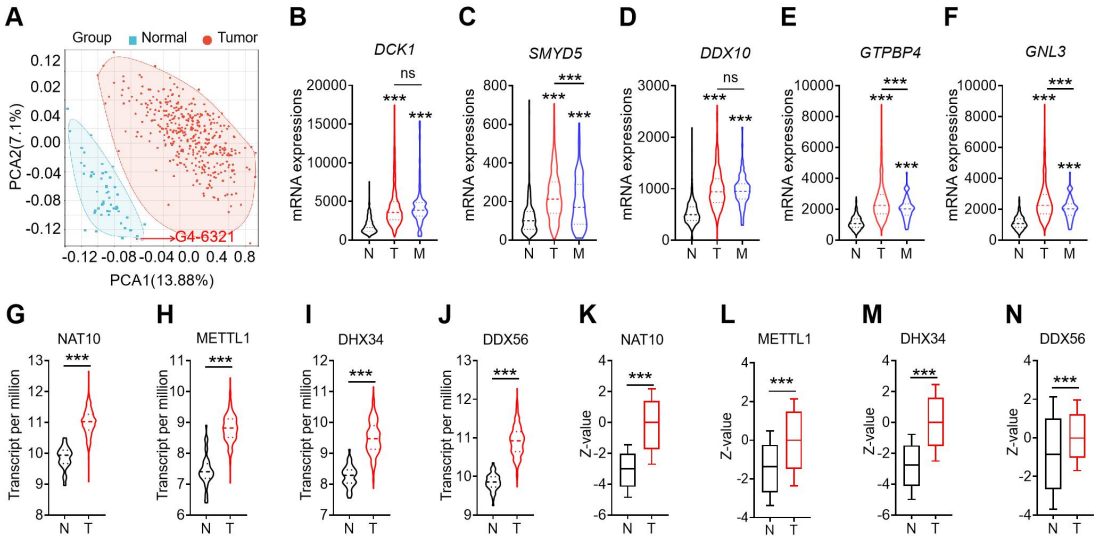
Supplementary data for

NAT10 triggers colorectal cancer progression via promoting PPAN-regulated DNA damage repair

Figure S1



Supplementary Figure 1. NAT10 is significantly upregulated in CRC and correlated with clinical progression.

(A) Principal Component Analysis (PCA) of TCGA colorectal cancer (CRC) expression profiles;

(B-F) The relative mRNA expression of *DCK1* (B), *SMYD5* (C), *DDX10* (D), *GTPBP4* (E), and *GNL3* (F) in individual cancer stages and normal tissues based on data of CRC available from TNMplot database;

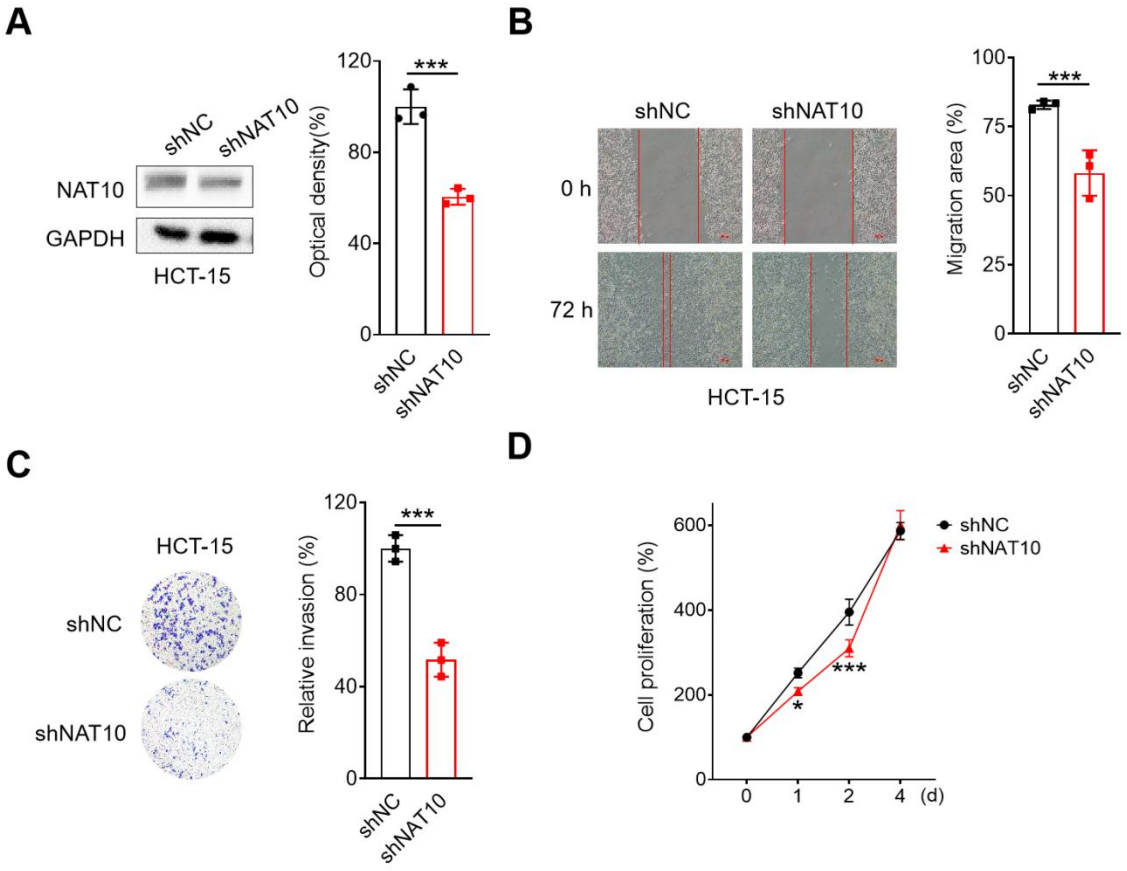
(G-J) The relative mRNA expression of *NAT10* (G), *METTL1*(H), *DDX34* (I), and *DDX56* (J) in tumor and normal tissues based on data of CRC available from the TCGA database;

(K-N) The relative protein expression of NAT10 (K), METTL1(L), DDX34 (M), and DDX56 (N) in tumor and normal tissues based on data of CRC available from the CPTAC database.

Data are presented as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ns, no significance, by Student's t-test between two groups and by one-way ANOVA followed by Bonferroni test for multiple comparisons;

Related to Figure 1.

Figure S2



Supplementary Figure 2. NAT10 facilitates the development of CRC via ac4C transferase activity.

(A) The protein expression of NAT10 was measured in shNAT10 HCT-15 and control cells, and statistical analysis was measured (right);

(B) In vitro wound healing assay of shNAT10 HCT-15 and control cells (left, scale bar, 100 μm), and statistical analysis was measured (right);

(C) In vitro, invasion assay of shNAT10 HCT-15 and control cells (left), and statistical analysis was measured (right);

(D) The relative cell proliferation was measured by CCK-8 kit in shNAT10 HCT-15 and control cells, and statistical analysis was measured (right);

Data are presented as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ns, no significance, by Student's t test between two groups and by one-way ANOVA

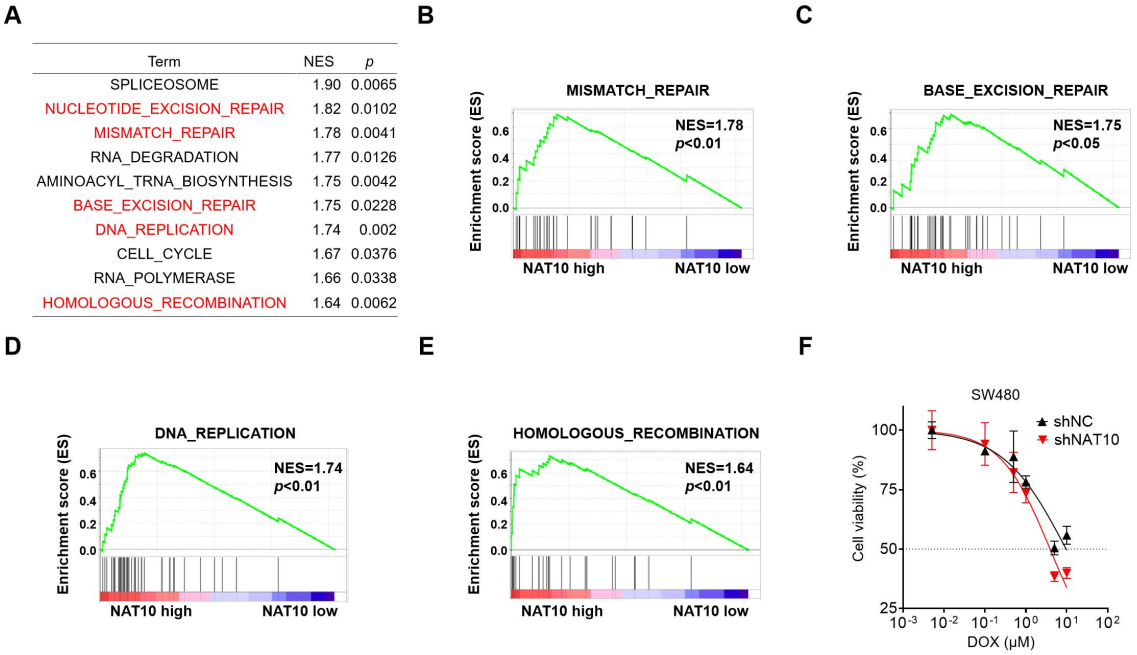
followed by Bonferroni test for multiple comparison.

Related to Figure 2.

Figure S3

A

Term	NES	*p*
SPLICEOSOME	1.90	0.0065
NUCLEOTIDE_EXCISION_REPAIR	1.82	0.0102
MISMATCH_REPAIR	1.78	0.0041
RNA_DEGRADATION	1.77	0.0126
AMINOACYL_TRNA_BIOSYNTHESIS	1.75	0.0042
BASE_EXCISION_REPAIR	1.75	0.0228
DNA_REPLICATION	1.74	0.002
CELL_CYCLE	1.67	0.0376
RNA_POLYMERASE	1.66	0.0338
HOMOLOGOUS_RECOMBINATION	1.64	0.0062



Supplementary Figure 3. NAT10 facilitates the development of CRC by promoting DNA damage repair.

(A) GSEA reveals positive enrichment of gene sets of NAT10 expression;

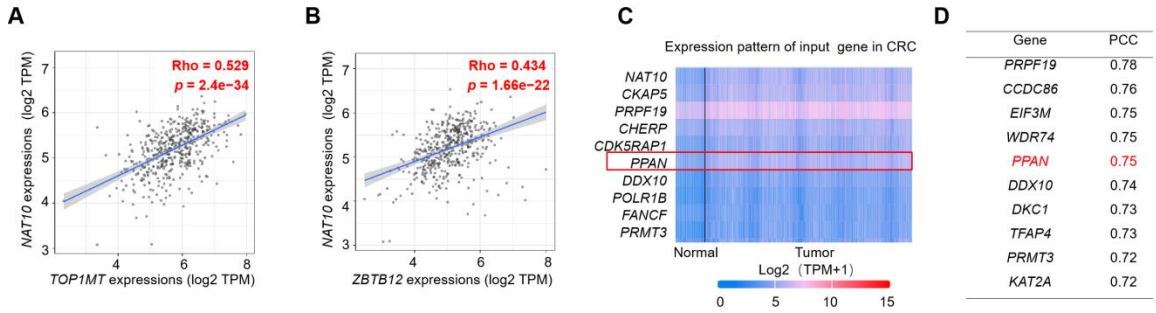
(B-E) GSEA reveals positive enrichment of genes in MISMATCH_REPAIR sets (B), BASE_EXCISION_REPAIR sets (C), DNA_REPLICATION sets (D) and HOMOLOGOUS_RECOMBINATION sets (E) of *NAT10* expression;

(F) WT and shNAT10 SW480 cells were treated with increasing concentrations of DOX for 48 h. Cell proliferation was tested by CCK-8 kit, and statistical analysis was measured (right).

Data are presented as mean ± SD from three independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.001, ns, no significance, by Student's *t* test between two groups and by one-way ANOVA followed by Bonferroni test for multiple comparison.

Related to Figure 3.

Figure S4



Gene	PCC
PRPF19	0.78
CCDC86	0.76
EIF3M	0.75
WDR74	0.75
PPAN	0.75
DDX10	0.74
DKC1	0.73
TFAP4	0.73
PRMT3	0.72
KAT2A	0.72

Supplementary Figure 4. PPAN mediates the oncogenic roles of NAT10 in CRC.

(A) Correlation analysis of relative mRNA expression levels between *NAT10* and *TOP1MT* in CRC and adjacent normal tissues based on data of CRC available from TIME 2.0 database;

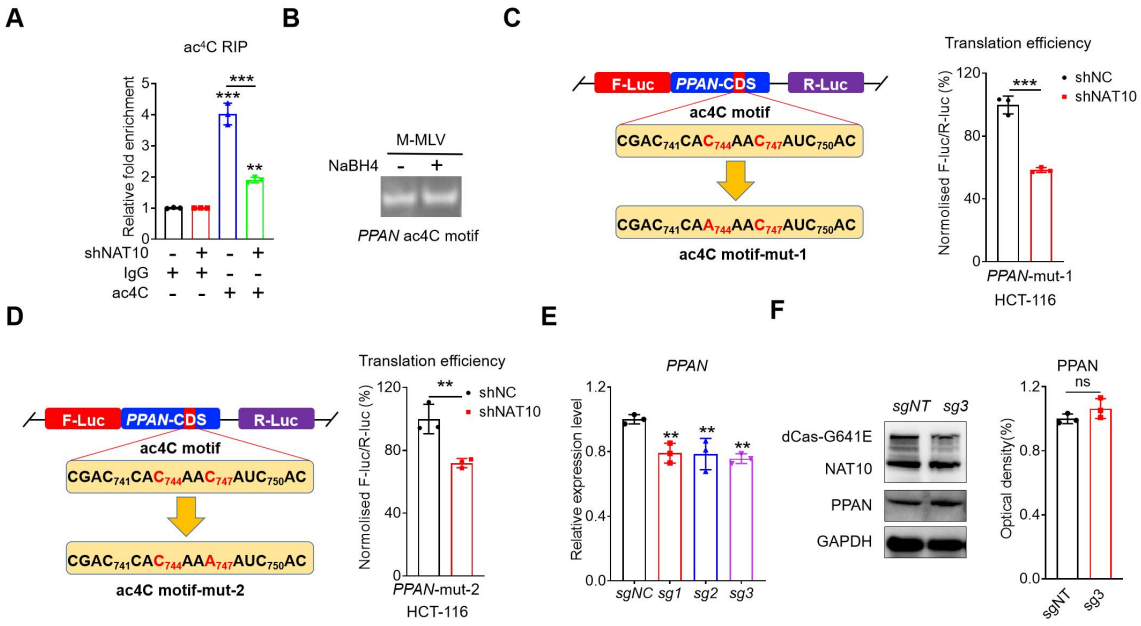
(B) Correlation analysis of relative mRNA expression levels between *NAT10* and *ZBTB12* in CRC and adjacent normal tissues based on data of CRC available from TIME 2.0 database;

(C) Similar genes detection with *NAT10* based on data of CRC available from UALCAN database;

(D) Similar genes detection with *NAT10* based on data of CRC available from gepia2 database.

Related to Figure 4.

Figure S5



Supplementary Figure 5. NAT10 regulates the translation efficiency of PPAN via the RNA acetylation at C744 and C747.

(A) ac4C-RIP-qPCR analysis of PPAN mRNA in SW480 cells, and statistical analysis was measured;

(B) Primer extension analysis of ac4C-containing RNAs following NaBH4 treatment and M-MLV RT;

(C) WT and shNAT10 HCT-116 cells were transfected with F-Luc/R-Luc of pmirGLO-*PPAN*-CDS-WT or pmirGLO-*PPAN*-CDS-C744-mut reporter (*PPAN*-mut-1) for 24 h. The translation outcome was determined as a relative signal of F-luc divided by R-luc, the mRNA abundance was determined by RT-qPCR of F-luc and R-luc, and the translation efficiency of *PPAN* is defined as the quotient of reporter protein production (F-luc/R-luc) divided by mRNA abundance, and statistical analysis was measured;

(D) WT and shNAT10 HCT-116 cells were transfected with F-Luc/R-Luc of pmirGLO-*PPAN*-CDS-WT or pmirGLO-*PPAN*-CDS-C747-mut reporter (*PPAN*-mut-2) for 24 h. The translation outcome was determined as a relative signal of F-luc divided by R-luc,

the mRNA abundance was determined by RT-qPCR of F-luc and R-luc, and the translation efficiency of *PPAN* is defined as the quotient of reporter protein production (F-luc/R-luc) divided by mRNA abundance, and statistical analysis was measured;

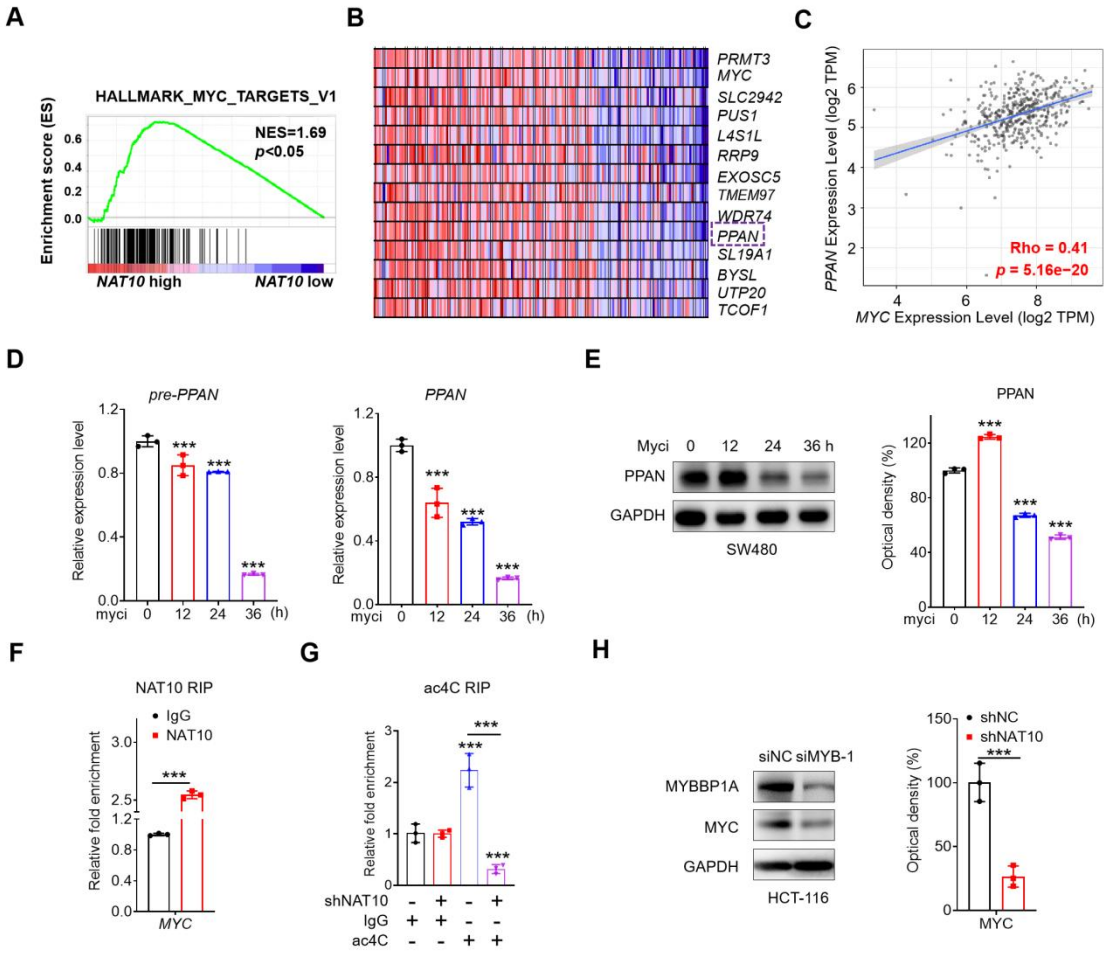
(E) The mRNA expression of *PPAN* in HCT-116 cells transfected with Cas13b combined with gRNA control or gRNA3, respectively, for 24 h, and statistical analysis was measured;

(F) The protein expression of NAT10, dcas-G641E, and PPAN in respectively, for 24 h (left), and statistical analysis was measured (right);

Data are presented as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ns, no significance, by Student's t test between two groups and by one-way ANOVA followed by Bonferroni test for multiple comparison.

Related to Figure 6.

Figure S6



Supplementary Figure 6. MYC mediates NAT10-regulated transcription of PPAN in CRC cells.

(A) GSEA reveals positive enrichment of genes in MYC targetsV1 sets of *NAT10* expression;

(B) GSEA reveals positive enrichment of genes in MYC targetsV2 sets of *NAT10* expression;

(C) Correlation analysis of relative mRNA expression levels between *PPAN* and *MYC* in CRC and adjacent normal tissues based on data of CRC available from TIME 2.0 database;

(D) The SW480 cells were treated with MYCi for 0 h 12 h, 24 h, 48 h. The precursor and mature mRNA of PPAN were checked, and statistical analysis was measured;

(E) The SW480 cells were treated with MYCi for 0 h, 12 h, 24 h, 48 h. The protein of PPAN were checked (left), and statistical analysis was measured (right);

(F) NAT10 RIP-qPCR analysis of *MYC* mRNA in SW480 cells, and statistical analysis was measured;

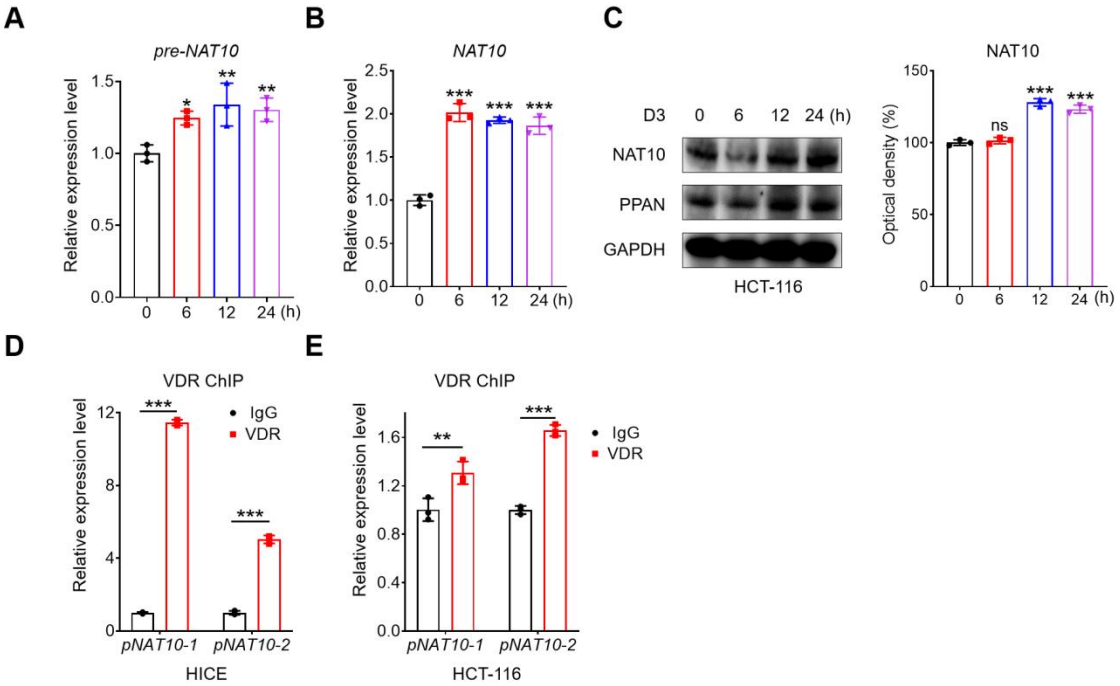
(G) ac4C RIP-qPCR analysis of *MYC* mRNA in SW480 cells, and statistical analysis was measured;

(H) WT and shNAT10 HCT-116 cells were transfected with siNC or siMYBBP1A-1, for 24 h, protein levels of MYC and MYBBP1A were checked (left), and statistical analysis was measured(right).

Data are presented as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ns, no significance, by Student's t test between two groups and by one-way ANOVA followed by Bonferroni test for multiple comparison.

Related to Figure 8.

Figure S7



Supplementary Figure 7. VDR is responsible for the upregulation of NAT10 in CRC cells.

(A) HCT-116 cells were treated with 1α,25(OH)2D3 for 0h, 6h, 12h, 24h. The precursor mRNA of *NAT10* were checked, and statistical analysis was measured;

(B) HCT-116 cells were treated with 1α,25(OH)2D3 for 0h, 6h, 12h, 24h. The mature mRNA of *NAT10* were checked, and statistical analysis was measured;

(C) HCT-116 cells were treated with 1α,25(OH)2D3 for 0h, 6h, 12h, 24h. The protein of NAT10 and PPAN was checked (left), and statistical analysis was measured (right);

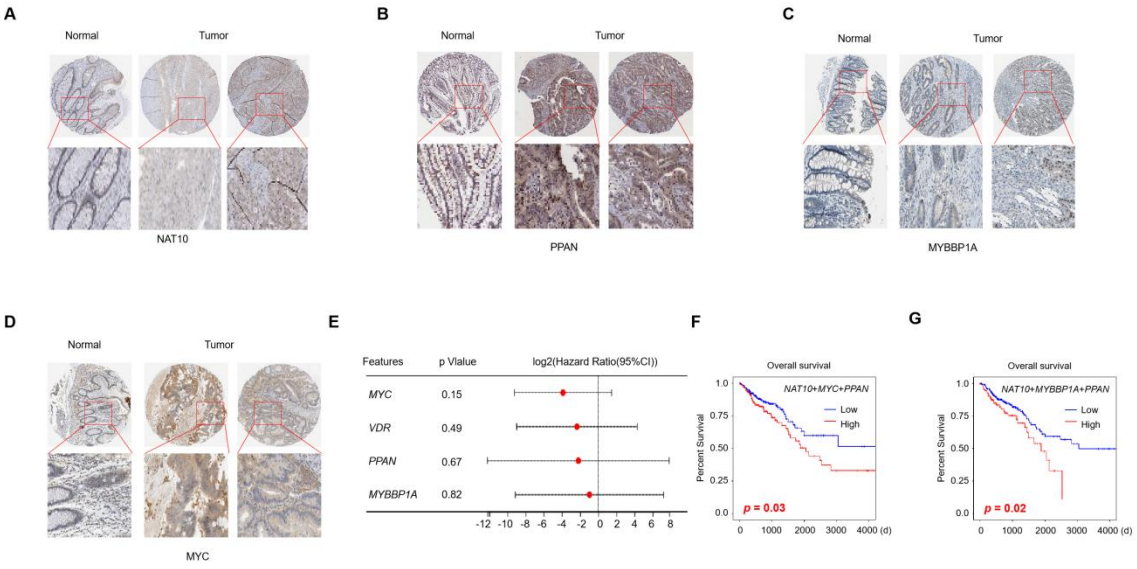
(D) The binding between VDR and promoter of *NAT10* in HIEC cells was checked by ChIP-PCR using IgG or VDR antibody, and statistical analysis was measured;

(E) The binding between VDR and promoter of *NAT10* in HCT-116 cells was checked by ChIP-PCR using IgG or VDR antibody, and statistical analysis was measured.

Data are presented as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ns, no significance, by Student's t test between two groups and by one-way ANOVA followed by Bonferroni test for multiple comparison.

Related to Figure 9.

Figure S8



Supplementary Figure 8. Oncogenic roles of NAT10-PPAN axis in CRC progression.

(A-D) The IHC staining of NAT10 (A), PPAN (B), MYBBP1A (C) and MYC (D) in CRC and adjacent normal tissues based on data of CRC available from The Human Protein Atlas [1];

(E) The forest plot illustrates the impact of *MYC*, *VDR*, *PPAN*, and *MYBBP* on OS based on data from CRC samples available in the TCGA database;

(F) The Kaplan-Meier survival curves of OS based on *NAT10*, *MYC* and *PPAN* expression in CRC cancer patients from TCGA database;

(G) The Kaplan-Meier survival curves of OS based on *NAT10*, *MYBBP1A* and *PPAN* expression in CRC cancer patients from TCGA database.

Data are presented as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ns, no significance, by Student's t test between two groups and by one-way ANOVA followed by Bonferroni test for multiple comparison.

Related to Figure 10.

Materials and methods

1. Plasmid, siRNA, shRNA, and generation of stable cell lines

The CDS of *NAT10*, *NAT10* mutant (*G641E*), *PPAN* and *MYC* were cloned into pPB to generate over expression plasmid. pPB was used as the vector control for analysis. The CDS of *PPAN* were cloned into pcDNA-2x*MS2BS* plasmid to generate over-expression plasmid. pcDNA-2x*MS2BS* was used as the vector control for analysis. For MYBBP1A knockdown, two synthesized duplex RNAi oligos targeting human mRNA sequences from Sigma were used. A scrambled duplex RNA oligo (5' -UUCUCCGAACGUGUCACGU) was used as RNA control. Twenty-four hours before transfection, the medium was replaced with fresh medium and transfected using Lipofectamine 2000 reagent (Invitrogen) with vector control, plasmid construct, siRNA negative control (siNC), or siRNAs according to the manufacturer's instructions. The working concentration of siRNA was 50 nM.

The shNC and shNAT10 CRC cells were generated by transfection of lentiviral shRNA constructs (MISSION lentiviral transduction particles, Genechem) according to manufacturer's instructions. The transfected cells were selected with Puromycin (2 μg/ ml) for two weeks. The survived cells were picked out and seeded into 96-well plate for formation of cell clones and further expansion.

To generate dCas13b-NAT10 and dCas13b-G641E fusion protein, the original PspCas13b plasmid (Addgene plasmid #103866), gRNA plasmid (Addgene plasmid #103854) and non-targeting gRNA plasmid (Addgene plasmid #103868) were obtained from Addgene.

2. Experimental animals and xenograft models

BALB/c nude mice (4-6 weeks old) were used to construct the animal models in this study. The mice were housed according to the protocol approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University [IACUC Approval No. SYSU-IACUC-2024-001583].

To determine the regulatory effect of NAT10 on CRC proliferation, shNAT10 HCT-116 and control cells (5×10^6) were injected subcutaneously into the axilla of the mice (n=5), establishing

a subcutaneous tumor model. One week after injection, tumor volumes were measured every two days. When the subcutaneous tumor diameter reached approximately 1000 mm^3, the mice were euthanized using CO_2 followed by cervical dislocation. Tumor size was analyzed, and the expression levels of Ki67 were assessed by IHC staining.

To determine the regulatory effect of NAT10 on CRC metastasis, shNAT10 HCT-116 and control cells (5×10^6) were injected into the tail vein of the mice (n = 5), establishing a metastatic tumor model. Three weeks after injection, the mice were euthanized using CO_2 followed by cervical dislocation. The presence of metastatic tumors in the lungs was analyzed, and the number of metastatic foci was quantified using HE staining.

To determine the regulatory effect of the NAT10-PPAN axis on the CRC DNA damage repair, we used HCT-116 cells to construct a mouse model. The groups included Mock + DMSO, Mock + Remodelin, Remodelin + DOX, and oe*PPAN* + Remodelin + DOX, with the Mock + DMSO group serving as the control group. Each group consisted of 8 mice. When the subcutaneous tumor diameter reached approximately 100 mm^3, the mice received intraperitoneal injections of Remodelin (5 mg/kg) and DOX (5 mg/kg) every two days. Tumor volumes were measured every two days. When the subcutaneous tumor diameter reached approximately 1000 mm^3, the mice were euthanized using CO_2 followed by cervical dislocation. Tumor size was analyzed, and the expression levels of PPAN and γH2AX were assessed by IHC staining.

The IHC staining of CRC tissues was stained by Pinuofei Biological Technology Co., Ltd, Wuhan, 430200, China. The xenografts slides were deparaffinized and rehydrated through an alcohol gradient followed by antigen retrieval with sodium citrate buffer. Tumor sections were blocked with 5% normal goat serum (Vector) with 0.1% Triton X-100 and 3% H_2O_2 in PBS for 60 min at room temperature and then incubated with primary antibodies 4°C overnight. IHC staining was performed with horseradish peroxidase (HRP) conjugates using DAB detection. Images were taken with Nikon microscopy.

3. Human primary CRC samples

CRC tissues and corresponding non-tumor normal tissues were purchased from Shanghai YEPCOME Biotechnology (Item No.: YP-cocSur2201). The expression levels of VDR, NAT10, PPAN, MYC, MYBBP1A, and Ki67 were analyzed by IHC staining.

4. Data collection and bioinformatics analysis

CRC data were collected from the TCGA database. Initially, we conducted PCA analysis on the CRC gene expression profile, identifying an outlier sample, G4-6321. Subsequently, we performed differential gene expression analysis on the CRC gene expression profile, followed by WGCNA analysis and clinical data analysis to screen for the core protein NAT10, which promotes the occurrence and progression of CRC.

To explore the downstream regulatory genes of NAT10, we divided the CRC gene expression profiles into two groups: samples in the top quartile for *NAT10* expression were designated as the *NAT10* high-expression group (*NAT10* H), while those in the bottom quartile were designated as the *NAT10* low-expression group (*NAT10* L). We then performed WGCNA analysis to identify core genes (NAT10 Hubs) that were significantly positively correlated with both *NAT10* and CRC. Following this, we conducted GSEA analysis and clinical data analysis to determine the regulatory pathways of *NAT10* and the core functional gene *PPAN*.

The Sanger BOX WGCNA analysis tool was employed to investigate gene expression profiles of CRC obtained from TCGA database. Genes that showed no differential expression were excluded based on initial gene expression analysis. Subsequently, WGCNA analysis was performed on the CRC expression profiles. Sample clustering was conducted first, after which a soft-threshold power of 7 was selected to convert adjacency relationships into a topological overlap matrix (TOM), with corresponding dissimilarity (1-TOM) calculated. The TOM matrix quantitatively describes node similarity by assessing the weighted correlation between two nodes relative to their correlations with other nodes. Genes with similar expression patterns were grouped into gene modules, with a minimum module size of 30 genes, sensitivity set to 3, and a module merging threshold of 0.25. The module most strongly correlated with CRC and/or *NAT10*

was selected, and hub genes were identified based on a Module Membership (MM) threshold ≥ 0.75, Gene Significance (GS) threshold ≥ 0.1, and weight threshold ≥ 0.1. Hub genes were intersected with known RNA modification recognition and regulatory proteins or potential targets of *NAT10*, isolating those RNA modification regulators or NAT10 Hubs most closely associated with CRC progression.

We utilized GSEA 4.1.0 software to conduct GSEA on NAT10 Hubs The expression profiles of NAT10 Hubs were extracted from CRC data in The TCGA for GSEA. Gene sets from the Hallmark and KEGG collections that showed significant positive correlations with NAT10 were identified and extracted. These gene sets were then selected for further experimental validation and analysis.

We utilized the Sanger BOX platform to conduct multigene survival analysis on the core genes within the NAT10-PPAN axis. Survival analysis and visualization were performed using R.

5. Cell viability and drug sensitivity assay

For the cell proliferation assay, WT or shNAT10 HCT-116 cells were transfected with vector control or *NAT10* or *G641E* or *PPAN* constructs for 24 h, and then the above cells (2000 cells/well) were seeded in 96-well plates and cultured in DMEM containing 10% FBS. Cell proliferation was assessed at 0, 1, 2, 3, and 4 days using the Cell Counting Kit-8 (CCK8, C6005, Ncmbio) to determine the proliferation capacity. A microplate reader was used to measure the absorbance at 450 nm. Further data analysis and visualization were performed using R and other plotting software.

For the drug sensitivity assay, WT or shNAT10 HCT-116 cells were transfected with vector control or *NAT10* or *G641E* or *PPAN* constructs for 24 h, and then the above cells (20, 000 cells/well) were seeded in 96-well plates and cultured in serum-free DMEM containing gradient concentrations of DOX or Zeocin. Cell viability was tested after 48 h using the Cell Counting Kit-8 (CCK8, C6005, Ncmbio) to determine the drug sensitivity of the cells. The specific detection procedure was carried out according to the manufacturer's instructions. A microplate

reader was used to measure the absorbance at 450 nm. Further data analysis and visualization were performed using R and other plotting software.

6. Wound healing assay

CRC cells were seeded in 6-well plates and allowed to grow to confluence. After reaching confluence, the cells were washed with PBS, and a uniform scratch was made across the cell monolayer. The initial width of the scratch was recorded by photographing. The cells were then cultured in serum-free DMEM for 2 days, after which the scratch was photographed again. The change in scratch width was analyzed to assess the cell migration ability.

7. In vitro migration and invasion assays

Cells starved for 24 h (5000 cells/chamber) were seeded into the upper chamber of Transwell inserts (REF3422, Corning, USA) that were either uncoated or coated with Matrigel (356234, dilution ratio 1:8, Corning, USA). The upper chambers were filled with serum-free DMEM, while the lower chambers contained DMEM with 10% FBS in a 12-well plate. After 2 days of incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with 0.05% crystal violet. Images of the cells were captured and analyzed to assess cell migration and invasion abilities.

8. Colony formation assays

CRC cells (1000 cells/well) were seeded in 6-well plates and cultured for 2 weeks. After the incubation period, the cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with 0.05% crystal violet. Images of the cell colonies were captured, and the number of colonies was analyzed to assess cell proliferation capacity.

9. Homologous recombination (HR) and non-homologous end joining (NHEJ) assays

Homologous and non-homologous DNA damage repair reporter plasmids (DR/EJ5) were

digested with I-SceI endonuclease (R0694S, NEB, USA). The digested plasmids (2 μg each) were then transfected into approximately 1 x 10^6 shNC and shNAT10 HCT-116 cells seeded in 6-well plates using Lipofectamine 2000 (11668030, Thermo Fisher, USA). After 48 hours of transfection, the cells were collected and washed once with PBS. The proportion of GFP-positive/negative cells was analyzed by flow cytometry to evaluate the efficiency of HR and NHEJ repair. Further data analysis and visualization were performed using R and other plotting software.

10. Protein extraction and western blot analysis

Total cellular proteins were extracted using IP lysis buffer (P0013, Beyotime, China). The protein samples were loaded onto SDS-PAGE gels and electrophoretically transferred to PVDF membranes. The membranes were then blocked with 5% skim milk in PBST for 2 hours and incubated overnight at 4°C with primary antibodies against NAT10 (ab194297, ABCAM, UK), PPAN (A304-598A-T, Thermo Fisher, USA), MYC (18583, Cell Signaling, USA), YY1 (ab109237, ABCAM, UK), MYBBP1A (14524-1-AP, Proteintech, China), VDR (12550, Cell Signaling, USA), γH2AX (ab81299, ABCAM, UK), and GAPDH (abs132004, ABSIN, China) at a dilution of 1:2000. Following incubation, the membranes were washed with PBST (three times for 5 minutes each) and then incubated with secondary antibodies (ab6721, ABCAM, UK) for 2 hours at room temperature. After further washing with PBST (three times for 5 minutes each), protein bands were visualized and quantified using an imaging system.

11. Immunofluorescence

For the Immunofluorescence, WT or shNAT10 HCT-116 cells (20000 cells/well) were seeded in glass bottom dishes and cultured in DMEM containing 10% FBS. After DOX treatment for 4h, the cells were washed with PBS, fixed with 4% formaldehyde for 20 minutes at room temperature then washed with 1x PBS solution for three times at room temperature (RT). Subsequently, the cells were permeabilized with 1 % Triton X-100 in PBS solution for 5 min, rinsed, and then blocked with Normal Goat Serum (Ready-to-use, BOSTER, AR0009) at RT for 1

h. The glass bottom dishes were then incubated with anti-γH2AX (ab81299, ABCAM, UK (1:200)) diluted in blocking solution at 4 ℃ overnight. The glass bottom dishes were washed with 1x PBS and then incubated with the secondary antibodies for Alexa Fluor® 594 (1:200, Donkey anti-sheep, abcam) at RT for 1 h. The glass bottom dishes were stained with 1 mg/mL DAPI (Sigma) for 5 min and washed three times for 5 min with 1x PBS. The foci detection of γH2AX were imaged with an inverted OLYMPUS FV3000 microscope.

12. ac4C detection by dot blot

RNA was denatured at 95°C for 5 minutes and immediately placed on ice for 1 minute. The denatured RNA was then gradient diluted and dropped onto nylon membranes at 1 cm intervals. The membranes were crosslinked with 254 nm UV light at 150 mJ/cm^2 for 30 minutes. After staining with methylene blue and photography, the membranes were blocked with 5% skim milk in PBST for 2 hours and then incubated overnight at 4°C with primary antibodies against ac4C (A18806, ABclonal, China). Subsequently, the membranes were washed with PBST (three times for 5 minutes each) and then incubated with secondary antibodies (ab6721, ABCAM, UK) for 2 hours at room temperature. After further washing with PBST (three times for 5 minutes each), protein bands were visualized and quantified to assess ac4C levels.

13. Luciferase reporter assay

To assess the impact of NAT10 or MYC on the transcription of *PPAN*, we cloned the *PPAN*-promoter (−2000/+1) or p*PPAN*-mut into pGL-Basic-Vector (Promega, USA) for promoter activity measurement. For the Luciferase reporter assay of PPAN transcription, WT or shNAT10 HCT-116 cells were transfected with vector control or *NAT10* or *G641E* or *MYC* for 24 h, and then the above cells were co-transfected with pGL-basic-promoter or *pPPAN*-promoter or *pPPAN*-mut and TK-Rluc reporter in a 6-well plate for 24 h, and the Dual-Luciferase Reporter Gene Assay Kit (Beyotime, China) was used to measure transcriptional activity. Renilla Luciferase (R-Luc) served as an internal control to normalize firefly luciferase (F-Luc) activity.

To assess the impact of VDR on the transcription of *NAT10*, we cloned the *NAT10*-promoter (−2000/+1) or *mut-1* or *mut-2* into pGL-Basic-Vector (Promega, USA) for promoter activity measurement. For the Luciferase reporter assay of *NAT10* transcription, HCT-116 cells were co-transfected with *NAT10*-promoter or *mut-1* or *mut-2* and TK-Rluc reporter in a 6-well plate for 24 h, and then were treated with 1α,25(OH)2D3 for 24 h. The Dual-Luciferase Reporter Gene Assay Kit (Beyotime, China) was used to measure transcriptional activity. Renilla Luciferase (R-Luc) served as an internal control to normalize firefly luciferase (F-Luc) activity.

To assess the impact of NAT10 or MYBBP1A on the translation of *PPAN* and *MYC*, we cloned the *PPAN*-CDS or *PPAN*-mut or *MYC*-5' UTR into pmiGLO-Basic-Vector (Promega, USA) for translation activity measurement. For the Luciferase reporter assay of *PPAN* or *MYC* transcription, WT or shNAT10 HCT-116 cells were transfected with vector control or *NAT10* or *G641E* or siNC or siMYBBP1A-1 or siMYBBP1A-2 for 24 h, and then the above cells were transfected with pmiGLO-basic-promoter or *PPAN*-CDS or *PPAN*-mut or *MYC*-5' UTR in a 6-well plate for 24 h, and the Dual-Luciferase Reporter Gene Assay Kit (Beyotime, China) was used to measure transcriptional activity. Renilla Luciferase (R-Luc) served as an internal control to normalize firefly luciferase (F-Luc) activity.

14. Polysome profiling

The fraction of ribosome was separated by centrifugation in a sucrose gradient according to our previous study [2]. The fraction of ribosome was separated by centrifugation in a sucrose gradient. Cells pretreated with 100 μg/ml cycloheximide were lysed in 1 ml lysis buffer (10 mM Tris, pH 7.4, 150 mM KCl, 5 mM MgCl2, 100 μg ml-1 CHX, 0.5% Triton-X-100, freshly add 1:100 protease inhibitor, 40 U ml - 1 SUPERasin). After centrifugation at 15,000 g for 15 min, the supernatant was separated by 5/50% w/v sucrose gradient at 4 °C for 4 h at 140, 000 g (Beckman, rotor SW28). The sample was then fractioned and analyzed by Gradient Station (BioCamp) equipped with ECONO UV monitor (BioRad) and fraction collector (FC203B, Gilson). The fractions resulting from sucrose gradient were used for RNA extraction and RT-qPCR.

15. RIP-qPCR

First, approximately 2 × 10^7 cells were subjected to two washes with PBS followed by fixation with 37% formaldehyde (8187081000, Sigma, Germany) for 30 minutes, and then quenched with 1.25M 10X Glycine (1275, BIOFROXX, China). The cells were collected by centrifugation at 4°C for 5 minutes at 500 g after adding 6 mL of pre-chilled PBS-PMSF cocktail (100:1). The cell pellet was resuspended in Cell Lysis buffer containing 1% Cocktail (200-664-3, Sigma, Germany) and PMSF and incubated on ice for 10 minutes. Subsequently, the cells were sonicated, and the supernatant was collected after centrifugation at 4°C for 10 minutes at 14000 rpm.

In the next step, the supernatant was divided, and one portion was used as Input after adding Dilution buffer, while the other portion was incubated with 60μl of Protein G beads (88847, Thermo Fisher, USA) pre-washed with Reaction Buffer. After incubation, the beads were washed and incubated with NAT10 or MYBBP1A antibodies. Following another round of washing, the immunoprecipitated complexes were eluted with Elution buffer and RNase inhibitor, and the DNA was extracted using Trizol-chloroform method.

Finally, the RNA was precipitated with isopropanol, washed with 75% ethanol-DEPC water, air-dried, and quantified using the QubitTM kit (Q32854, Thermo Fisher, USA). Reverse transcription and RT-qPCR were performed to assess the level of ac4C modification on RNA.

16. MS2-MS2BS RNA pull-down and Mass spectrometry (MS) analysis

Initially, approximately 2 × 10^7 cells overexpressing HA-MS2 and MS2BS-PPAN-CDS were washed twice with PBS. Then, 200 μl of IP lysis buffer (P0013, Beyotime, China) supplemented with PMSF (ST505, Beyotime, China) and RNase inhibitor was added to the cells, and cell lysis was performed for 20 minutes. The cell lysates were collected in a 6 cm cell culture dish and crosslinked with 254 nm UV light at 150 mJ/cm^2 for 30 minutes. A 1% portion of the crosslinked material was saved as Input.

The remaining crosslinked material was incubated with 20 μl of protein G magnetic beads for 2 hours and then discarded. The remaining cell samples were divided into two portions. One portion was incubated overnight with 2 μg of HA antibody (81290-1-RR, Proteintech, USA), while the other portion was incubated with an equivalent amount of species-matched IgG as a negative control. After overnight incubation, 25 μl of protein G magnetic beads was added to each sample and incubated together at 4°C for 4 hours.

Finally, the immunoprecipitated complexes were retrieved, washed three times with RIPA lysis buffer (P0013B, Beyotime, China) supplemented with PMSF (1:100), and eluted by adding 50 μl of 1 x loading buffer. The eluted proteins were then denatured at 100 °C for 20 minutes and subjected to mass spectrometry analysis. Proteins identified in the HA pulldown group but absent in the IgG pulldown group were considered as potential binding partners of MS2BS-PPAN-CDS. The mass spectrometry identified 451 proteins (Table S6) that were overlapped with the acetylation-related proteins identified by previous researchers (Table S7). The results showed that MYBBP1A was the only acetylation-related protein binding with PPAN mRNA except NAT10. MYBBP1A in HA pulldown group and IgG pulldown group were detected by Western blotting analysis.

17. Reverse transcription and misincorporation analysis of ac4C RNAs

We utilized the ac4C method described by David Bartee et al. (2018) to identify ac4C modification sites. Briefly, we first treated the extracted RNA (10 μg) with NaBH4 (100 mM in H_2O) or solvent (H_2O) to a final volume of 100 μl and incubated the mixture at 37°C for 60 minutes. The reaction was stopped by adding 15 μl of 1 M HCl, followed by neutralization with 15 μl of 1 M Tris-Cl [pH 8.0]. The resulting 130 μl solution was then brought to 200 μl with water, and RNA was precipitated using 1 mL of 70% ethanol (pre-cooled at 4°C) and centrifuged at 12,000 g for 1 minute. After several washes and drying, the RNA was resuspended in water, and its concentration was measured using Nanodrop. Equal amounts of purified RNA were used for in vitro reverse transcription with RNA primers (3.5 pmol) and reverse transcriptase (AMV

(M0277L, NEB, USA) or M-MLV (AG11734, Agbio, China)) in a 50 μl system. The resulting 50 μl reaction mixture was then diluted to 200 μl with water, and DNA was extracted using phenol: chloroform, desalted with 70% cold ethanol, and resuspended in 20 μl of water for PCR. Finally, 2 μl of the PCR product was used for amplification, followed by PAGE gel electrophoresis, imaging, and band extraction for Sanger sequencing analysis.

Supplementary References

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