Supporting Materials:

RNA-binding protein RALY reprograms mitochondrial metabolism via mediating miRNA processing in colorectal cancer

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Supporting Methods:

Colorectal cancer patient samples, and tissue culture

The colorectal tumor and normal tissues were obtained at the First Affiliated Hospital of Sun Yat-Sen University and were approved by the institutional review board of the hospital. The study was compliant with all relevant ethical regulations regarding research involving human participants. For fresh tissues, colorectal tumors and adjacent normal tissues were separately dissected at the time of surgery and immediately transferred to RNAlater (R0901, Sigma). The paraffin-embedded specimens were collected from at the Third Affiliated Hospital of Sun Yat-Sen University (No. [2016]2-145). HCT116, HCT15, RKO, SW480, SW620, HT29, HT55, MC38, CT26, HEK293T and U2OS cell lines were obtained from American Type Culture Collection (ATCC). CT26 and SW620 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, Corning, USA) with 10% FBS at 37°C in 5% CO_2. HCT116, HCT15, RKO, SW480, HT29, HT55, MC38 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Corning, USA), with 10% FBS at 37°C in 5% CO_2. All cell lines were stored in multiple backup upon receipt to reduce risk of phenotypic drift, and tested with mycoplasma free by Mycoplasma Stain Assay Kit (C0296, Beyotime, China). Cell line authentication was validated by the STR analysis using GenePrint 10 System according to the manufacturer's instruction (B9510, Promega, USA).

Plasmid constructions, cell transfection, and virus infection

Stable knockdowns of target genes were generated by lentiviral-based shRNA. The plasmids pLKO.1-puromycin or pLKO.1-hygromycin was constructed by using primers listed in Table S1. For overexpressing constructs, RALY cDNA (NM_016732), miR-483 (MI0002467), miR-676 (MI0016436) and miR-877 (MI0005561) were cloned into pCDH puro lentiviral vector (CD510B-1, System Biosciences). For stable knockdown assay, pLKO.1 vector or pCDH vector together with packing and helper plasmids PAX2 and MD2G were co-transfected into HEK-293T cells by Calcium Phosphate Transfection Kit (CAPHOS-1KT, Sigma). Viruses were produced, filtered, and titrated according to the instruction, and infected the desired cells with 8 mg/mL Polybrene (TR-1003, Sigma). After screening by puromycin (1 ug/mL) or hygromycin (100 ug/mL) in corresponding concentration for 3-5 days, the stable knockdown or overexpression cells were harvested.

Antibodies

Anti-RALY (WB 1:1000; IHC 1:100; ab170105), Anti-METTL3 (WB 1:1000; IHC 1:500;

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ab195352), and goat anti-rabbit IgG (WB 1:2000; ab6721) antibodies were purchased from Abcam, USA. Anti-ATP5I (WB 1:1000; IHC 1:100; CSB-PA000954) and anti-ATP5G1 (WB 1:1000; CSB-PA030012) antibodies were purchased from Cusabio, China. Anti-ATP5G3 (WB 1:1000; IHC 1:100; A18278), anti-CYC1 (WB 1:1000; A10449), anti-Caspase 3 (WB 1:1000; IHC 1:100; A11953), anti-Ki67 (IHC 1:100; A11390), anti-GAPDH (WB 1:1000; AC027); anti-tubulin (WB 1:5000; AC021) and anti-β-actin (WB 1:50000; AC026) antibodies were purchased from Abclonal, China. Western blot analyses were performed by standard methods described previously.[1]

Quantitative RT-PCR

Total RNA was isolated using Trizol reagent (15596018, ThermoFisher, USA) according to the manufacturer's instruction and cDNA was synthesized by using the PrimeScript RT reagent Kit (RR036A, Takara, Japan). The resulting cDNA was used for quantitative RT-PCR by using SYBR-Green Master mix (RR820B, Takara, Japan) in 7500 apparatus (Applied Biosystems). Mature and pri-miRNAs were quantified by Clontech miRNA and pri-miRNA assays (638314, Takara, Japan). U6 snRNA and GAPDH mRNA are the housekeeping genes used to normalize the expression of miRNAs and mRNAs, respectively. RT-PCR primer sequences were listed in supplemental table 5.

Animal experiments

All mouse procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Sun Yat-Sen University (No.[2017]03-07). We used both male and female mice (1:1) for all our mouse experiments when possible. For subcutaneous implantation mouse models, 8 4-week-age BALB/c-nu/nu mice were randomly grouped (8 mice (4M + 4F) per group) and injected with 1×10^6 shCTRL, shRALY#1 or shRALY#2 HCT116 cells. 20 4-week-age C57BL/6 mice were injected with 1×10^5 inducible-RALY knockdown MC38 cells, half of the mice (10 mice (5M + 5F) per group) were randomly fed with Doxycycline water (1% sucrose water + 2 mg/mL Doxycycline, changed every 2 days) or regular water. Tumors were measured with a caliper every 3 or 7 days to analyze tumor growth. Mice were generally sacrificed when tumors became necrotic or their volume reached about 1000 mm^3. For orthotopic colorectal xenograft mouse model, 5×10^5 inducible-RALY knockdown LOVO cells were injected into the cecal wall of 12 4-week old NOD/SCID mice after given an anaesthetic. Tet systems of half mice (6 mice (3M + 3F) per group) were induced by non-invasive methods of Dox delivery *in vivo* in drinking water containing sucrose Doxycycline water (1% sucrose water + 2 mg/mL Doxycycline, changed every 2 days). Mice were sacrificed at one month following the start of treatment. For the PDX model, colorectal tumors and normal tissues were obtained from the First Affiliated Hospital of Sun Yat-Sen University and were approved by the institutional review board of the hospital. Colorectal tumor tissues were subcutaneously implanted into NOD/SCID mice for generation of PDX mouse models. All the mice were randomly divided into 2 or 3 groups (3 mice (2M + 1F) per group in PDX#1, 4 mice (2M + 2F) per group in PDX#2, PDX#24 and PDX#70, 5 mice (3M + 2F) per group in PDX#7) when the tumor size reached about $100mm^3$, and treated with siRALY-nano-gold particles and with siCTRL-nano-gold particles, respectively. siRNAs for *in vivo* delivery were encapsulated into CTAB-nanoparticles. They were mixed at a ratio of 1:1(mol/mol) siRNA:CTAB-nanoparticles. Before *in vivo* delivery, this

preparation was hydrated with PBS at room temperature at a concentration of 500μg siRNA per kilogram per injection twice/week intervals (each mouse received 200 μl of nano-gold particles-siRNA-PBS solution by the intraperitoneal injection). At the experimental endpoint, tumor tissues were harvested and fixed with 4% PFA for paraffin-embedded section, or kept in liquid nitrogen for protein level detection.

Immunohistochemistry

Formalin fixed paraffin embedded samples of tumors and organoids were cut 5 μm thick on polarized glass. Antigen retrieval was performed for 40minutes in 95-100°C 1x Citrate Buffer, pH=6.0. Tumor and organoid sections were blocked with 5% normal goat serum (Vector) with 0.1% Triton X-100 and 3% H_2O_2 in PBS. All incubations with primary antibodies were carried out overnight at 4°C. Incubation with Alexafluor-conjugated secondary antibodies was performed for 1 hour at room temperature. Nuclei were visualized with DAPI. Images were taken with Nikon (Japan).

RNA Immunoprecipitation (RIP)

RIP assay was performed using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (17-700, Merck Millipore, USA) in accordance with manufacture's protocol. Stable RALY-overexpressed HEK293T cells were collected and RNA was obtained by re-suspending and dispersing the cell pellet in complete RIP Lysis Buffer. Antibodies of RALY (ab170105, Abcam) and negative control Normal Rabbit IgG (PP64B) were added in magnetic beads to pull down specific RNAs. Enrichment of microRNAs were sent for high-throughput NGS sequencing and validated by quantitative RT-PCR.

Luciferase reporter assays and mutagenesis assay

The dual-luciferase vector pmiGLO was purchased from Promega (C838A, Promega, USA). The 3'UTRs of ETC genes including ATP5I, ATP5G3, ATP5G1 and CYC1 were amplified by PCR using the genomic DNA from HCT116 cells as a template. The indicated deletion mutants were generated by QuikChange II Site-Directed Mutagenesis Kit (200,523, Agilent, USA) according to the instruction. Luciferase activity was measured by Dual Luciferase Reporter Gene Assay Kit (RG028, Beyotime, China) in GM2000 (Promega). Experiments were performed in triplicates. The firefly luciferase activity values were normalized to the Renilla luciferase activity values that reflect expression efficiency.

ATP and NAD+/NADH assay

ATP and NAD+/NADH were measured respectively using ATP Assay Kit (S0026, Beyotime, China) and NAD+/NADH Assay Kit with WST-8 (S0175, Beyotime, China) in accordance with the manufacturer's protocol. The protein supernatant samples were quantified using BCA protein assay kit (P0011, Beyotime, China) to calculate the level of ATP or NAD+/NADH per microgramme protein.

ROS detection assay

Cells were plated in a 6-well plate overnight for ROS level detection using Reactive Oxygen Species Assay Kit (S0033, Beyotime, China). DCFH-DA was diluted at 1:1000 with serum-free

medium to a final concentration of 10 μM and added into a 6-well plate about 1 mL volume incubating in the CO_2 incubator in dark at 37°C for 20 minutes. The cells were washed with serum-free cell culture solution three times to fully remove the DCFH-DA that did not enter the cells. Cells were then collected for flow cytometry detection.

Metabolic detection

ECAR and OCR were monitored using a Seahorse Bioscience Analyzer (XF96) according to the manufacturer's instructions. Cells were seeded in 96-well plates at a density of 5–8×10^3 cells per well with growth medium for at least 18 h. Next, medium was changed to base medium (DMEM, 143 mM NaCl, phenol red, pH 7.35). For ECAR analysis, cells were added with medium (2 mM glutamine, pH 7.35) and monitored every 3 min following successive administration of 10 mM of glucose, and inhibitors (1 μM oligomycin and 50 mM 2-deoxyglucose). For OCR analysis, cells were added with mito stress-test base medium (10 mM glucose, 2 mM glutamine, 1 mM pyruvate, pH 7.4) and monitored every 3 min following successive administration of inhibitors (2 μM oligomycin, 2 μM FCCP, or 0.5 μM rotenone/antimycinA).[2]

Organoids generation

For mice organoids, DOX-induced MC38 tumors tissues were carefully dissected avoiding adjacent other tissue contamination. For human organoids, patient derived tumor tissues were dissected without any normal colon tissue by doctors, the tissue was kept in a fresh culture medium in ice less than 1 hour before experimental treatment to maintain maximum cancer cell activity. The tumor tissues were cut as much of the tumor as possible using surgical scissors, and then incubated in digestion buffer (DMEM, 1% FBS, penicillin, streptomycin, 0.125 mg/mL Collagenase Type XI, 0.125 mg/mL Dispase II) for 1.5 hr at 37°C with gentle rocking. Digested tissues were further digested with TrypLE for 10 min at 37°C with gentle agitation. After digestion, the tissue suspension was filtered with 40 μm cell strainer to obtain the single-cell suspension. Dissociated tissues were spun down at 350 g for 5 min, resuspended in 10 mL of PBS, and spun down again until the supernatant is clear. Single cell suspensions were initially seeded at high density and reseeded at a lower density after ~1 week. Organoids were passed at a 1:2-3 dilution every 2–3 weeks. To generate stocks, organoids were frozen in 90% FBS and 10% DMSO and stored in liquid nitrogen. Cryopreserved stocks have been successfully recovered for up to at least 18 months after freezing. Mice DOX-induced MC38 organoids were treated with DOX (500 ng/mL) to induce expression of the RALY knockdown, then sorted 7 days later to analyze organoid survival. Patient-derived organoids were transfected with RALY siRNA for 3 days to analyze organoid survival and H&E staining.

Bioinformatics analysis

KEGG pathway enrichment analysis was performed by applying the online tool of DAVID Bioinformatics Resources 6.8 (http://david.ncifcrf.gov). Fold-change (FC) of gene expression was calculated with a cut-off of log2FC≥1, log2FC<-1 and P value<0.05. A website of Gene Expression Profiling Interaction Analysis (GEPIA, https:// http://gepia.cancer-pku.cn/index.html) was applied to determine the clinical survival of the related genes. MiRNA-mRNA relationship was analyzed and checked by four websites (http://www.targetscan.org/vert_72/;

http://www.mirbase.org/; http://www.microrna.org/microrna/home.do and http://mirtrail.bioinf.uni-sb.de/). The gene expression profile dataset was downloaded from Gene Expression Omnibus (GEO) database (shown in Table S4) and The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov), and subsequently analyzed by R (Version 3.4, http://www.bioconductor.org) with edgeR package. The interaction network of RALY and miRNAs was performed by Funrich3.13 (http://www.funrich.org).

Statistical analysis

Statistical significance for all experiments was evaluated by comparing p values determined by Prism 8.0 software with unpaired two-tailed Student t test. P values are indicated on plots (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). Results are expressed as mean ± standard error (SD).

Reference

1. Niu Y, Lin Z, Wan A, et al. RNA N6-methyladenosine demethylase FTO promotes breast tumor progression through inhibiting BNIP3. *Molecular cancer* 2019;18(1):46. doi: 10.1186/s12943-019-1004-4
2. Lee J, Yesilkanal AE, Wynne JP, et al. Effective breast cancer combination therapy targeting BACH1 and mitochondrial metabolism. *Nature* 2019;568(7751):254-58. doi: 10.1038/s41586-019-1005-x

Supporting supplemental figure legends:

Supplementary figure legends

Supplementary Figure 1

Immunohistochemistry (IHC) of 130 pairs of CRC tumors FFPE with specific antibodies against RALY.

Supplementary Figure 2

A. Up-regulation of RALY is significantly associated with poorer overall survival rates in local CRC patients (cut-off 50% vs 50%).

B. The expression of RALY is significantly correlated with univariate clinical factors including stage and distant metastasis (M) (TCGA-COAD & TCGA-READ).

C. RALY is co-localized with Drosha in HCT116 cells by immunofluorescent staining.

Supplementary Figure 3

A. Stable knockdown of RALY in HCT116, HCT15 and LOVO cell lines by lentiviral shRNA sequences (shRALY#1, shRALY#2). The knockdown effect is verified in protein level.

B. Stable overexpression of RALY in HCT116, HCT15 and LOVO cell lines and was verified at protein level.

C. Various levels of RALY affected ECAR value and glycolytic capacity in HCT116 cells.

D. Various levels of RALY affected OCR value and mitochondrial respiratory capacity in HCT116 cells.

E. Various levels of RALY affected ECAR value and glycolytic capacity in HCT15 cells.
F. Various levels of RALY affected OCR value and mitochondrial respiratory capacity in HCT15 cells.
G-J. Various levels of RALY regulated ATP production (G), ROS production (H) and NAD+/NADH level (I, J) in HCT116 and HCT15 cells.
Data information: In all relevant panels, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; two-tailed t-test. Data are presented as mean±SD and are representative of three independent experiments.

Supplementary Figure 4
A-B. Stable knockdown of ATP5I, ATP5G1, ATP5G3, CYC1 in HCT116, HCT15 and LOVO cell lines by lentiviral shRNA sequences (shATP5I, shATP5G1, shATP5G3, shCYC1), respectively. The knockdown effect was verified at mRNA level (A) and protein level (B).
C. Knockdown of RALY-dependent ATP5I, ATP5G1, ATP5G3 and CYC1 decreased ROS levels in RALY-depleting HCT116 and HCT15 cells.
D. Potential binding sites on the 3'UTR of ATP5I, ATP5G1, ATP5G3 and CYC1 by RALY-dependent miRNAs. The binding predictions were performed by various tools including Targetscan, MiRanda and Microcosm. Δ: deletion of miRNA binding site.
E. Wild-type or miRNA-binding site deletion mutant of ETC 3'UTR is fused with firefly luciferase reporter.
F. Relative luciferase activity of the wild-type and deletion mutant ETC 3'UTR reporter vectors treated with miRNA oligos or antagomirRNAs in HCT116 cells, respectively.
Data information: In all relevant panels, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; two-tailed t-test. Data are presented as mean±SD and are representative of three independent experiments.

Supplementary Figure 5
A-B. Validation of RALY-dependent miRNAs in targeting mitochondrial metabolism-related genes at mRNA level (A) and at protein level (B) in HCT116 cells.
C-D. Validation of RALY-dependent miRNAs in targeting mitochondrial metabolism-related genes at mRNA level (C) and at protein level (D) in HCT15 cells.
E-G. Overexpression of RALY-dependent miRNAs rescued ECAR level, glycolytic capacity, OCR level and mitochondrial respiratory capacity in RALY-knockdown LOVO (E), HCT116 (F) and HCT15 (G) cells.
H-J. Overexpression of RALY-dependent miRNAs rescued NAD+/NADH level in RALY-knockdown LOVO (H), HCT116 (I) and HCT15 (J) cells.
K-L. Overexpression of RALY-dependent miRNAs decreased ROS levels in RALY-depleting HCT116 (K) and HCT15 (L) cells.
Data information: In all relevant panels, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; two-tailed t-test. Data are presented as mean±SD and are representative of three independent experiments.

Supplementary Figure 6
A. Cell proliferation with RALY knockdown was measured by cell number counting and CCK8

assay in HCT15 cells.
B. Cell proliferation with RALY knockdown was measured by cell number counting and CCK8 assay in LOVO cells.

Supplementary Figure 7
A. Five representative miRNAs including miR-148a, miR-301a, miR-483, miR-676 and miR-877 are regulated by METTL3 and RALY. The RALY-binding motif (red) and the m^6A motif mediated by METTL3 (cyan) are shown in the precursor sequences.
B. Knockdown of METTL3 decreased RALY-dependent miRNAs in LOVO cells.
C. The enhanced interaction between RALY and A3-m^6A methylated region was verified by RNA pull down assay and Western blotting assay. Quantification is shown at the right panel.
Data information: In all relevant panels, *P<0.05; **P<0.01; ***P<0.001; two-tailed t-test. Data are presented as mean±SD and are representative of three independent experiments.

Supplementary Figure 8
A. Knockdown of METTL3 increased expression of RALY-dependent ATP5I, ATP5G1, ATP5G3 and CYC1 by RT-PCR.
B. Knockdown of METTL3 decreased cell proliferation in HCT116, HCT15 and LOVO cells by crystal violet staining assay.
C. Overexpression of miR-877 rescued ECAR value and OCR level in METTL3-knockdown HCT15 and LOVO cells.
D. Overexpression of miR-877 rescued glycolytic capacity and mitochondrial respiratory capacity in METTL3-knockdown HCT15 and LOVO cells.
E-G. Overexpression of miR-877 rescued ATP production (E), ROS production (F), and NAD+/NADH level (G) in METTL3-knockdown HCT15 and LOVO cells.
Data information: In all relevant panels, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; two-tailed t-test. Data are presented as mean±SD and are representative of three independent experiments.

Supplementary Figure 9
A. Tumor images of orthotopic xenograft mice model.
B. H&E staining of orthotopic xenograft tumors and adjacent normal tissues.
C. Quantitative of RALY and ATP5I expression levels in knockdown of RALY by overexpression of miR-676 in HCT116 cells derived xenografts from Western blotting analysis.
Data information: In all relevant panels, *P<0.05; two-tailed t-test. Data are presented as mean ±SD and are representative of three independent experiments.

Supplementary Figure 10
A. Various protein expression levels of RALY in CRC patient tumors.
B. Knockdown of RALY by siRNAs suppressed growth of CRC patient-derived tumor organoids with various levels of RALY.
C-E. Gross tumor images (C), tumor weight (D) and tumor growth curve (E) of PDX models with various levels of RALY treated with nano-gold siCTRL and nano-gold siRALYs with administration strategy as indicated. The nano-gold particles (20 mg/kg) were administrated

every 3 days for 3 weeks.

Data information: In all relevant panels, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; two-tailed t-test. Data are presented as mean±SD.

Supplementary Figure 11

A. Gene expression levels of RALY, METTL3, ATP5I, ATP5G1, ATP5G3 and CYC1 in colon primary tumors compared to normal tissues (TCGA-COAD).

B-C. Correlations between RALY and miRNAs in CRC datasets from supplementary table 4 were analyzed by FunRich3.13 (B). The details of RALY-dependent miRNAs are shown (C).

D. Up-regulation of miR-148a and miR-877 are significantly associated with shorter overall survival rates in CRC patients (TCGA-COAD).

E. Up-regulation of miR-877, miR-96 and miR-483 are significantly associated with shorter overall survival rates in CRC patients (GSE29623).

Supplementary tables

Table 1

Demographic characteristics of 130 patients with colorectal cancer.

Table 2

Demographic characteristics of TCGA CRC patients.

Table 3

Differentially expressed genes from RALY-RIP sequencing, RALY-KD miRNA sequencing and RALY-KD mRNA sequencing.

Table 4

Dataset of clinical samples for correlation analysis of miRNAs and RALY.

Table 5

Primer lists used in this study.

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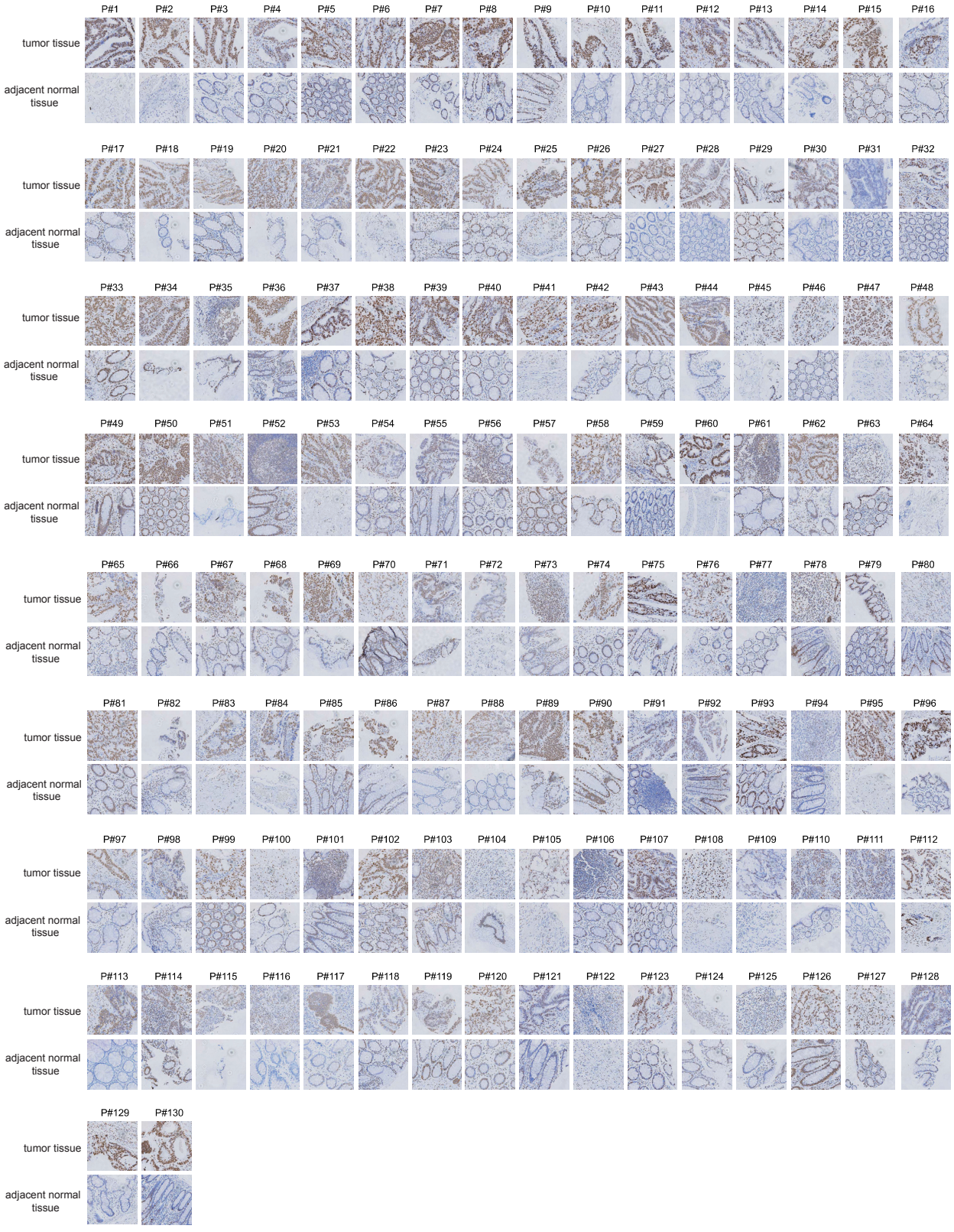
Supplementary Figure 1



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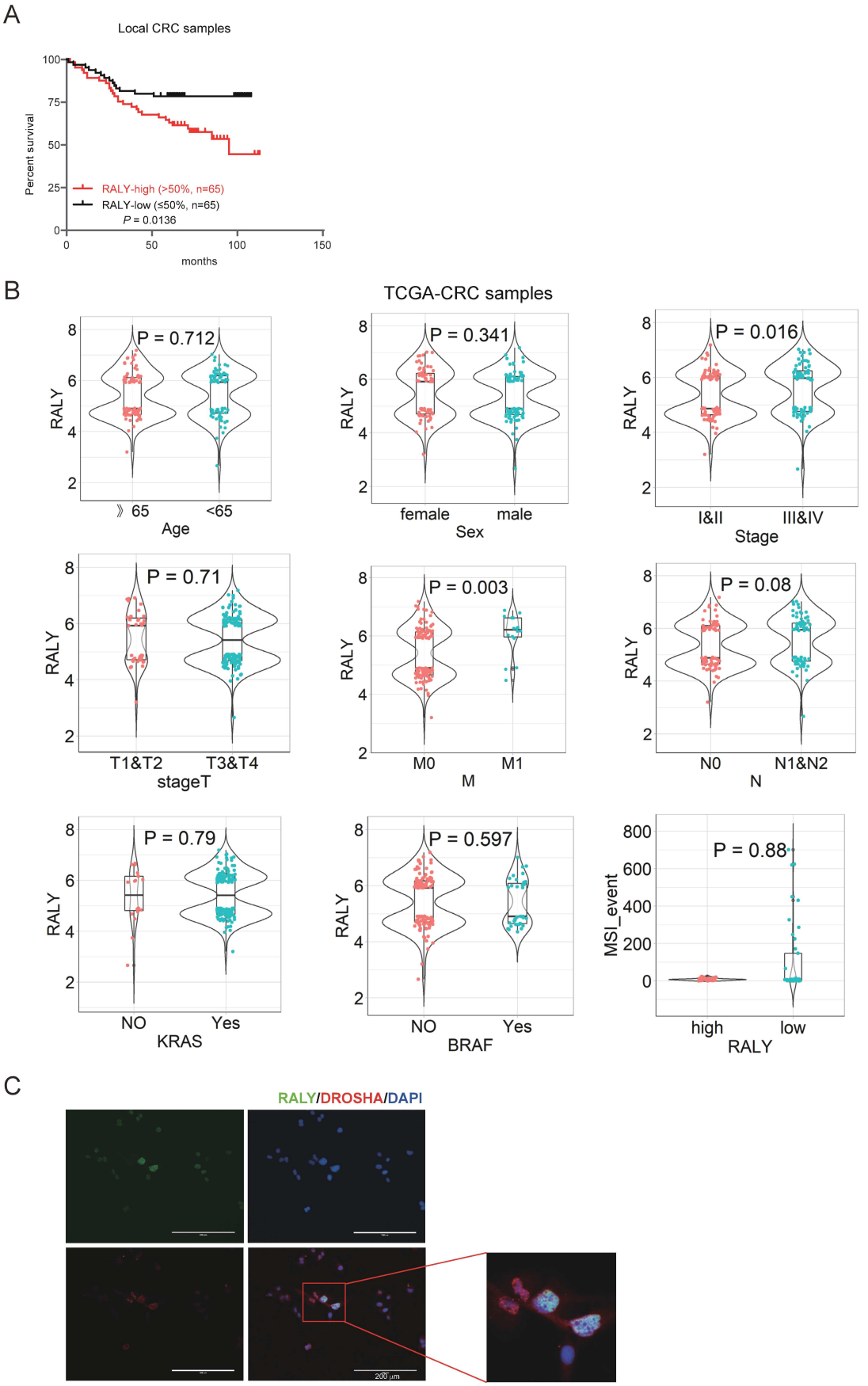

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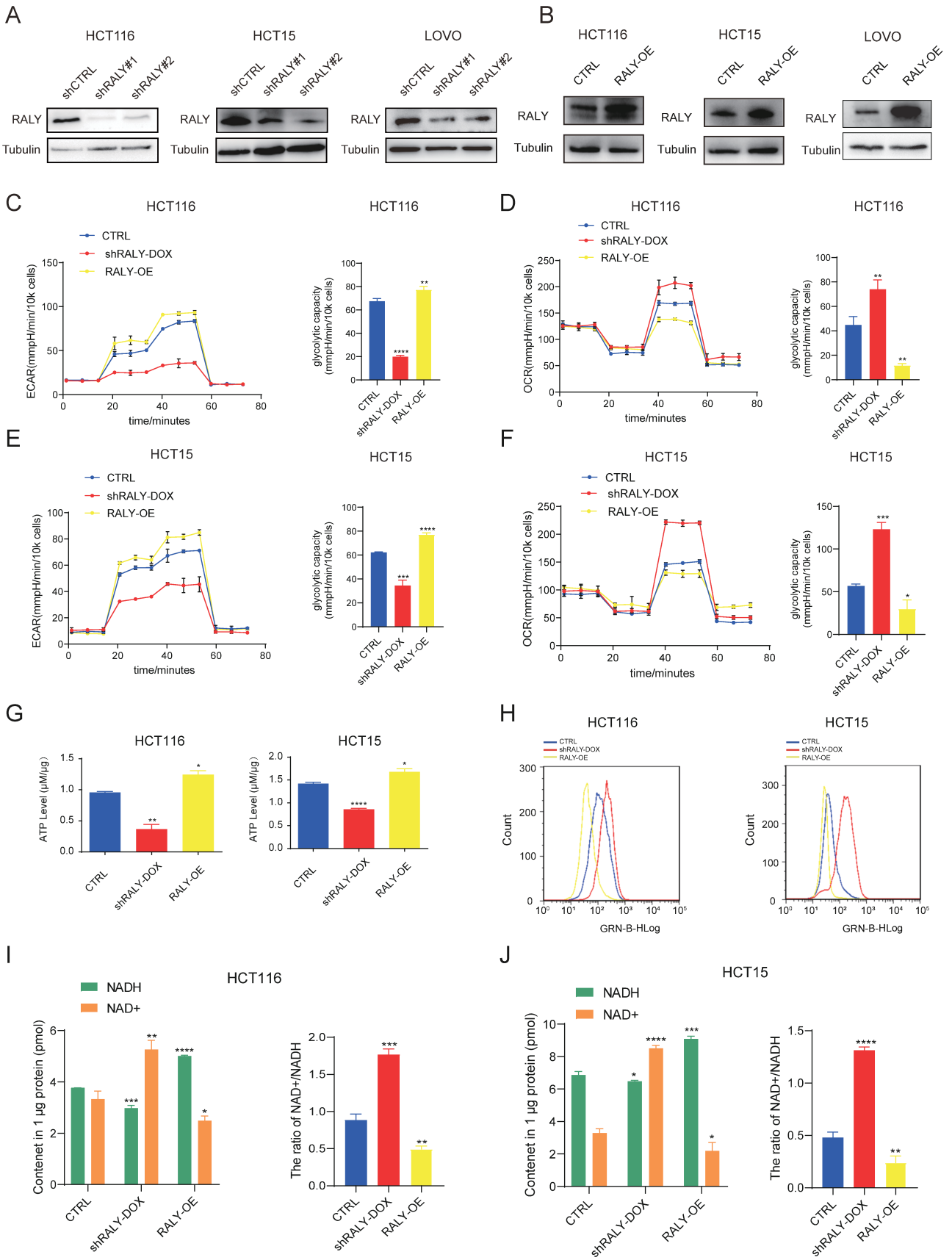

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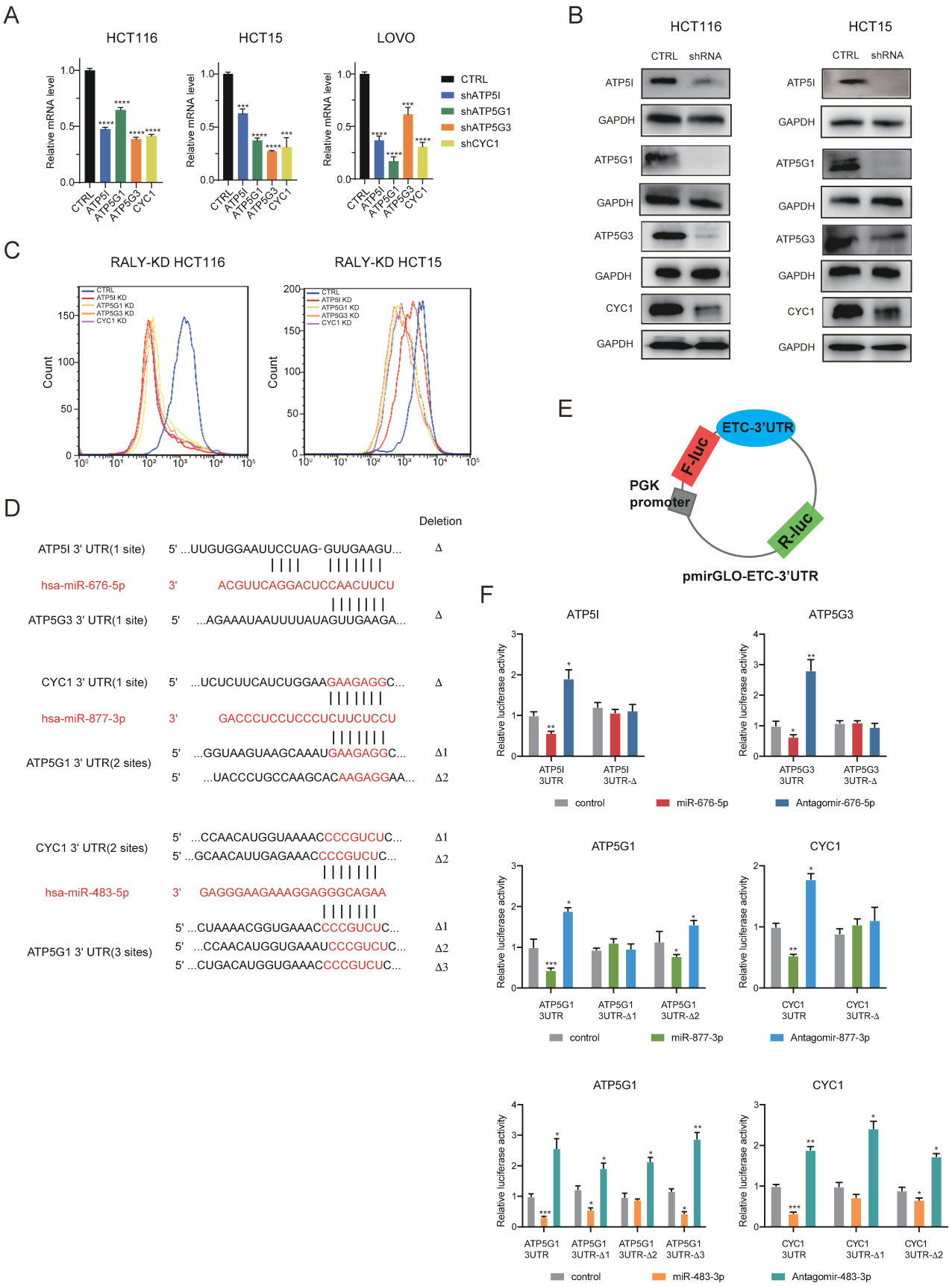

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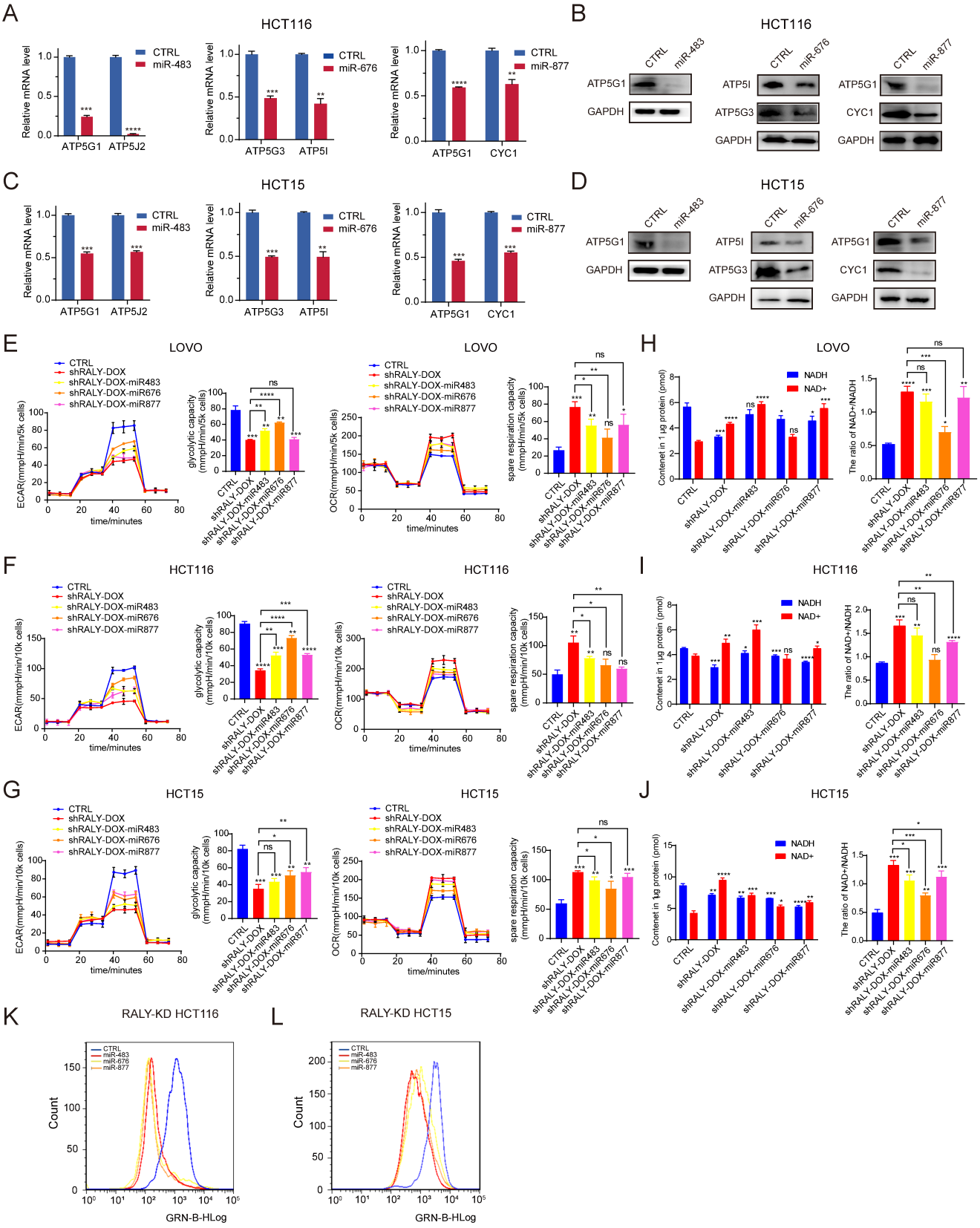

Supplementary Figure 6

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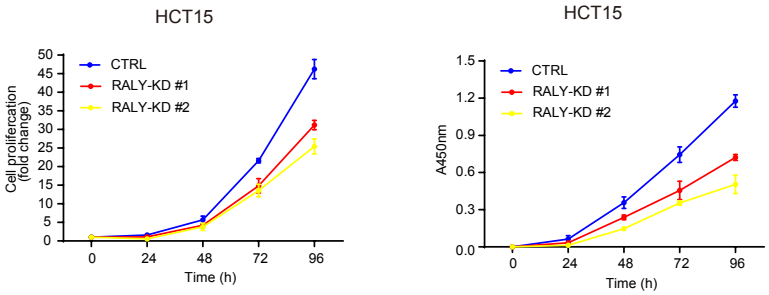

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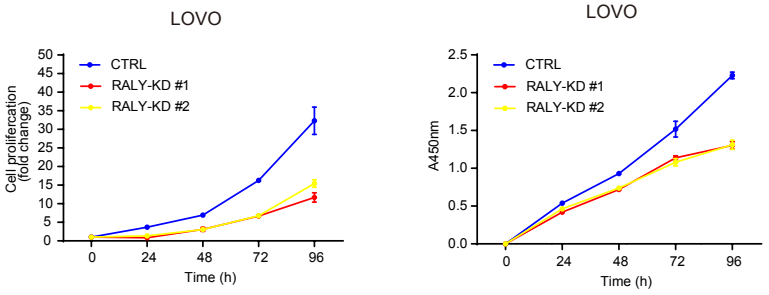
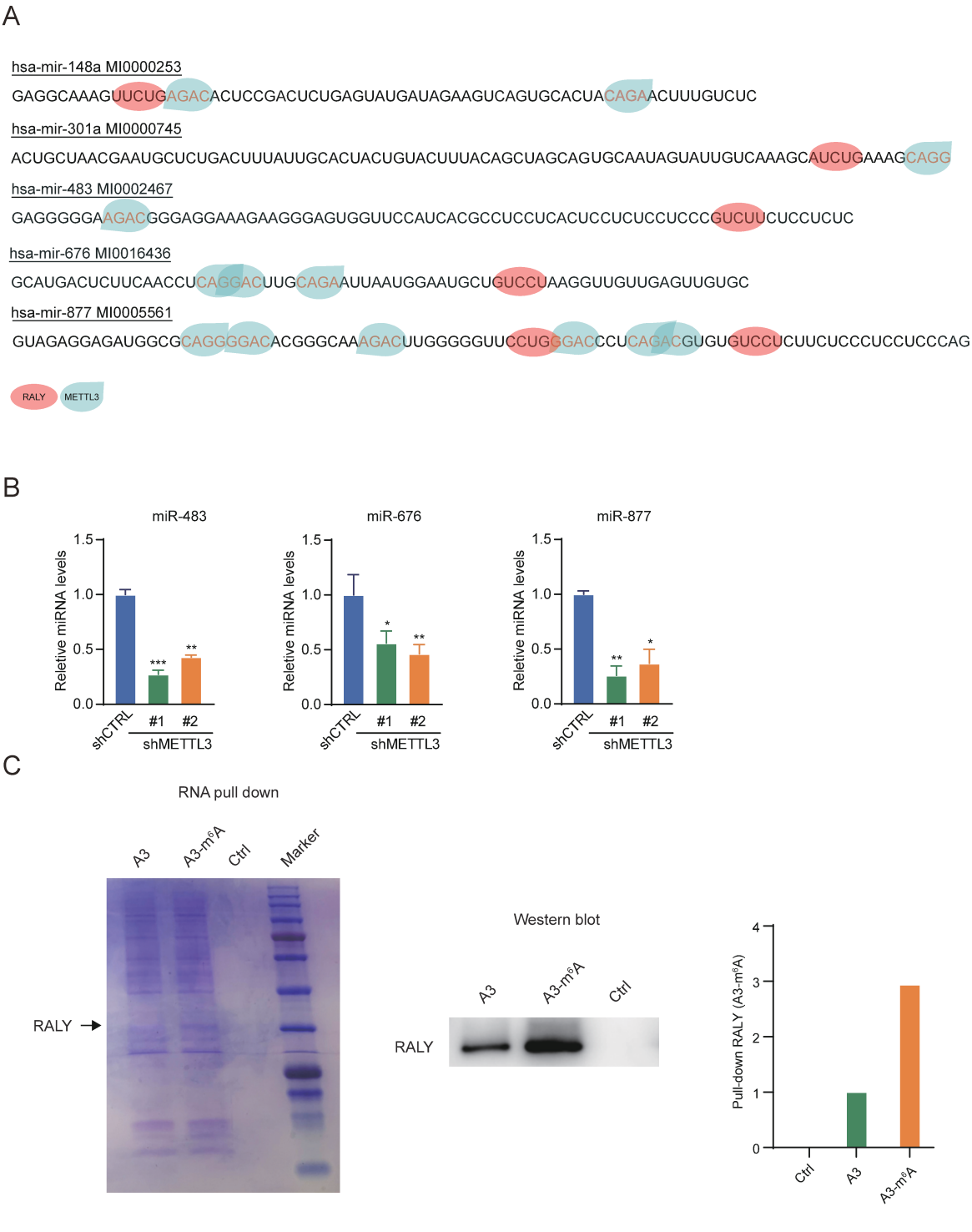


Supplementary Figure 7



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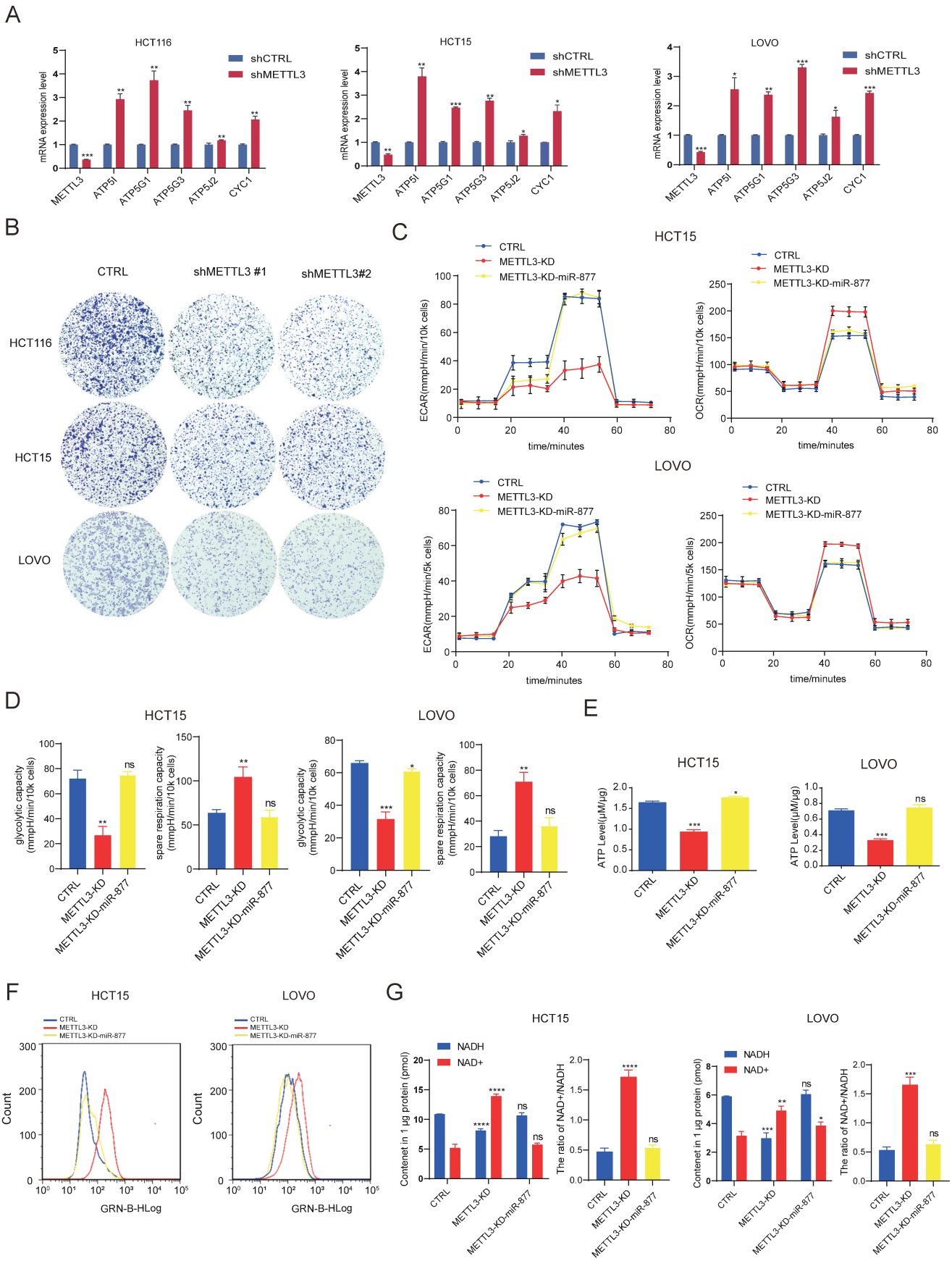

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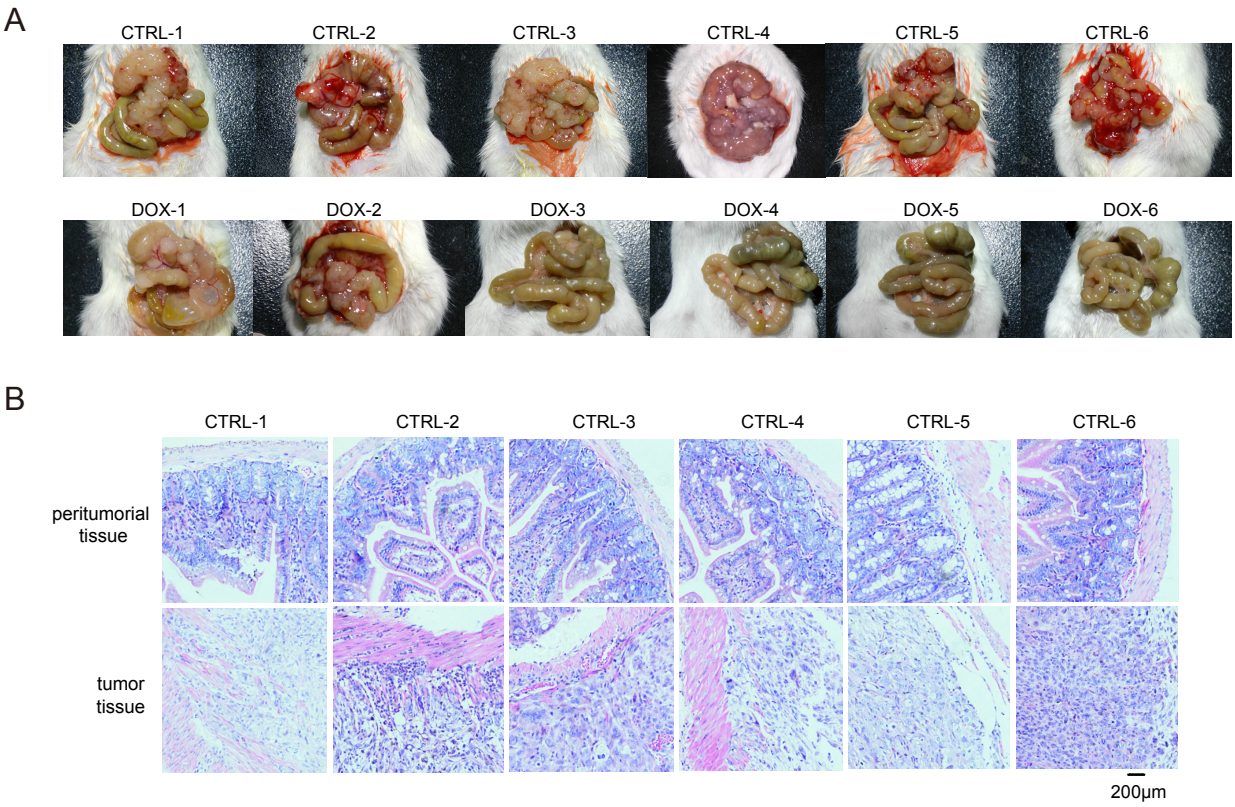

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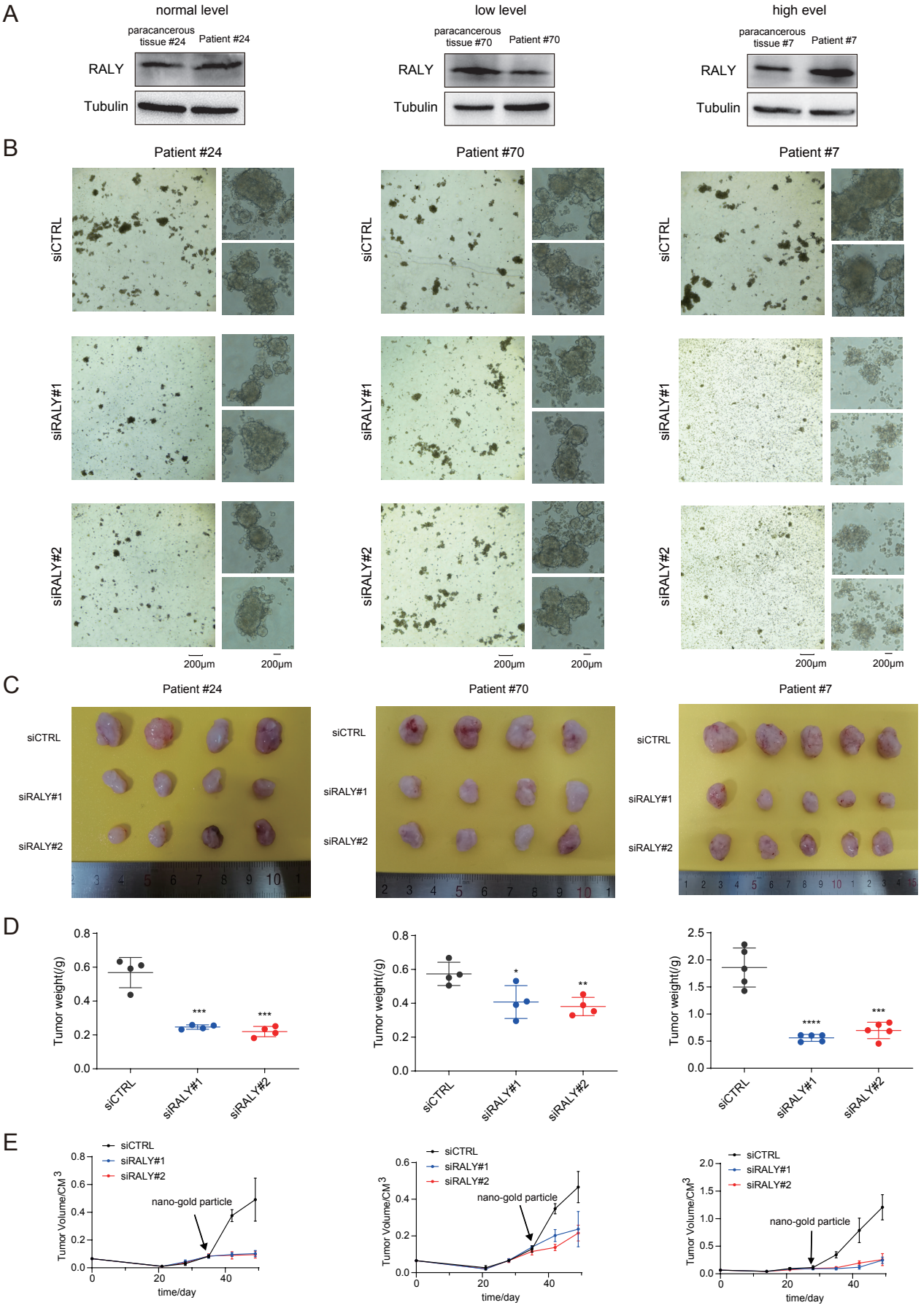

Supplementary Figure 11

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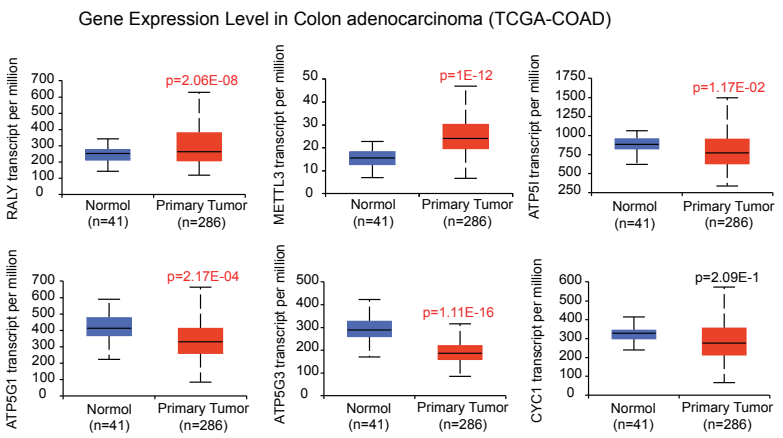

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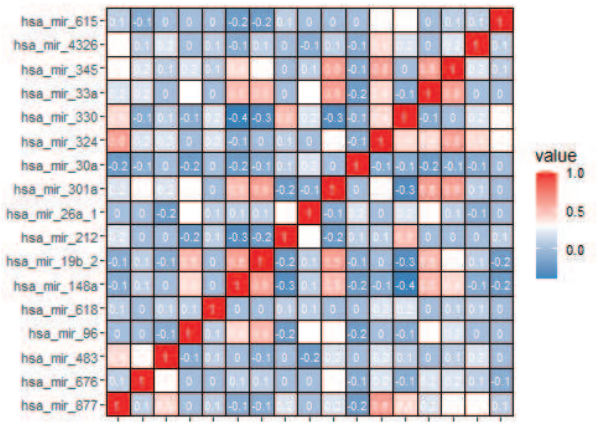
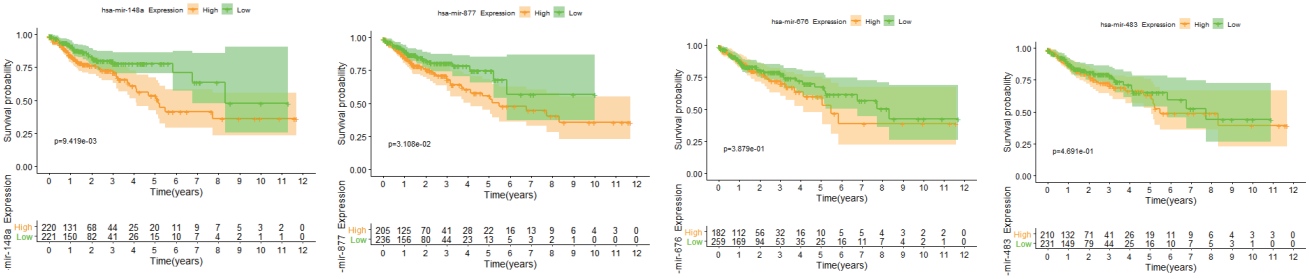
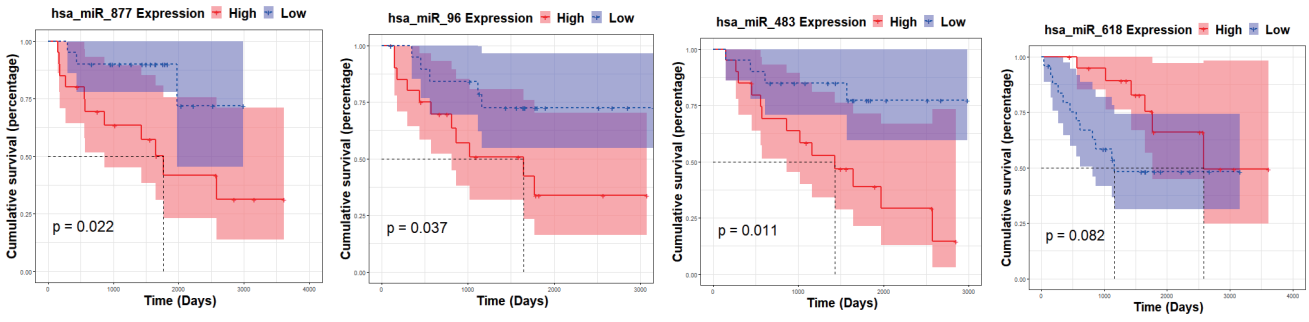


C

Analysis by FunRich3.13

Gene	cor	ConI	pval
RALY	1	1-1	0.00E+00
hsa_mir_877	-0.093	-0.193-0.01	0.076
hsa_mir_676	0.234	0.135-0.329	5.98E-06
hsa_mir_483	0.04	-0.063-0.142	0.446
hsa_mir_96	0.141	0.04-0.24	0.007
hsa_mir_618	-0.07	-0.171-0.033	0.184
hsa_mir_148a	0.222	0.122-0.317	1.80E-05
hsa_mir_19b_2	0.341	0.247-0.428	2.09E-11
hsa_mir_212	-0.182	-0.279- -0.081	4.74E-04
hsa_mir_26a_1	0.001	-0.101-0.104	0.981
hsa_mir_301a	0.2	0.099-0.296	1.19E-04
hsa_mir_30a	-0.007	-0.11-0.095	0.892
hsa_mir_324	-0.039	-0.141-0.064	0.457
hsa_mir_330	-0.293	-0.384--0.197	1.05E-08
hsa_mir_33a	0.186	0.085-0.283	3.58E-04
hsa_mir_345	0.165	0.063-0.263	0.002
hsa_mir_4326	0.156	0.055-0.255	0.003
hsa_mir_615	-0.298	-0.388--0.201	6.44E-09

D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