancer cells showed that USP9X knockdown significantly suppressed cell migration and invasion in MDA-MB231 and SUM159 lines in vitro (**Figures 4j and 4k**). Notably, introducing the K162Q mutant into USP9X-knockdown cells rescued migration and invasion more effectively than re-expression of wild-type RHOA (**Figures 4l–4o**). These results collectively establish USP9X as a deubiquitinase that acts on RHOA.



b)



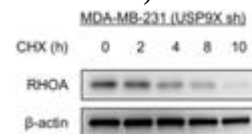
c)



d)



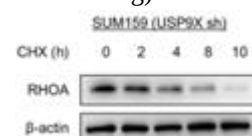
e)



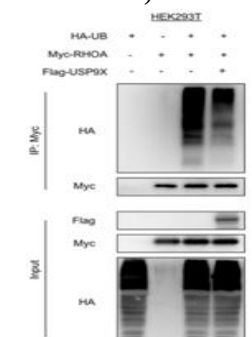
f)



g)



h)



i)

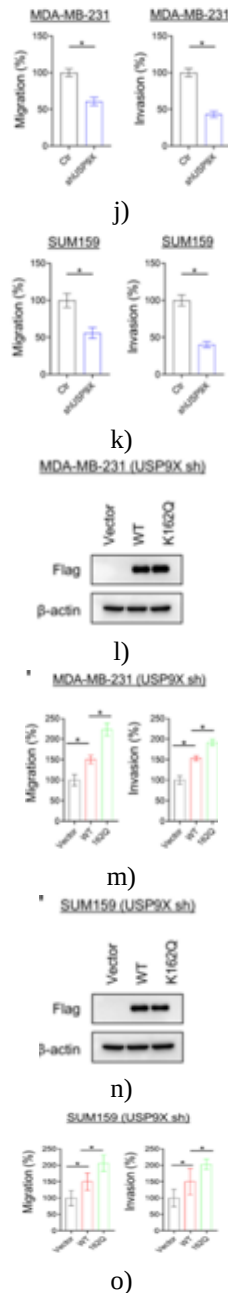


Figure 4. USP9X promotes deubiquitination of RHOA.

Panel (a) presents analysis of the TCGA dataset showing the relationship between RHOA and USP9X expression. Relative protein levels of RHOA were plotted against those of USP9X, with correlations evaluated using both Pearson's correlation and Spearman's rank correlation methods. In panel (b), lysates from HEK293T cells expressing WT-RHOA and/or USP9X were immunoprecipitated with Flag-M2 agarose, and protein interactions were examined by Western blotting. Panels

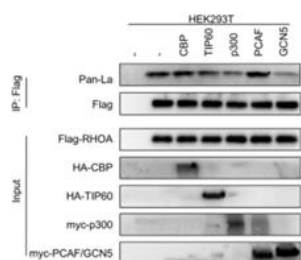
(c) and (d) show Western blot analysis of RHOA and USP9X levels in MDA-MB-231 (c) and SUM159 (d) cells stably expressing either empty vector or USP9X-targeting shRNA. For the CHX chase experiments in panels e–h, MDA-MB-231 (e, f) and SUM159 (g, h) cells with stable control or USP9X knockdown were treated with 25 µg/mL CHX for the indicated periods, followed by Western blotting to assess RHOA protein levels. Panel (i) involved co-transfection of HEK293T cells with the specified plasmids, after which RHOA ubiquitination was quantified using a ubiquitination assay. Panels (j) and (k) evaluated cell migration and invasion abilities of MDA-MB231 (j) and SUM159 (k) cells expressing a control vector or USP9X shRNA. The percentages of migratory and invasive cells are displayed in bar graphs (mean ± SD from three independent experiments), with statistical significance indicated by * $P < 0.05$ using Student's t-test. Panels (l) and (n) show Western blot analysis of RHOA and K162Q expression in MDA-MB-231 (l) and SUM159 (n) cells with stable USP9X knockdown. Panels m and o assessed migration and invasion in shUSP9X-expressing MDA-MB231 (m) and SUM159 (o) cells rescued with empty vector, WT-RHOA, or the K162Q mutant. Percentages of migratory and invasive cells are shown in bar graphs (mean ± SD from three separate experiments), with * $P < 0.05$ by Student's t-test.

PCAF adds lactyl groups to RHOA, while HDAC3 removes them

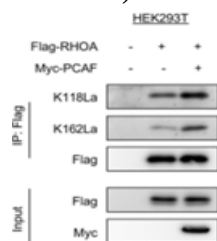
To identify the enzymes responsible for RHOA lactylation, five different acyltransferases — CREB-binding protein (CBP), TIP60 (KAT5), p300 (E1A-binding protein, 300 kDa), PCAF (KAT2B), and GCN5 (KAT2A) — were individually expressed in HEK293T cells. Among them, only ectopic PCAF expression led to a substantial rise in RHOA lactylation. In contrast, the others showed minimal or no effect (**Figure 5a**). Further experiments demonstrated that increasing PCAF levels enhanced lactylation at both K118 and K162 sites on RHOA, while reducing PCAF expression lowered lactylation at these same residues (**Figures 5b and 5c**). In vitro lactylation reactions confirmed that PCAF effectively modified wild-type RHOA at K118 and K162 but displayed no activity toward K-to-R, K-to-Q, or K-to-N mutants at these positions, highlighting its site-specific enzymatic action (**Figure 5d**). These findings support PCAF as a key lysine lactyltransferase for RHOA. Molecular docking of lactyl-CoA into the PCAF

structure (PDB: 1CM0) showed that the molecule fits well into the cofactor-binding pocket, resembling the binding mode observed in the CoA-PCAF complex (**Figure 5e**).

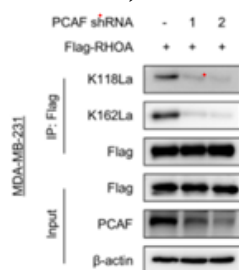
Aminoacyl-tRNA synthetase 1 (AARS1), previously reported to generate lactate-AMP intermediates for lysine modification [13], was also tested. However, AARS1 expression had no impact on RHOA lactylation levels. This suggests that lactylation of different target proteins is likely controlled by distinct sets of acyltransferases.



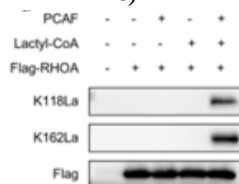
a)



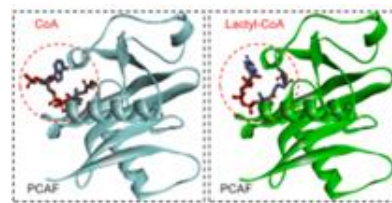
b)



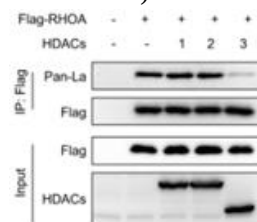
c)



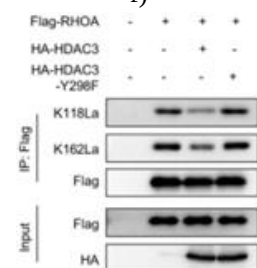
d)



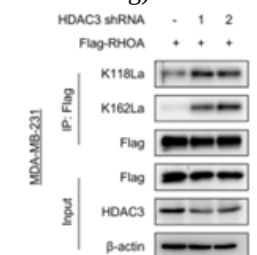
e)



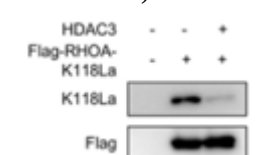
f)



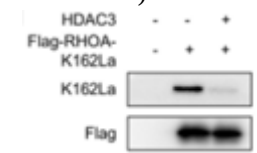
g)



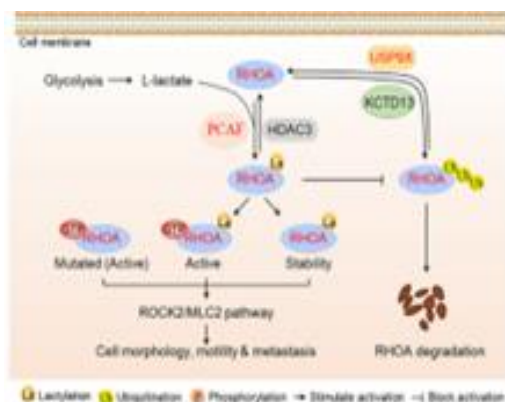
h)



i)



j)



k)

Figure 5. PCAF functions as a lactyltransferase for RHOA, while HDAC3 acts as its delactylase.

Panel (a): HEK293T cells were co-transfected with the indicated constructs, and overall RHOA lactylation was detected using a pan-Kla antibody. Panel (b): HEK293T cells expressing RHOA with or without PCAF were analyzed for site-specific lactylation on RHOA using targeted antibodies. Panel (c): MDA-MB231 cells co-expressing RHOA and/or shPCAF underwent immunoprecipitation, followed by assessment of RHOA lactylation with site-specific antibodies. Panel (d) shows an in vitro lactylation reaction demonstrating PCAF's ability to modify RHOA at K118 and K162. Panel (e) illustrates the cofactor-binding pocket of PCAF (PDB: 1CM0) occupied by CoA (left) or lactyl-CoA (right), with PCAF rendered in cartoon representation and the transferred groups highlighted by red circles. Panel (f): HEK293T cells were co-expressed with RHOA and various HDAC family members, and RHOA lactylation was evaluated using a pan-Kla antibody. Panel (g): HEK293T cells co-expressing RHOA with either HDAC3 or the catalytically inactive HDAC3-Y298F mutant were tested for site-specific lactylation on RHOA. Panel (h): MDA-MB231 cells expressing RHOA with or without shHDAC3 were immunoprecipitated and analyzed for site-specific RHOA lactylation. Panels (i) and (j) present in vitro delactylation assays showing HDAC3's ability to remove lactyl groups from RHOA-K118La (i) and RHOA-K162La (j). Panel (k): HEK293T cells were co-transfected with the indicated plasmids, and RHOA ubiquitination levels were measured. Panel (l) provides a schematic model summarizing how lactylation regulates RHOA activity and stability (see Discussion).

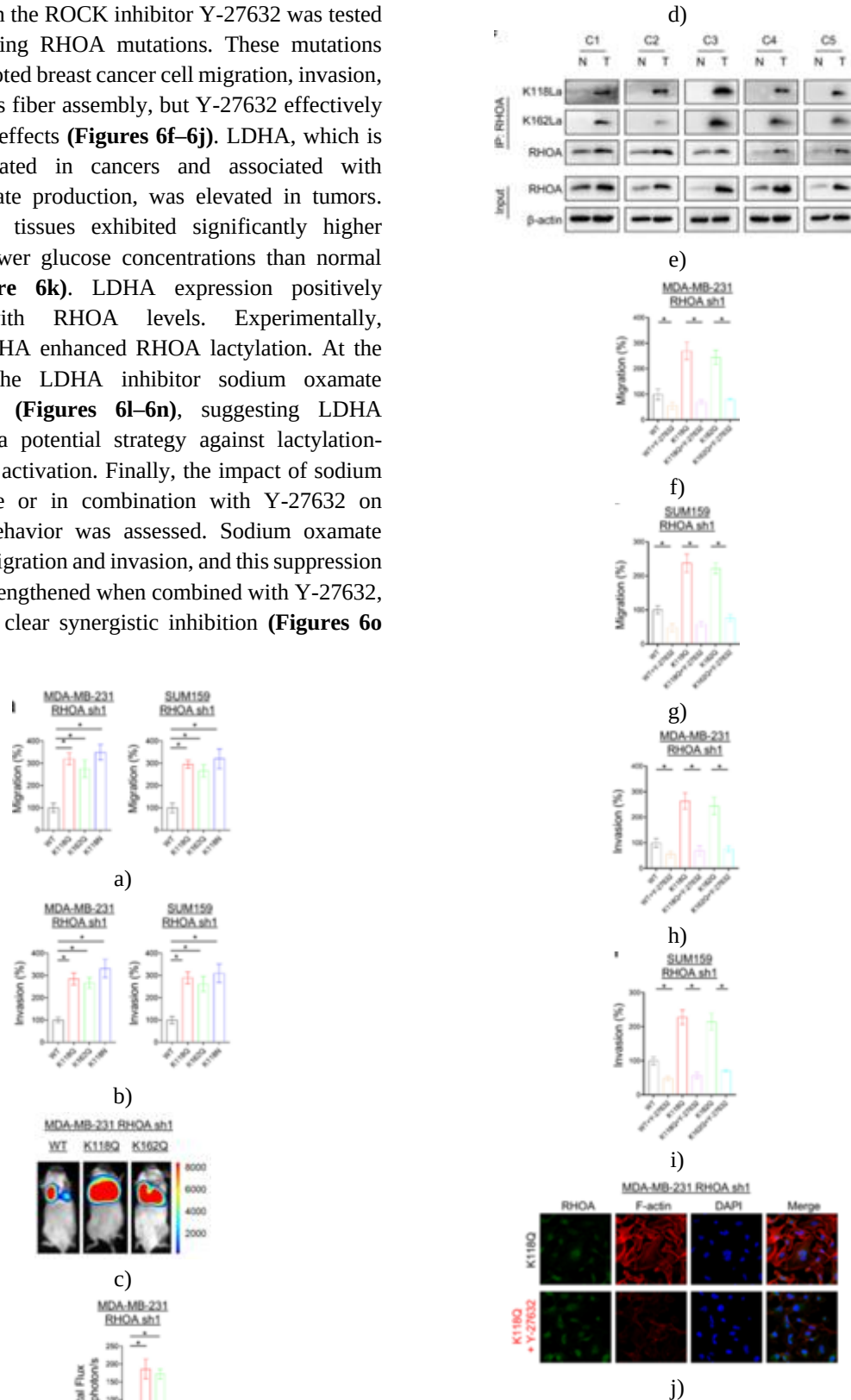
To find the enzyme responsible for removing lactyl groups from RHOA, the protein was co-expressed with several HDAC family members (HDAC1–3) and SIRT family members (SIRT1–5). Only HDAC3 markedly lowered RHOA lactylation levels, while the other HDACs and SIRTs had little or no effect (**Figure 5f**). The wild-type HDAC3 strongly decreased RHOA lactylation, but the enzymatically inactive HDAC3-Y298F mutant did not (**Figure 5g**). In addition, reducing HDAC3 expression through knockdown increased lactylation at both K118 and K162 sites on RHOA (**Figure 5h**). In vitro delactylation experiments verified that HDAC3 can directly remove lactyl modifications from K118 and K162 on RHOA (**Figures 5i and 5j**). Furthermore, overexpression of HDAC3 increased RHOA ubiquitination (**Figure 5k**). Clinical data indicated that higher HDAC3 expression is associated with poorer patient survival. Collectively, these observations identify HDAC3 as the primary deacetylase acting on RHOA.

RHOA lactylation drives metastasis both in vitro and in vivo, and combining inhibitors of RHOA lactylation with agents targeting RHOA mutations produces synergistic anti-tumor effects

To investigate the biological roles of RHOA lactylation and its cancer-associated mutations, lactylation-mimicking mutants (K118Q and K162Q) and a tumor-derived mutant (K118N) were introduced into various cancer cell lines. Compared with cells expressing wild-type RHOA, cells with K118Q, K118N, or K162Q showed enhanced colony formation, migration, and invasion abilities in culture (**Figures 6a and 6b**). In vivo lung metastasis assays using xenograft models further demonstrated that K118Q and K162Q mutants significantly increased metastatic burden compared with WT-RHOA (**Figures 6c and 6d**).

RHOA-knockout (KO) cell lines were also created and subsequently reconstituted with either wild-type or mutant RHOA. Cells expressing the K118Q or K162Q mutants in the KO background displayed stronger metastatic potential than those reconstituted with WT-RHOA. Examination of fresh clinical breast cancer samples revealed that both RHOA protein levels and its lactylation were markedly elevated in tumor tissues relative to matched adjacent normal tissues (**Figure 6e**). The increase in RHOA lactylation was particularly pronounced compared with the rise in total RHOA (**Figure 6e**).

Treatment with the ROCK inhibitor Y-27632 was tested on cells carrying RHOA mutations. These mutations strongly promoted breast cancer cell migration, invasion, and actin stress fiber assembly, but Y-27632 effectively blocked these effects (**Figures 6f–6j**). LDHA, which is often upregulated in cancers and associated with increased lactate production, was elevated in tumors. Breast cancer tissues exhibited significantly higher lactate and lower glucose concentrations than normal tissues (**Figure 6k**). LDHA expression positively correlated with RHOA levels. Experimentally, increasing LDHA enhanced RHOA lactylation. At the same time, the LDHA inhibitor sodium oxamate suppressed it (**Figures 6l–6n**), suggesting LDHA inhibition as a potential strategy against lactylation-driven RHOA activation. Finally, the impact of sodium oxamate alone or in combination with Y-27632 on cancer cell behavior was assessed. Sodium oxamate reduced cell migration and invasion, and this suppression was further strengthened when combined with Y-27632, demonstrating clear synergistic inhibition (**Figures 6o and 6p**).



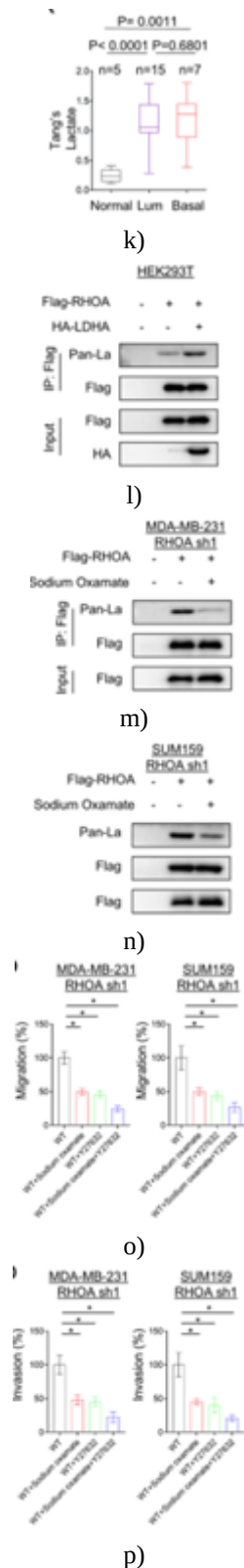


Figure 6. RHOA lactylation drives metastasis in both cell culture and living organisms, while pharmacological agents that block RHOA

lactylation, combined with those targeting RHOA mutations, exert synergistic anticancer effects.

Panels (a) and (b) evaluated migratory capacity (a) and invasive capacity (b) of shRHOA-expressing MDA-MB231 and SUM159 cells stably reconstituted with WT-RHOA, K118Q, K118N, or K162Q mutants. Percentages of migrated and invaded cells are presented as bar graphs (mean $\pm$ SD from three independent experiments), with statistical significance marked by $*P < 0.05$ using Student's t-test. In panels (c) and (d), shRHOA-expressing MDA-MB231 cells stably expressing WT-RHOA, K118Q, or K162Q were injected into nude mice through the tail vein. Lung metastatic burden was assessed 4 weeks later via bioluminescence imaging (c) and quantified by photon flux measurements (mean $\pm$ SEM across 6 animals) (d). Images from three representative mice per group are displayed (c). Panel (e): Paired human breast cancer samples (T for tumor, N for adjacent normal tissue) were lysed and analyzed directly by Western blotting for total RHOA. For lactylation analysis, lysates were first immunoprecipitated with an anti-RHOA antibody and then probed with site-specific K118la or K162la antibodies. Panels (f)–(i) examined migratory ability (f, g) and invasiveness (h, i) of shRHOA-expressing MDA-MB231 and SUM159 cells stably expressing WT-RHOA, K118Q, or K162Q after treatment with or without the ROCK inhibitor Y-27632 (10 μ M). Results are shown as bar graphs of percentages (mean $\pm$ SD from three experiments), $*P < 0.05$ by Student's t-test. Panel (j) shows F-actin staining with Rhodamine Phalloidin (red) in shRHOA-MDA-MB231 cells expressing the K118Q mutant, with or without Y-27632 (10 μ M) treatment.

Panel (k) reports lactate concentrations in normal breast tissue, luminal, and basal-like tumors based on Tang's metabolomics dataset. Panel (l): HEK293T cells co-expressing Flag-RHOA and/or HA-LDHA were subjected to immunoprecipitation, followed by Western blot analysis of K1a levels. Panels (m) and (n): shRHOA-expressing MDA-MB231 (m) and SUM159 (n) cells transfected with Flag-RHOA were treated with or without sodium oxamate (20 mM) for 24 h, then immunoprecipitated and analyzed for K1a by Western blot. Panels (o) and (p) assessed migratory ability (o) and invasiveness (p) of shRHOA-expressing MDA-MB231 and SUM159 cells with stable WT-RHOA after 24 h treatment with Y-27632 (10 μ M), sodium oxamate (20

mM), or both. Percentages are shown in bar graphs (mean $\pm$ SD from three experiments), * $P < 0.05$ by Student's t-test.

This work demonstrates that lactylation of RHOA at K118 and K162 augments its activity and stability, in a manner reminiscent of oncogenic mutations. It positions lactylation as a reversible “epi-mutation” process and underscores its importance in cancer biology (**Figure 5I**).

Lactylation at mutation-prone sites on RHOA accelerates tumor progression by emulating mutational activation and stabilization

The study uncovers a lactate-PCAF/HDAC3 regulatory axis that controls RHOA lactylation: PCAF adds lactyl groups to K118 and K162, while HDAC3 removes them. This connects the Warburg effect (aerobic glycolysis) directly to RHOA pathway activation. Notably, PCAF increased RHOA lactylation, whereas AARS1 had no influence, emphasizing that lactylation is regulated in a protein-specific manner. HDAC3 serves as a metabolic sensor, fine-tuning lactylation levels in response to the tumor microenvironment to modulate RHOA signaling. Experiments showed that lactylation or N/R substitution at K118 (but not K162) shifts RHOA toward its active GTP-bound conformation. Within the G4 motif (XKXD), the positively charged ϵ -amino group of K118 normally forms hydrogen bonds with the ribose of GTP [3, 20]. Attachment of a lactyl group to this amino group disrupts that interaction, thereby affecting GTP binding and GTPase activity. Although crystal structures indicate highly similar overall conformations between wild-type and K118-mutated RHOA in the GTP-binding region, molecular dynamics simulations revealed that K118 lactylation-mimicking or cancer-associated mutations weaken key hydrogen bonds involved in GTPase function without altering overall binding flexibility to GTP. Given that intracellular GTP levels are approximately tenfold higher than GDP [25], reduced hydrolysis favors accumulation of active GTP-RHOA, replicating the effect of constitutive activating mutations. These changes likely arise from (1) steric interference with proper GTP positioning and (2) disruption of the catalytic hydrogen-bond network.

Previous studies have noted that certain lysine residues prone to oncogenic mutations can also undergo ubiquitination [26, 27]. Here, lactylation at K162 in the G5 motif (SAK) competitively interferes with KCTD13-mediated K48-linked ubiquitination, thereby preventing proteasomal degradation. This mirrors the protective

effect of cancer-associated K162N/R mutations. USP9X further supports RHOA stabilization through deubiquitination. Consequently, modifications at the two sites work together: K118 primarily boosts activity, while K162 enhances protein longevity.

Functionally, lactylated RHOA promotes aggressive cancer behaviors such as increased cell motility and distant metastasis in both in vitro and in vivo settings. In patient samples, RHOA lactylation is substantially higher in breast tumors than in paired normal tissues. These results highlight RHOA lactylation as an important metabolic mechanism that phenocopies oncogenic mutations to drive cancer development.

In summary, lactylation at K118 (which activates RHOA) and K162 (which stabilizes it) functionally mimics genetic mutations. The PCAF/HDAC3 axis bridges glycolytic metabolism to oncoprotein regulation, establishing lactylation as a metabolic substitute for mutations in tumor progression.

Therapeutic implications for tumors exhibiting RHOA lactylation

LDHA-driven lactate production is closely tied to protein lactylation [9-11]. The current data confirm a positive association between lactate levels and RHOA lactylation and show that the LDHA inhibitor oxamate suppresses migration and invasion in cells with elevated RHOA lactylation, suggesting that lactate reduction is a viable treatment option for such tumors. Existing strategies for addressing RHOA mutations include blocking RHOA expression or its downstream signaling [28, 29]. The ROCK inhibitor Y27632 effectively suppressed phenotypes caused by both lactylation-mimicking variants and cancer mutations. Importantly, combining the ROCK inhibitor with the LDHA inhibitor produced synergistic inhibition. Because lactylation and mutation at these sites share mechanistic and functional similarities, dual targeting of lactylation and RHOA signaling offers a promising combined therapeutic avenue.

This study introduces lactylation as a reversible “epi-mutation” mechanism

We introduce the concept of post-translational lactylation at mutation hotspot residues as a reversible “epi-mutation” system that functionally replicates the effects of genetic mutations at the same positions. This allows tumors to rapidly and reversibly hyperactivate oncoproteins in response to metabolic cues, without

altering the genome. The findings blur traditional boundaries between genetic and epigenetic drivers of cancer and help explain RHOA hyperactivation in tumors lacking mutations. Use of the site-specific lactylation incorporation system was essential, as it avoids artifacts associated with mutagenesis and directly proves that lactylation itself is sufficient to produce mutation-like effects.

This framework likely applies more broadly: other oncoproteins and different post-translational modifications may utilize analogous “epi-mutation” strategies at shared regulatory sites. Discovering this reversible, metabolism-driven mutation-mimicking system significantly advances our understanding of how tumors exploit non-genetic mechanisms to gain oncogenic advantages. It carries important consequences for precision medicine and the design of new therapies.

Conclusion

In conclusion, RHOA lactylation serves as a metabolic counterpart to oncogenic mutations. Modifications at K118 (activation) and K162 (stabilization) sustain persistent RHOA signaling and tumor progression. The lactate-PCAF/HDAC3 axis connects altered metabolism to oncoprotein control. Lactylation acts as a dynamic, reversible “epi-mutation” system that enables mutation-like phenotypes while preserving adaptability. Therapeutically, simultaneous inhibition of lactate production and RHOA pathway activity emerges as a strong strategy against lactylation-dependent cancers, integrating metabolic and signaling-targeted approaches in precision oncology.

Limitations of the study

Although the site-specific lactylation system allows accurate placement of lactyl-lysine, its transient nature limits its use in long-term experiments. K-to-R mutants at K118/K162 were inappropriate as controls because they possess intrinsic oncogenic properties; therefore, K-to-Q substitutions were used instead, as they better replicate the polar nature of lactylated lysine (-CH₃CHOH) compared with the amino group of glutamine (-NH₂) and faithfully reproduce both mechanistic and phenotypic outcomes. Detection of endogenous lactylation in clinical tissues required prior immunoprecipitation with RHOA antibody to minimize cross-reactivity arising from conserved K118/K162 residues across RHO family members. These constraints

highlight the need for advanced tools to study protein modifications, yet multiple complementary approaches including site-specific lactylation, targeted mutagenesis, and molecular dynamics simulations robustly support the conclusions.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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