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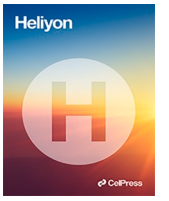

Research article

NAT10-mediated ac^{4}C modification promotes stemness and chemoresistance of colon cancer by stabilizing NANOGP8☆

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ABSTRACT

Background: Colon cancer (CC) stem cells can self-renew as well as expand, thereby promoting tumor progression and conferring resistance to chemotherapeutic agents. The acetyltransferase NAT10 mediates N4-acetylcytidine (ac^{4}C) modification, which in turn drives tumorigenesis, metastasis, stemness properties maintenance, and cell fate decisions. Nonetheless, the specific involvement of ac^{4}C modification mediated by NAT10 in regulating stemness and chemosensitivity in CC remains undetermined.

Methods: The levels of NAT10 in normal colon and chemoresistant CC tissues were determined utilizing quantitative real-time polymerase chain reaction alongside immunohistochemistry. Assessing cancer cell stemness and chemosensitivity was conducted by various methods including spheroid and colony formation, western blotting, and flow cytometry. RNA-Seq was used to identify target genes, and RNA immunoprecipitation analysis was used to explore the potential mechanisms.

Results: We observed NAT10 overexpression and increased ac^{4}C modification levels in chemoresistant CC tissues. The *in vivo* and *in vitro* analysis findings suggested that NAT10 promoted CC cell stemness while suppressing their chemosensitivity. Conversely, Remodelin, a NAT10-specific inhibitor, enhanced CC cell chemosensitivity. Mechanistically, NAT10 increased the level of *NANOGP8* ac^{4}C modification and promoted *NANOGP8* mRNA stability.

Conclusions: NAT10 promotes the maintenance of stemness and chemoresistance in CC cells by augmenting the mRNA stability of *NANOGP8*. The inhibition of NAT10 via Remodelin improves chemotherapeutic efficacy and impedes CC progression.

Abbreviations: CC, colon cancer; CSCs, cancer stem cells; ac^{4}C, N4-acetylcytidine; RIP, RNA immunoprecipitation; IgG, immunoglobulin G; qPCR, quantitative polymerase chain reaction; RT-qPCR, reverse transcription qPCR; ACTB, beta-actin; BSA, bovine serum albumin; TCGA, The Cancer Genome Atlas.

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1. Introduction

Colon cancer (CC) represents a major contributor to global cancer-associated deaths, with approximately half of the patients experiencing metastasis, recurrence, and even death within 5 years of treatment [1–4]. Despite advancements in screening and treatment strategies for CC, its prognosis remains poor, primarily because the mechanisms underlying its development are not well understood [5–7]. Accumulating evidence indicates that CC harbors a population of cancer stem cells (CSCs) demonstrating "stemness" properties [8,9]. CSCs exhibit self-renewal and expansion properties that facilitate tumor progression and confer resistance to chemotherapeutic agents [10,11]. Therefore, an urgent need exists to elucidate the underlying mechanism by which CSCs maintain stemness, as this may provide valuable insights regarding potential therapeutic targets.

Nucleoside modifications of RNA are involved in RNA structure, biogenesis, and function [12–14]. N4-acetylcytidine (ac^4C) constitutes a novel and exceedingly conserved mRNA modification catalyzed by acetyltransferase NAT10 [15]. Arango et al. demonstrated that mRNA acetylation promotes mRNA stability and translation efficiency [16]. Recent reports have indicated that NAT10-mediated ac^4C modification regulates tumor proliferation, metastasis, stemness maintenance, and cell fate decisions [7,15,17,18]. In particular, NAT10 promotes *BCL9L*, *SOX4*, and *AKT1* translation efficiency and stability through ac^4C modification, which is essential for maintaining stem cell-like characteristics in bladder cancer cell lines [15]. Nevertheless, the involvement of NAT10 and ac^4C modifications in CC cell stemness and chemosensitivity is not yet understood.

This study revealed that NAT10 exhibited higher expression levels, and there was an elevation in ac^4C modification levels in chemoresistant CC tissues. The *in vivo* and *in vitro* analysis findings suggested that NAT10 promotes the stemness of CC cells while suppressing their chemosensitivity. Conversely, the NAT10-specific inhibitor Remodelin enhanced CC cell chemosensitivity. Mechanistically, NAT10 enhanced *NANOGP8* ac^4C modification and facilitated *NANOGP8* mRNA stability. Overall, this study reveals that the NAT10/NANOGP8 axis is crucial for maintaining stemness and chemoresistance of CSCs and could be an encouraging therapeutic strategy to treat CC.

2. Materials and methods

2.1. Patient samples

The Lanzhou University Second Hospital Medical Ethics Committee reviewed and approved this study (approval number: 2021A-516). All patients provided written informed consent. This study had a cohort of 40 patients diagnosed with metastatic CC, ranging in

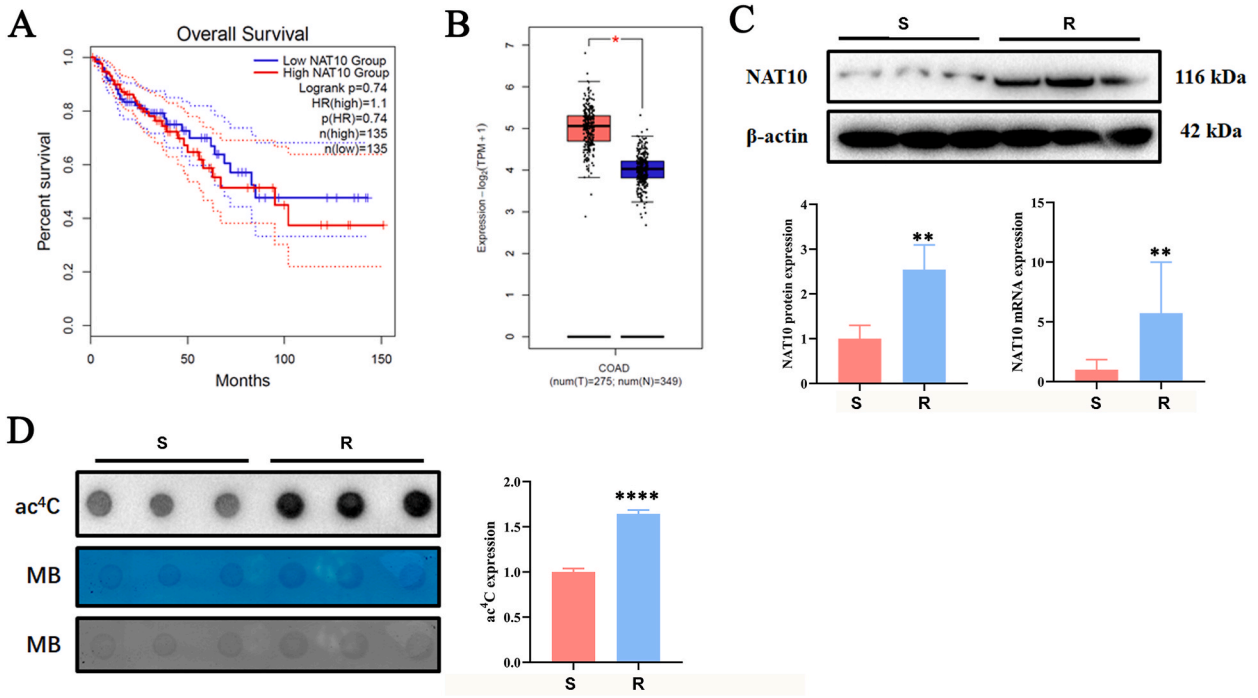

Fig. 1. NAT10 protein expression is examined in the public database or CC tissues. **A**, The graph shows the results of the Kaplan-Meier analysis of the overall survival (OS) rate in CC patients with high or low expression of NAT10. **B**, NAT10 protein expression was assessed using data from the TCGA and GTEx databases. **C**, The expression of NAT10 proteins and mRNA was assessed by protein scores in chemoresistant CC tissues and chemosensitive CC tissues. **D**, Dot blot images showing ac^4C abundance in chemoresistant CC tissues and chemosensitive CC tissues. Data are shown as the mean ± SD of three replicates (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$). S, chemosensitive. R, chemoresistance.

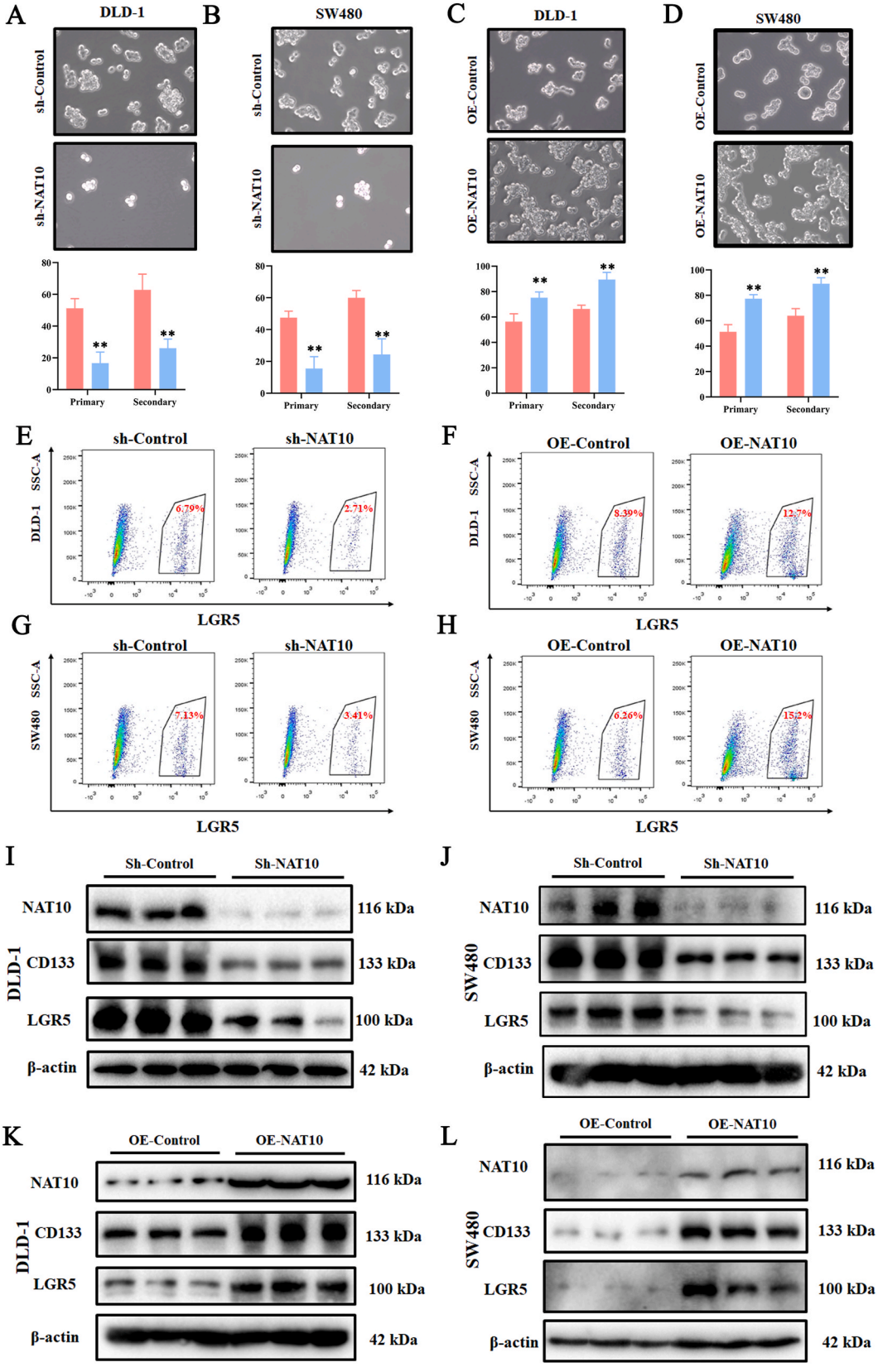

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Fig. 2. NAT10 maintains the stemness of CC cells. **A-D**, Representative images of sphere formation induced by the transfection of sh-NAT10 into DLD-1 and SW480 cells or the transfection of a NAT10 overexpression into DLD-1 and SW480 cells. The surviving colonies were measured for the number of tumorspheres. **E-H**, Flow cytometry was used to assess the percentage of $LGR5^{high}$ cells in CC cells with NAT10 depletion or overexpression. **I-J**, The expression levels of CSC markers, including CD133 and LGR5, were examined in sh-NAT10-transfected CC cells by Western blotting. **K-L**, The expression levels of CSC markers, including CD133 and LGR5, were examined in NAT10 overexpression plasmid-transfected CC cells by Western blotting. Data are shown as the mean ± SD of three replicates (**, $P < 0.01$).

age from 30 to 80 years. The Department of General Surgery administered oxaliplatin as the initial chemotherapy treatment to all patients from 2016 to 2018. Out of these patients, 13 received surgery either pre or post-receiving chemotherapy. The Tumor Regression Grade Classification System was used to assess the responses to chemotherapy. Our study categorized patients into chemoresistant (R) and chemosensitive (S) groups based on their response to chemotherapy.

2.2. Animals

Male BALB/c nude mice (six weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and raised in a controlled environment free from specific pathogens. Moreover, we subcutaneously injected Sh-NAT10 or OE-NAT10 transfected cancer cells, together with control cells, into the mice flank regions (six mice/group). Tumor development in the mice was monitored for a duration of 4 weeks by quantifying the volume of the tumors. The tumors were surgically removed, and their weight was measured. The Lanzhou University Second Hospital Animal Welfare Ethics Review Committee reviewed and approved the animal experiments (approval number: D2021-272).

2.3. Cell lines and cell culture

The CC cell lines (SW480 and DLD-1 cells) procured from the American Type Culture Collection (Manassas, VA. USA) were subjected to culture in RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) containing 10 % fetal bovine serum along with 1 % antibiotic/antimycotic solution (Gibco, USA) and incubated at 37 °C in 5 % CO_2.

2.4. Cell transfection

Plasmids for NAT10 knockdown (sh-NAT10) and overexpression (OE-NAT10) were synthesized directly (GenePharma, Shanghai, China). sh-NAT10 and OE-NAT10 vectors were delivered via lentiviral infection into SW480 and DLD-1 cells. Furthermore, cells that were infected using lentiviruses with scrambled plasmids were utilized as negative controls. After a span of 48 h, the cells were collected in order to conduct additional studies.

2.5. Spheroid formation assay

Overall, 1000 CC cells were subjected to plating on ultra-low attachment plates, followed by 10-day culture in RPMI-1640 medium (Invitrogen, 81 Wyman Street, Waltham, MA, USA) that contained 4 mg/mL insulin (Sigma-Aldrich, St. Louis, Missouri, USA), B27 (1:50, Gibco), 20 ng/mL epidermal growth factor (Sigma-Aldrich), and 20 ng/mL basic fibroblast growth factor (Sigma-Aldrich). For serial passaging, the primary spheres were gathered, separated using trypsin, resuspended in RPMI-1640 medium containing the mentioned supplements, and then placed on a plate to produce secondary spheroids. The sphere number was enumerated using a microscope.

2.6. RNA immunoprecipitation (RIP)

We carried out RIP using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) per the instructions. In brief, 5 μg of anti-NAT10 antibody-coated magnetic beads (ab194297, Abcam, Cambridge, UK) went through overnight incubation with prepared cell lysates at 4 °C. Subsequently, we rinsed the RNA-protein complexes six times subsequent with incubation using proteinase K digestion buffer. Standard phenol-chloroform RNA extraction method were performed for RNA extraction while utilizing quantitative polymerase chain reaction (qPCR) to determine relative RNA expression and normalize to the input.

2.7. ac^4C-RIP

Total RNA purification from cancer cells (150 μg) was conducted through an RNA extraction kit, fragmenting these purified samples into nearly 200 nt pieces using 10 × fragmentation buffer (100 mM Tris-HCl, 100 mM $ZnCl_2$ in nuclease-free H_2O) followed by

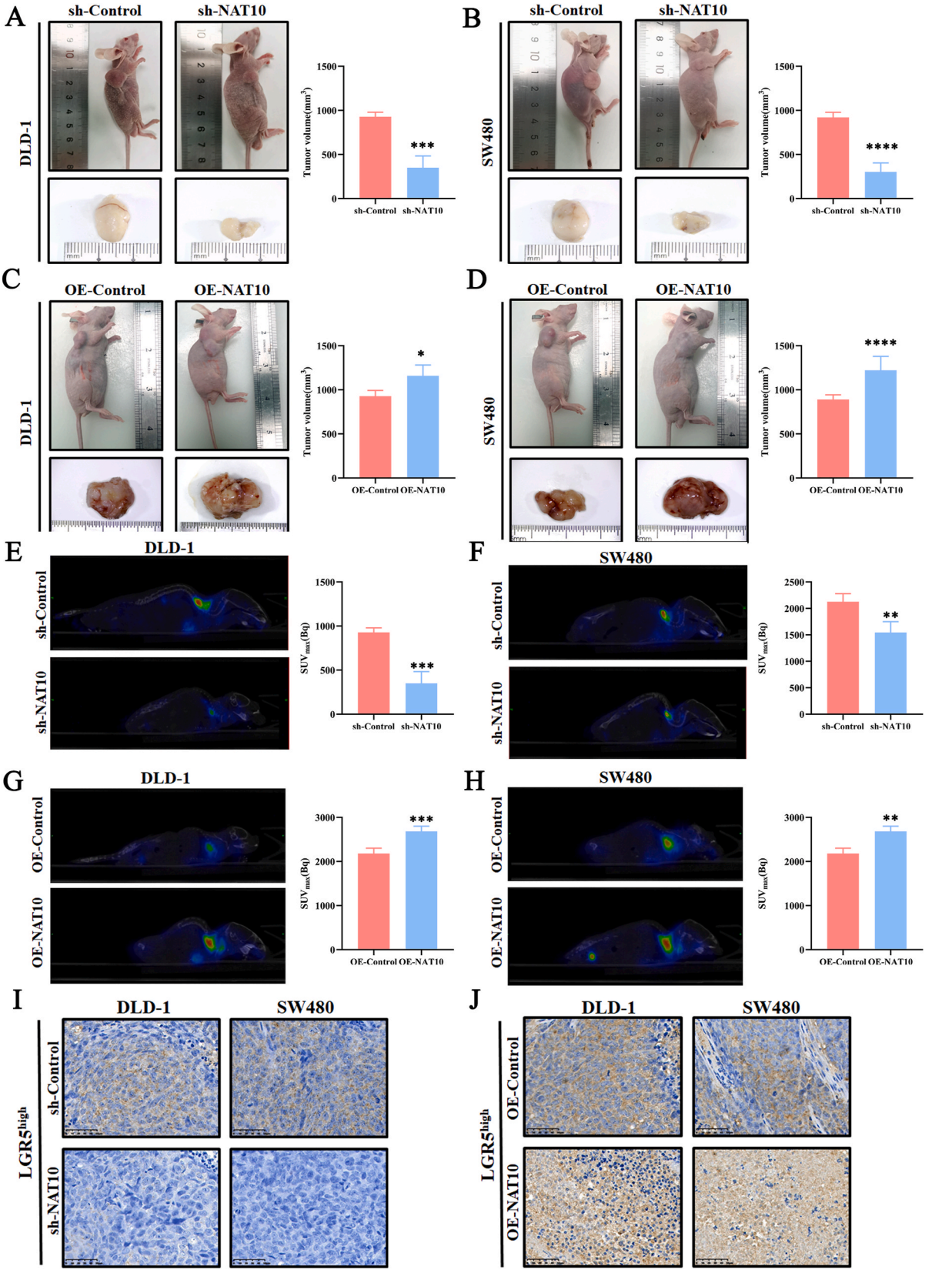

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Fig. 3. NAT10 promotes CC cell-mediated tumor formation. **A-D**, Tumor formation in nude mice injected with sh-NAT10-transfected DLD-1 and SW480 cells (5×10^4 cells per mouse) or the transfection of a NAT10 overexpression into DLD-1 and SW480 cells. The incidence of tumor formation was monitored for 40 days. **E-H**, Representative ^{18}F-FDG micro-PET/CT images in nude mice injected with sh-NAT10-transfected DLD-1 and SW480 cells or the transfection of a NAT10 overexpression into DLD-1 and SW480 cells. SUV_{max}, maximum standard uptake value. **I-J**, The levels of $LRG5^{high}$ expression were assessed by IHC in different groups. Data are shown as the mean ± SD of three or four replicates (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

incubation for 6 min at 70 °C. Ethylenediaminetetraacetic acid was introduced immediately thereafter to cease the reaction. Our study employed a Zymo RNA clean and concentrator-25 kit (Irvine, CA, USA) for further purification and recovery of the fragmented RNA. Herein, we utilized anti-ac^{4}C (ab252215, Abcam) and control rabbit immunoglobulin G (IgG) antibodies, aiming at interaction with the RNA's ac^{4}C modification sites. RIP was conducted through Pierce™ Protein A/G Magnetic Beads (88,802, Thermo Fisher Scientific, Waltham, MA, USA) per the protocols.

2.8. mRNA stability assay

An actinomycin D assay was conducted aiming at assessing mRNA stability. Herein, we utilized cells cultured in a complete medium as the control group, adding 200 nM actinomycin D (a transcription inhibitor) to the culture medium for co-culturing with the cells. Upon subjecting the cells to actinomycin D treatment for 0, 2, 4, and 6 h, we proceeded to harvest the cells to extract RNA. Reverse transcription qPCR (RT-qPCR) was carried out on total RNA to determine NANOGP8 mRNA levels at each time point; the mRNA half-life ($t_{1/2}$) was determined via measuring RNA initial and final quantities.

2.9. RNA isolation and RT-qPCR

Our study isolated total RNA from murine colon cells and tissues through TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the protocols. For reverse transcription, when utilized the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan). Furthermore, complementary DNA was generated from microRNAs (miRNAs) employing the Mir-X miRNA First-Strand Synthesis Kit (Takara, Kyoto, Japan). Herein, we deployed TB Green Premix Ex Taq II (Takara Bio) for conducting RT-qPCR, setting 18S as an endogenous control. Supplementary Table summarizes primer sequences.

2.10. ac^{4}C dot blot

The experimental group-collected total RNA samples were diluted to an equal concentration and subjected to heat at 95 °C for 3 min, thereby uniformly distributing 2 μl of the diluted RNA onto a Hybond-N+ membrane (#RPN203B, GE Healthcare, Chicago, IL, USA) and cross-linking by employing a Stratalinker 2400 UV Crosslinker (1,200 μJ, 5 min; Agilent Technologies, Santa Clara, CA, USA). The membrane went through blockage utilizing 5 % bovine serum albumin (BSA) besides overnight incubation at 4 °C with an anti-ac^{4}C antibody (ab252215, Abcam). Afterward, the membrane was subjected to a 2-h incubation period with a secondary antibody, then processed and captured using imaging techniques.

2.11. Immunohistochemistry

The tissue sections underwent deparaffinization in xylene and were subsequently rehydrated utilizing a graded ethanol series. The sections were immersed in a solution containing 0.3 % peroxidase and methanol for a duration of 30 min in order to suppress the endogenous peroxidase activity. Following this, the sections were pretreated using citrate buffer for 15 min in a microwave oven at 100 °C to facilitate antigen retrieval and were subjected to overnight incubation at 4 °C with antibodies targeting caspase 3 (1:1000, ab184787, Abcam) and LGR5 (1:300, ab219107, Abcam), and subsequently underwent secondary antibody incubation. The sections that had stains were further stained with hematoxylin, and images were taken with an Olympus BX51 microscope (Tokyo, Japan).

2.12. Western blotting

Our study homogenized cells in ice-cold lysis buffer and centrifuged at 12,000×*g* for 20 min and at 4 °C. Lysates (50 μg protein/lane) went through separation by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA. USA). Subsequently, we blocked the membranes utilizing 5 % BSA for 1 h, cut them per each protein molecular weight, and incubated them incubation at 4 °C with the corresponding primary antibodies for a whole night. Our study employed these primary antibodies: anti-LGR5 antibody (ab75850, Abcam), anti-CD133 (ab284389, Abcam), anti-ac^{4}C (ab252215, Abcam), anti-beta Actin (ab8226, Abcam), and anti-NAT10 (ab194297, Abcam) antibodies. Subsequently, the membranes were investigated utilizing horseradish peroxidase-labeled goat anti-rabbit IgG (H + L) (A0208, Beyotime, Jiangsu, China) at room

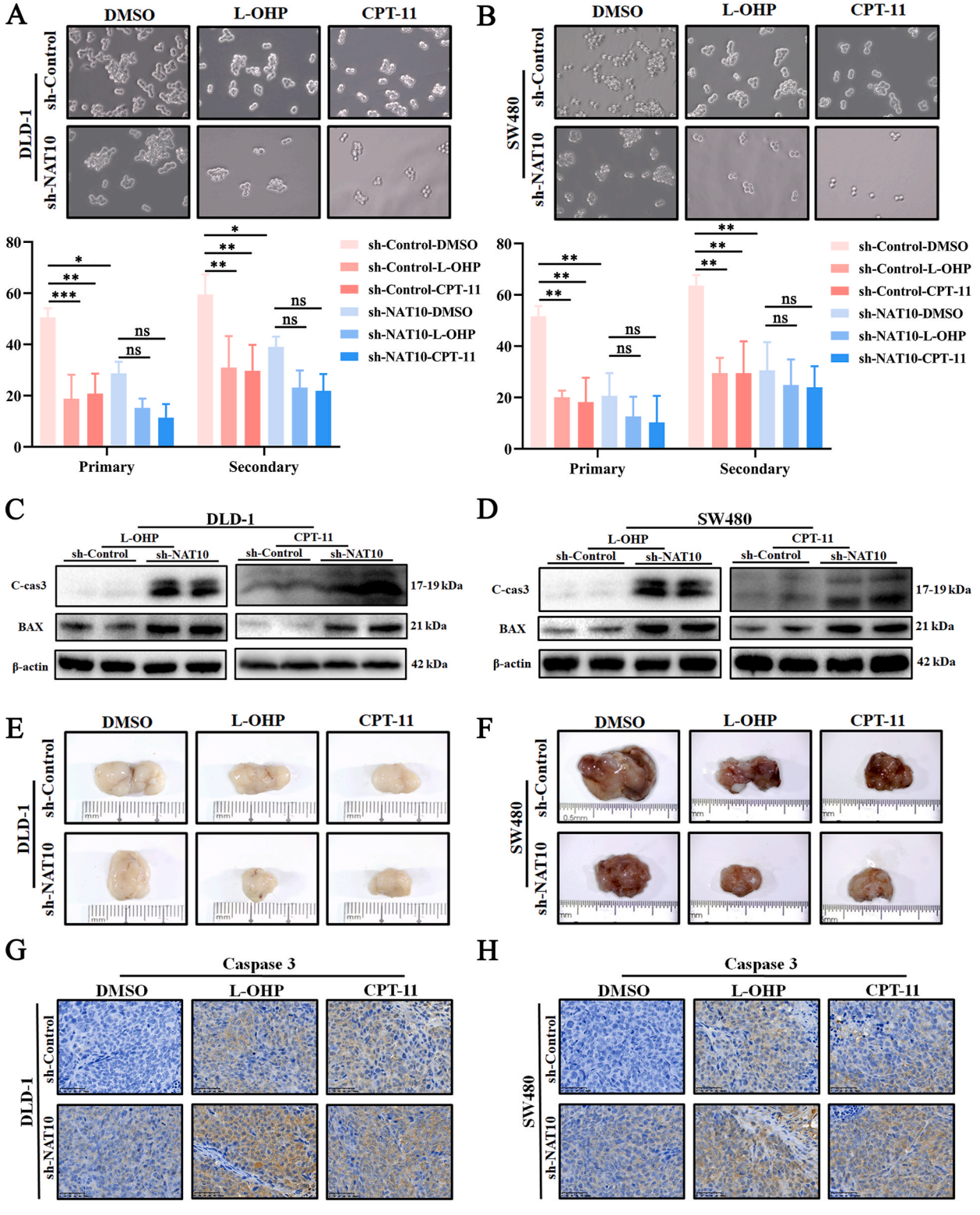

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Fig. 4. NAT10 suppresses the chemosensitivity of CC Cells to L-OHP or CPT-11. **A and B**, Colony formation in sh-NAT10-transfected CC cells after treatment with L-OHP or CPT-11 at a concentration of 10 μmol/L. **C**, The expression levels of apoptosis markers, including cleaved caspase-3 and BAX, were examined in sh-NAT10-transfected DLD-1 cells after treatment with L-OHP or CPT-11 by Western blotting. **D**, Western blotting was used to detect the expression levels of cleaved caspase-3 and BAX in sh-NAT10-transfected SW480 cells after treatment with L-OHP or CPT-11. **E-F**, Tumor formation in nude mice injected with sh-NAT10-transfected DLD-1 and SW480 cells after treatment with L-OHP or CPT-11. **G-H**, The levels of Caspase 3 expression were assessed by IHC in different groups. The incidence of tumor formation was monitored for 40 days. Data are shown as the mean ± SD of three replicates (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

temperature for 2 h employing an enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA), we visualized protein bands per the protocols. ACTB was utilized as a loading control, performing protein expression quantification using densitometry.

2.13. Statistical analysis

Statistical analyses were conducted through GraphPad Prism 8.0 (La Jolla, CA, USA) and SPSS 18.0 (Chicago, IL, USA), expressing data as mean ± SD of at least three biological replicates. Data normality was determined by employing the Shapiro–Wilk test. Furthermore, we evaluated differences between the two groups utilizing the Student's t-test and Mann–Whitney *U* test for normally and non-normally distributed data, respectively. $P < 0.05$ deemed statistical significance.

3. Results

3.1. NAT10 expression levels and ac^4C modification are increased in chemoresistant CC tissues

Herein, we accessed The Cancer Genome Atlas (TCGA) database for NAT10 mRNA expression profile analysis in normal colon (n = 349) and CC tissues (n = 275). NAT10 was significantly expressed in CC tissues (Fig. 1A). Moreover, patients with high NAT10 CC expression exhibited a decreased 150-month survival rate (Fig. 1B). Next, we investigated NAT10 expression and ac^4C modification in CC tissues according to chemoresistance status, revealing their elevation in chemoresistant than in chemosensitive CC tissues (Fig. 1C and D and Supplementary Fig. 1).

3.2. NAT10 sustains CC cell stemness properties

Given the intrinsic chemoresistance of CSCs, our study examined the NAT10 effect on CC cell stemness. Inhibiting NAT10 reduced the CC cell (DLD-1 and SW480) ability of primary and secondary spheroid formation in comparison to the control (Fig. 2A and B), which was conversed by overexpressing NAT10 (Fig. 2C and D). Downregulation of NAT10 decreased the proportion of $LGR5^{high}$ cells as determined using flow cytometry (Fig. 2E and F). However, NAT10 upregulation increased the proportion of $LGR5^{high}$ cells (Fig. 2G and H). Western blotting revealed that the stemness marker expressions CD133 and LGR5 were increased by NAT10 overexpression (Fig. 2I–J and Supplementary Fig. 2 A-D), whereas the suppression of NAT10 inhibited the expression of these stemness markers (Fig. 2K-L and Supplementary Fig. 2 E-H). In addition, these stemness marker expressions were examined through RT-PCR. The results further confirmed that NAT10 inhibition downregulated CD24/44/133/166 and LGR5 in CC cells (Supplementary Fig. 3A and B), while NAT10 overexpression promotes CD24/44/166 and LGR5 expression in CC cells (Supplementary Fig. 3C and D).

3.3. NAT10 promotes CC growth, metabolism, and activity

NAT10's involvement in CC cell stemness was investigated more deeply by subcutaneously inoculating cells into NOD/SCID mice. Tumor growth in NOD/SCID mice that received DLD-1 and SW480 cell injection with NAT10 knockdown was significantly decreased (Fig. 3A and B), whereas NAT10 overexpression promoted CC growth *in vivo* (Fig. 3C and D). Furthermore, CC metabolism and activity, assessed using 18F-FDG coincidence imaging, were decreased by NAT10 knockdown (Fig. 3E and F), whereas NAT10 overexpression promoted CC metabolism and activity (Fig. 3G and H). Immunohistochemical examination revealed decreased numbers of $LGR5^{high}$ cells in DLD-1 and SW480 cells upon NAT10 knockdown, albeit increased upon NAT10 overexpression (Fig. 3I and J).

3.4. Suppression of NAT10 alleviates CC cell chemoresistance to L-OHP and CPT-11

The decreased primary and secondary spheroid formation showed that NAT10 inhibition sensitized CC cells to L-OHP (10 μM) and CPT-11 (10 μM) (Fig. 4A and B). In addition, we observed NAT10 knocking down impact on L-OHP and CPT-11 therapeutic effects. L-OHP and CPT-11 treatment effects were up-regulated at the apoptosis level of DLD-1 (Fig. 4C and Supplementary Fig. 4A) and SW480

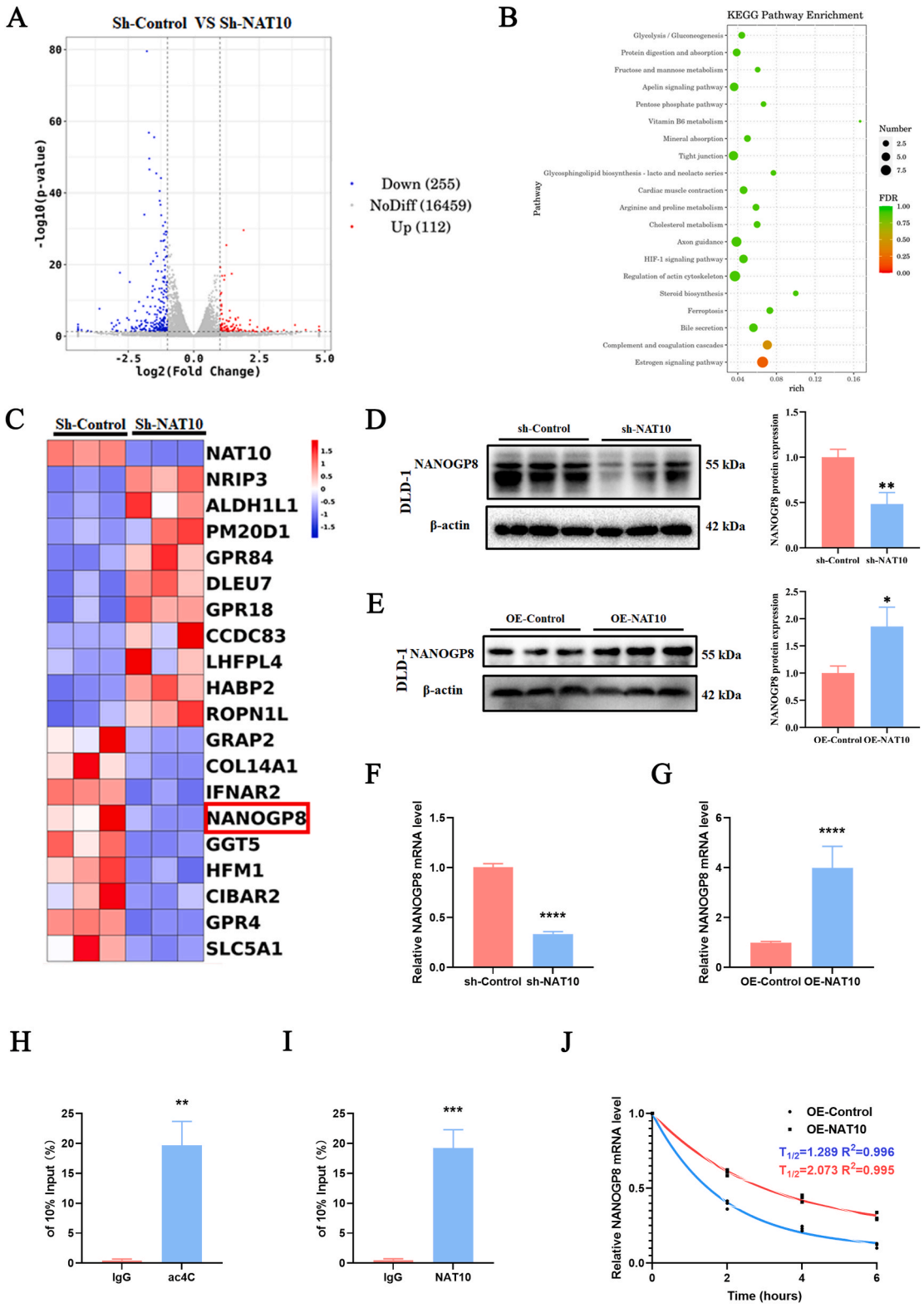

Fig. 5. RNA-seq revealed potential target genes of NAT10 in CC Cells. **A**, RNA-seq showed the differentially expressed genes after NAT10 depletion. **B**, RNA-seq statistics of in CC cells after NAT10 depletion-related genes KEGG Enrichment. **C**, The top 20 differentially expressed genes in CC cells after NAT10 deletion. **D and E**, The NANOGP8 protein expression levels of sphere formation induced by the transfection of sh-NAT10 into DLD-1 cells or the transfection of a NAT10 overexpression plasmid into DLD-1 cells. **F and G**, The expression level of NANOGP8 mRNA was induced by

transfection of sh-NAT10 into DLD-1 cells or transfection of NAT10 overexpression plasmid into DLD-1 cells. **H**, RIP analysis using ac^{4}C antibody of DLD-1 cells products by RT-qPCR. **I**, RIP analysis using NAT10 antibody of DLD-1 cells products by RT-qPCR. **J**, RT-qPCR showing the half-life of NANOGP8-mRNA by monitoring the transcript abundance after transcriptional inhibition with actinomycin D at different time points in NAT10 overexpression transfection (non-linear regression). Data are shown as the mean ± SD of three replicates (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

(Fig. 4D and Supplementary Fig. 4B) cells after NAT10 inhibition, further demonstrating that NAT10 inhibition raised CC cell sensitivity to L-OHP and CPT-11. Aiming to further investigate whether NAT10 knockdown promoted chemosensitivity, we injected mice with DLD-1 and SW480 cells that had been modified to either have NAT10 knockdown or not. The growth of the tumor was prevented by intraperitoneally injecting L-OHP or CPT-11. Additionally, when NAT10 was suppressed, the tumor volume was further reduced (Fig. 4E and F and Supplementary Fig. 5). Immunohistochemical examination revealed increased numbers of caspase 3^+ cells in DLD-1 and SW480 cells following L-OHP or CPT-11 treatment, with NAT10 knockdown further elevating their numbers (Fig. 4G and H).

3.5. NAT10-mediated ac^{4}C modification enhances NANOGP8 mRNA stability

To identify NAT10 targets, we conducted RNA-Seq on RNA isolated from sh-NC or sh-NAT10-transfected SW480 cells. Knocking down NAT10 upregulated and downregulated 112 and 255 genes, respectively (Fig. 5A). Kyoto Encyclopedia of Genes and Genomes analysis findings suggested that NAT10 knockdown significantly altered tumor metabolic pathways (Fig. 5B). Given the pivotal role of NAT10 in CSCs, it is plausible that the tumor stem gene NANOGP8 is a downstream target of NAT10 (Fig. 5C). OE-NAT10 upregulated NANOGP8 mRNA and protein expression, whereas sh-NAT10 downregulated NANOGP8 mRNA and protein (Fig. 5D–G, and Supplementary Fig. 6). Furthermore, ac^{4}CRIP was conducted on SW480 cells using an IgG control or anti-ac [4]C antibody. Immunoprecipitation with the anti-ac^{4}C antibody significantly elevated NANOGP8 mRNA levels compared to those obtained with the IgG control antibody (Fig. 5H). High enrichment of *NANOGP8* mRNA was also obtained following immunoprecipitation using the anti-NAT10 antibody (Fig. 5I). Following NAT10 overexpression, we assessed *NANOGP8* mRNA levels at 0, 2, 4, and 6 h post actinomycin D treatment to inhibit RNA polymerase activity. RT-qPCR analysis revealed that NAT10 overexpression extended *NANOGP8* mRNA half-life in comparison to that in the control group (Fig. 5J).

3.6. NAT10 promotes CC cell stemness and chemoresistance via NANOGP8

NANOGP8 inhibition effectively reversed the increased stemness induced by NAT10 in CC cells, as indicated by the decreased primary and secondary spheroid formation ability and downregulated expression of the stemness markers CD133 and LGR5 (Fig. 6A–D and Supplementary Fig. 7A). The findings of flow cytometry depicted that NAT10 overexpression elevated LGR5^{high} cell proportion, whereas NANOGP8 downregulation reversed this effect (Fig. 6E and F). NAT10 overexpression raised CC cell chemoresistance to L-OHP, as indicated by increased apoptotic protein expression. Conversely, the suppression of NANOGP8 alleviated chemoresistance (Fig. 6G–H and Supplementary Figs. 7B–C).

3.7. NAT10-specific inhibitor remodelin facilitates CC cell chemosensitivity

Remodelin inhibited CC cell stemness properties, as reflected by decreased primary and secondary spheroid formation capability and the proportions of LGR5^{high} cells (Fig. 7A–D). Remodelin promoted CC chemosensitivity to L-OHP and CPT-11 by reducing tumor volume (Fig. 7E and F) and increasing apoptosis-related protein expression (Fig. 7G–H and Supplementary Fig. 8). Additionally, the results of caspase 3 staining were consistent with those of the apoptotic proteins (Fig. 7I and J).

4. Discussion

CSCs are a self-renewing cancer cell subpopulation that drives tumor growth, metastasis, and chemoresistance [19,20]. Increasing evidence suggests that to overcome the limitations of current therapies, it is imperative to consider CSCs when designing CC treatments [21]. Our study manifested that NAT10 is involved in colon CSCs, thereby enhancing CSC stemness and chemoresistance. Mechanistically, NAT10-mediated ac^{4}C modification promoted the stability of *NANOGP8* mRNA, thereby upregulating NANOGP8 expression. Furthermore, Remodelin, a specific NAT10 inhibitor, effectively mitigated chemoresistance in CC and enhanced the tumor cytotoxicity of chemotherapy agents. Our data suggest that the NAT10/NANOGP8 axis could be a viable target for treating colon CSCs and overcoming chemoresistance.

NAT10 is associated with various poor tumor prognoses, including that of CC [7,15,22]. However, the mechanisms by which NAT10 maintains stemness and chemoresistance in CSCs remain unclear. NAT10 is highly expressed in CC tissues, according to data

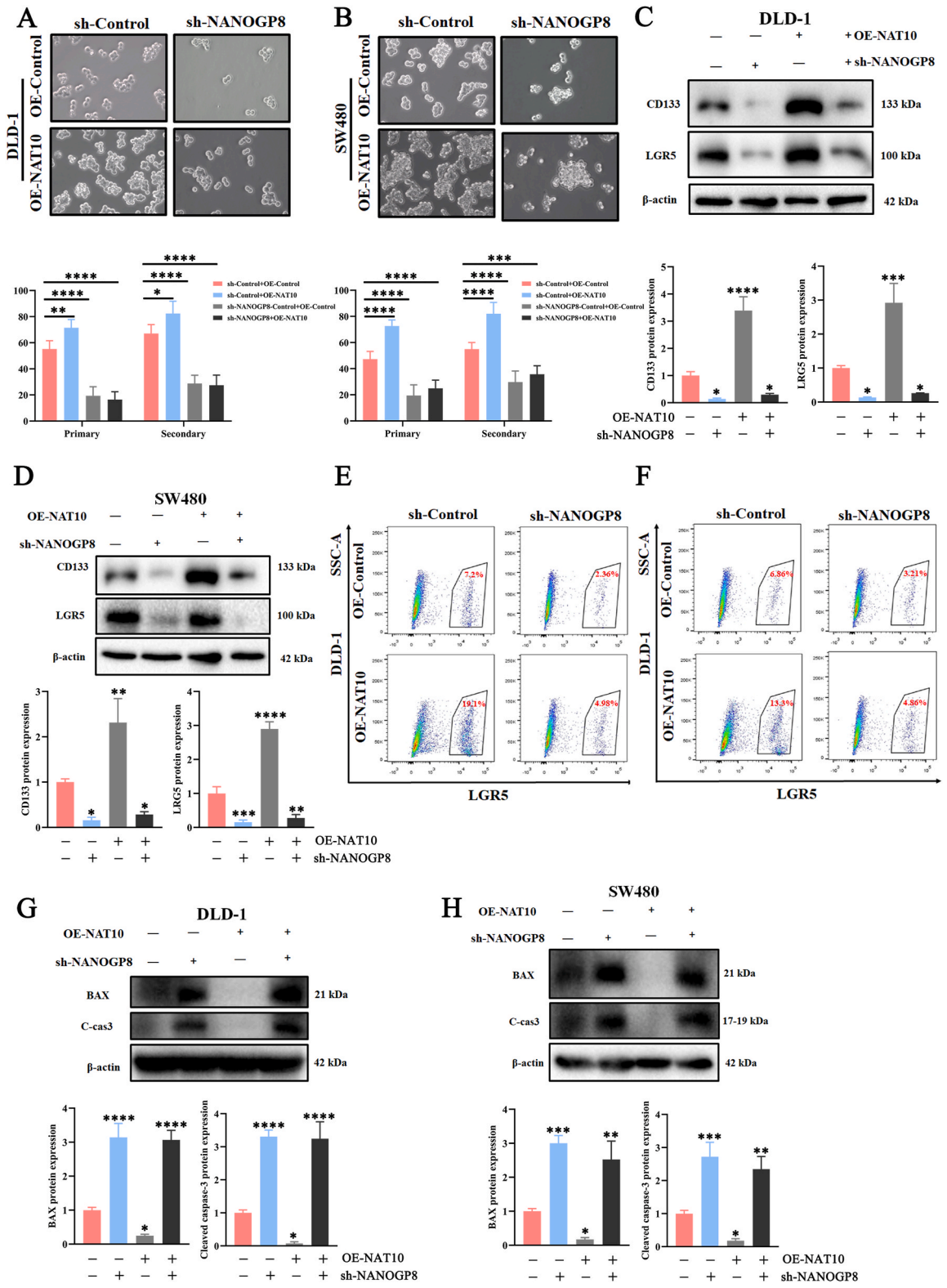

Fig. 6. NAT10 mediates LRG5 transcription by affecting the stability of NANOGP8. **A and B**, Representative images of sphere formation induced by the transfection of sh-NANOGP8 into DLD-1 and SW480 cells after NAT10 overexpression. The surviving colonies were measured for the number of tumorspheres. **C and D**, The expression levels of CSC markers, including CD133 and LGR5, were examined after sh-NANOGP8 transfection of sh-NANOGP8 and NAT10 overexpression into CC cells by Western blotting. **E and F**, Flow cytometry was used to assess the percentage of LGR5^{high} cells

in CC cells after sh-NANOGP8 transfection of sh-NANOGP8 and NAT10 overexpression. **G and H**, The expression levels of apoptosis markers, including cleaved caspase-3 and BAX, were examined in NANOGP8 and NAT10 overexpression-transfected CC cells by Western blotting. Data are shown as the mean ± SD of three replicates (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

from TCGA; additionally, patients with CC exhibiting high NAT10 expression demonstrate reduced overall survival time. Moreover, the expression of NAT10 was elevated within chemoresistant than in chemosensitive CC tissues. NAT10 increased the ability of CC cells to form primary and secondary spheroids, as well as the stemness marker expressions CD133 and LGR5, in SW480 and DLD-1 CC cell lines. Furthermore, chemoresistance is a hallmark of CSC stemness [23]. Consistent with this, NAT10 displayed a more robust resistance to apoptosis induced by L-OHP and CPT-11, which are commonly used in clinical settings against CC. *In vivo*, inhibition of NAT10 decreased tumor formation and metabolic ability and increased chemosensitivity. Notably, previous studies reported that NAT10 promotes CC progression by inhibiting ferroptosis [7] and microsatellite status [24]. Alternatively, we report here for the first time that NAT10 is closely related to the stemness and chemoresistance of CC. Thus, our findings supplement the NAT10's known roles in CC progression.

To acquire an in-depth knowledge of how NAT10 participates in colon CSC preservation, we compared the expression profiles between NAT10-knockdown and control SW480 cells. Transcriptomic analysis revealed a significant reduction in *NANOGP8* expression. *NANOGP8*, a pseudogene belonging to the NANOG gene family, exhibits exclusive expression in cancerous tissues, including glioblastoma multiforme, leukemia, hepatocellular carcinoma, prostate, lung, and colorectal cancers [25–28]. In gastric cancer cell lines, NANOGP8 overexpression significantly upregulates CSC markers, including LGR5, and enhances sphere formation, clonogenic capacity, and chemoresistance [25,29]. Our findings suggest that NAT10 functions as an enzyme mediating ac^4C modification through direct binding to *NANOGP8* sequence-characterized regions. NAT10 upregulated NANOGP8 expression by promoting *NANOGP8* mRNA stability. Conversely, NANOGP8 inhibition inverted NAT10 positive impacts on stemness maintenance and chemoresistance in CC cell lines. Our findings indicate that stabilizing *NANOGP8* transcripts through NAT10-mediated ac^4C modification is critical for colon CSC stemness. In comparison, Zheng et al. showcased that NAT10 enhances CC progression by impeding ferroptosis via ac^4C modification and FSP1 stabilization [7]. The different mechanisms of NAT10 in CC emphasize the significance of NAT10 as a therapeutic target. A limitation of this study was that we only evaluated *NANOGP8* mRNA stability as affected by ac^4C modification rather than by other pathways of RNA metabolism, such as mRNA translation.

To confirm the potential targetability of NAT10 in CC, we employed Remodelin, which suppresses the capabilities of the cell to proliferate and migrate besides inducing cell cycle arrest and apoptosis in multiple cancer cells [30]. Herein, Remodelin inhibited the maintenance of stemness and chemoresistance in CC cell lines and significantly suppressed CC tumor growth and chemoresistance *in vivo*. Moreover, Remodelin effectively prevented CC tumor progression and enhanced the therapeutic benefits of chemotherapy. Together, these findings provide empirical evidence for the future clinical implementation of NAT10 inhibition.

Collectively, we elucidated that NAT10 and ac^4C modifications are upregulated in chemoresistant CC tissues. NAT10 promoted stemness maintenance and chemoresistance in CC cells by increasing *NANOGP8* stability and expression. NAT10 inhibition by Remodelin synergistically enhanced the efficacy of chemotherapy and suppressed CC progression, suggesting its potential as an adjunct therapy for CC.

Ethical statement

Approval for this study wan obtained from The Lanzhou University Second Hospital Medical Ethics Committee (approval number: 2021A-516) and The Lanzhou University Second Hospital Animal Welfare Ethics Review Committee (approval number: D2021-272).

Data availability statements

The data are obtainable upon logical request from the corresponding authors.

CRediT authorship contribution statement

Li-ping Gao: Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Ting-dong Li:** Methodology, Formal analysis, Data curation. **Su-zhen Yang:** Methodology, Formal analysis, Data curation. **Hui-min Ma:** Methodology, Formal analysis, Data curation. **Xiang Wang:** Writing – review & editing, Writing – original draft. **De-kui Zhang:** Writing – review & editing, Writing – original draft, Funding acquisition, Data curation.

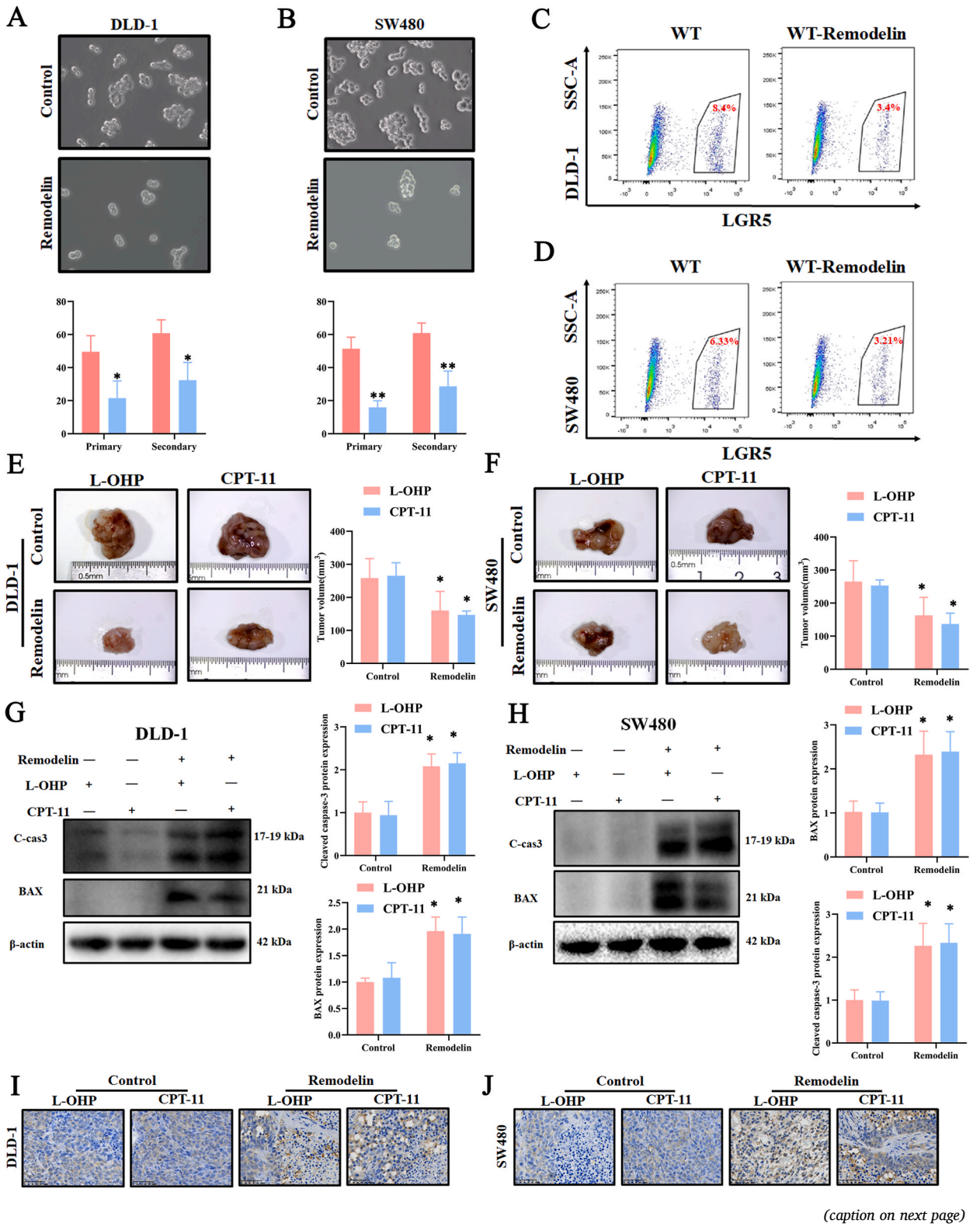

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Fig. 7. NAT10 inhibitor (Remodelin) inhibits the stemness of CC Cells. **A and B**, Representative images of sphere formation in DLD-1 and SW480 cells after remodelin treatment. The surviving colonies were measured for the number of tumorspheres. **C and D**, Flow cytometry was used to assess the percentage of $LGR5^{high}$ cells after treatment with remodelin. **E and F**, Tumor formation in nude mice injected with DLD-1 and SW480 cells after treatment with L-OHP, CPT-11, or remodelin. The incidence of tumor formation was monitored for 40 days. **G and H**, The expression levels of apoptosis markers, including cleaved caspase-3 and BAX after treatment with L-OHP, CPT-11, or remodelin by Western blotting. **I and J**, The levels of Caspase 3 expression were assessed by IHC in different groups. Data are shown as the mean ± SD of three replicates (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30330.

References

[1] J. Bien, A. Lin, A review of the diagnosis and treatment of metastatic colorectal cancer, JAMA 325 (23) (Jun 15 2021) 2404–2405, https://doi.org/10.1001/jama.2021.6021.
[2] J.H. Strickler, T. Yoshino, R.P. Graham, S. Siena, T. Bekaii-Saab, Diagnosis and treatment of ERBB2-positive metastatic colorectal cancer: a review, JAMA Oncol. 8 (5) (May 1 2022) 760–769, https://doi.org/10.1001/jamaoncol.2021.8196.
[3] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2020, CA A Cancer J. Clin. 70 (1) (Jan 2020) 7–30, https://doi.org/10.3322/caac.21590.
[4] Y.J. Chen, W.F. Hong, M.L. Liu, et al., An integrated bioinformatic investigation of mitochondrial solute carrier family 25 (SLC25) in colon cancer followed by preliminary validation of member 5 (SLC25A5) in tumorigenesis, Cell Death Dis. 13 (3) (Mar 14 2022) 237, https://doi.org/10.1038/s41419-022-04692-1.
[5] J. Goc, M. Lv, N.J. Bessman, et al., Dysregulation of ILC3s unleashes progression and immunotherapy resistance in colon cancer, Cell 184 (19) (Sep 16 2021) 5015–5030 e16, https://doi.org/10.1016/j.cell.2021.07.029.
[6] A. Osman, B. Yan, Y. Li, et al., TCF-1 controls T(reg) cell functions that regulate inflammation, CD8(+) T cell cytotoxicity and severity of colon cancer, Nat. Immunol. 22 (9) (Sep 2021) 1152–1162, https://doi.org/10.1038/s41590-021-00987-1.
[7] X. Zheng, Q. Wang, Y. Zhou, et al., N-acetyltransferase 10 promotes colon cancer progression by inhibiting ferroptosis through N4-acetylation and stabilization of ferroptosis suppressor protein 1 (FSP1) mRNA, Cancer Commun. 42 (12) (Dec 2022) 1347–1366, https://doi.org/10.1002/cac2.12363.
[8] N. Li, Y. Li, H. Gao, et al., Forkhead-box A3 (FOXA3) represses cancer stemness and partially potentiates chemosensitivity by targeting metastasis-associated in colon cancer 1 (MACC1) signaling pathway in colorectal cancer cells, Curr. Cancer Drug Targets (Dec 7 2020), https://doi.org/10.2174/1568009620666201207150632.
[9] L. Wang, J. Guo, J. Zhou, D. Wang, X. Kang, L. Zhou, NF-κB maintains the stemness of colon cancer cells by downregulating miR-195-5p/497-5p and upregulating MCM2, J. Exp. Clin. Cancer Res. : CR 39 (1) (Oct 28 2020) 225, https://doi.org/10.1186/s13046-020-01704-w.
[10] R. Bharti, G. Dey, M. Mandal, Cancer development, chemoresistance, epithelial to mesenchymal transition and stem cells: a snapshot of IL-6 mediated involvement, Cancer Lett. 375 (1) (May 28 2016) 51–61, https://doi.org/10.1016/j.canlet.2016.02.048.
[11] C. Rodriguez-Ramirez, Z. Zhang, K.A. Warner, et al., p53 inhibits Bmi-1-driven self-renewal and defines salivary gland cancer stemness, Clin. Cancer Res. 28 (21) (Nov 1 2022) 4757–4770, https://doi.org/10.1158/1078-0432.Ccr-22-1357.
[12] L. Bessler, L.M. Vogt, M. Lander, et al., A new bacterial adenosine-derived nucleoside as an example of RNA modification damage, Angew. Chem. 62 (11) (Mar 6 2023) e202217128, https://doi.org/10.1002/anie.202217128.
[13] A. Kim, G.G. Wang, R-loop and its functions at the regulatory interfaces between transcription and (epi)genome, Biochim. Biophys. Acta Gene Regul. Mech. 1864 (11–12) (2021) 194750, https://doi.org/10.1016/j.bbagrm.2021.194750.
[14] S. Leimkühler, M. Bühning, L. Beilschmidt, Shared sulfur mobilization routes for tRNA thiolation and molybdenum cofactor biosynthesis in prokaryotes and eukaryotes, Biomolecules 7 (1) (Jan 14 2017), https://doi.org/10.3390/biom7010005.
[15] G. Wang, M. Zhang, Y. Zhang, et al., NAT10-mediated mRNA N4-acetylcytidine modification promotes bladder cancer progression, Clin. Transl. Med. 12 (5) (May 2022) e738, https://doi.org/10.1002/ctm2.738.
[16] D. Arango, D. Sturgill, N. Alhusaini, et al., Acetylation of cytidine in mRNA promotes translation efficiency, Cell 175 (7) (Dec 13 2018) 1872–1886 e24, https://doi.org/10.1016/j.cell.2018.10.030.
[17] L. Liao, Y. He, S.J. Li, et al., Lysine 2-hydroxyisobutyrylation of NAT10 promotes cancer metastasis in an ac4C-dependent manner, Cell Res. (Mar 8 2023), https://doi.org/10.1038/s41422-023-00793-4.
[18] G. Jin, M. Xu, M. Zou, S. Duan, The processing, gene regulation, biological functions, and clinical relevance of N4-acetylcytidine on RNA: a systematic review, Mol. Ther. Nucleic Acids 20 (Jun 5 2020) 13–24, https://doi.org/10.1016/j.omtn.2020.01.037.
[19] M. Shimokawa, Y. Ohta, S. Nishikori, et al., Visualization and targeting of LGR5(+) human colon cancer stem cells, Nature 545 (7653) (May 11 2017) 187–192, https://doi.org/10.1038/nature22081.
[20] K. Xue, J.C. Wu, X.Y. Li, et al., Chidamide triggers BTG1-mediated autophagy and reverses the chemotherapy resistance in the relapsed/refractory B-cell lymphoma, Cell Death Dis. 12 (10) (Oct 1 2021) 900, https://doi.org/10.1038/s41419-021-04187-5.
[21] Z. Jahanafrooz, J. Mosafer, M. Akbari, M. Hashemzaei, A. Mokhtarzadeh, B. Baradaran, Colon cancer therapy by focusing on colon cancer stem cells and their tumor microenvironment, J. Cell. Physiol. 235 (5) (May 2020) 4153–4166, https://doi.org/10.1002/jcp.29337.
[22] H. Xu, B. Jiang, L. Meng, et al., N-alpha-acetyltransferase 10 protein inhibits apoptosis through RelA/p65-regulated MCL1 expression, Carcinogenesis 33 (6) (Jun 2012) 1193–1202, https://doi.org/10.1093/carcin/bgs144.
[23] K. Eun, S.W. Ham, H. Kim, Cancer stem cell heterogeneity: origin and new perspectives on CSC targeting, BMB Rep 50 (3) (Mar 2017) 117–125, https://doi.org/10.5483/bmbrep.2017.50.3.222.

[24] H. Zhang, W. Shan, Z. Yang, et al., NAT10 mediated mRNA acetylation modification patterns associated with colon cancer progression and microsatellite status, Epigenetics 18 (1) (Dec 2023) 2188667, https://doi.org/10.1080/15592294.2023.2188667.
[25] X. Ma, B. Wang, X. Wang, Y. Luo, W. Fan, NANOGP8 is the key regulator of stemness, EMT, Wnt pathway, chemoresistance, and other malignant phenotypes in gastric cancer cells, PLoS One 13 (4) (2018) e0192436, https://doi.org/10.1371/journal.pone.0192436.
[26] C.R. Jeter, B. Liu, X. Liu, et al., NANOG promotes cancer stem cell characteristics and prostate cancer resistance to androgen deprivation, Oncogene 30 (36) (Sep 8 2011) 3833–3845, https://doi.org/10.1038/onc.2011.114.
[27] N. Kawamura, K. Nimura, H. Nagano, S. Yamaguchi, N. Nonomura, Y. Kaneda, CRISPR/Cas9-mediated gene knockout of NANOG and NANOGP8 decreases the malignant potential of prostate cancer cells, Oncotarget 6 (26) (Sep 8 2015) 22361–22374, https://doi.org/10.18632/oncotarget.4293.
[28] M. Vaidya, J. Smith, M. Field, K. Sugaya, Analysis of regulatory sequences in exosomal DNA of NANOGP8, PLoS One 18 (1) (2023) e0280959, https://doi.org/10.1371/journal.pone.0280959.
[29] B. Wang, Q. Chen, Y. Cao, et al., LGR5 is a gastric cancer stem cell marker associated with stemness and the EMT signature genes NANOG, NANOGP8, PRRX1, TWIST1, and BMI1, PLoS One 11 (12) (2016) e0168904, https://doi.org/10.1371/journal.pone.0168904.
[30] R. Wei, X. Cui, J. Min, et al., NAT10 promotes cell proliferation by acetylating CEP170 mRNA to enhance translation efficiency in multiple myeloma, Acta Pharm. Sin. B 12 (8) (Aug 2022) 3313–3325, https://doi.org/10.1016/j.apsb.2022.01.015.