Supporting Information

An Optimized Enzyme-Nucleobase Pair Enables In Vivo RNA Metabolic Labeling with Improved Cell-Specificity

Monika K. Singha,[1] Jan Zimak[2], Samantha R. Levine[2], Nan Dai[5], Chan Hong[4], Cecily Anaraki[1], Mrityunjay Gupta[3], Christopher J. Halbrook[1], Scott X. Atwood[4], and Robert C. Spitale[123]*

(1) Department of Molecular Biology and Biochemistry University of California, Irvine. Irvine, CA 92697. (2) Department of Pharmaceutical Sciences University of California, Irvine. Irvine, CA 92697. (3) Department of Chemistry University of California, Irvine. Irvine, CA 92697. (4) Department of Cell and Developmental Biology University of California, Irvine. Irvine, CA 92697. (5) New England Biolabs Beverly, MA 01915

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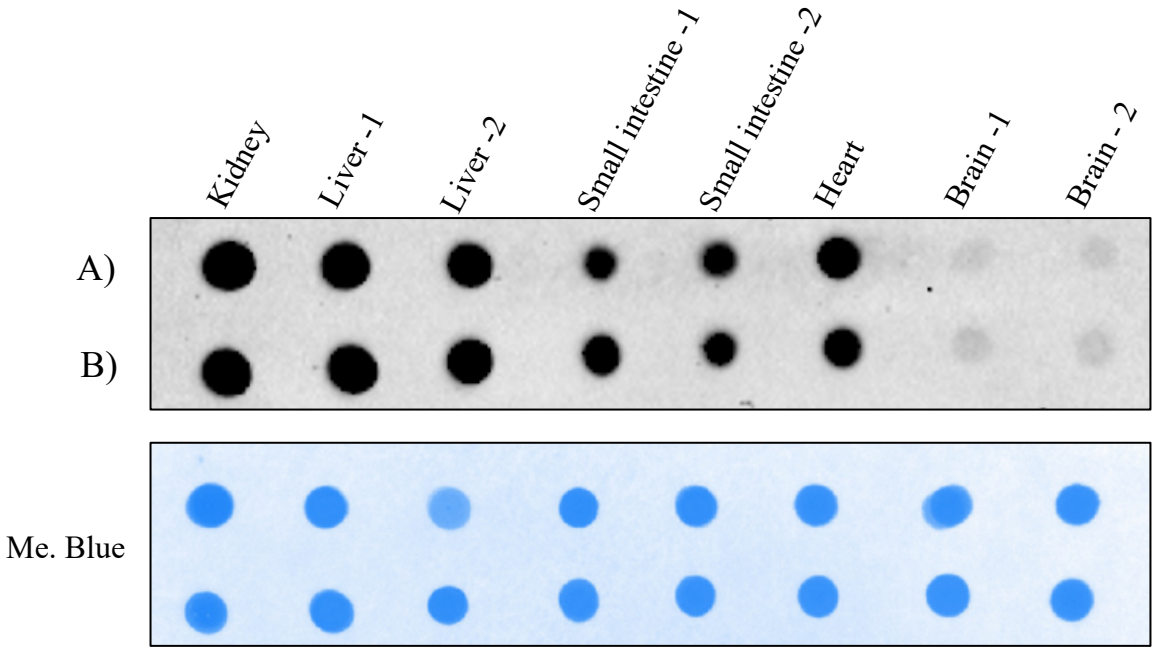


Figure S1: IP-injection of 5-vuD (+) into WT mice. Wild-type (WT) C57BL/6 mice were treated with 500 mM IP injections (300 mg/kg) of 5-vinyluridine over 5 hours prior to isolation of organs (two sections of liver, small intestine and brain were analyzed). RNA was reacted with tetrazine-biotin for dot blot analysis. A) and B) indicate biological replicates. 1 and 2 indicate technical replicates from organs.

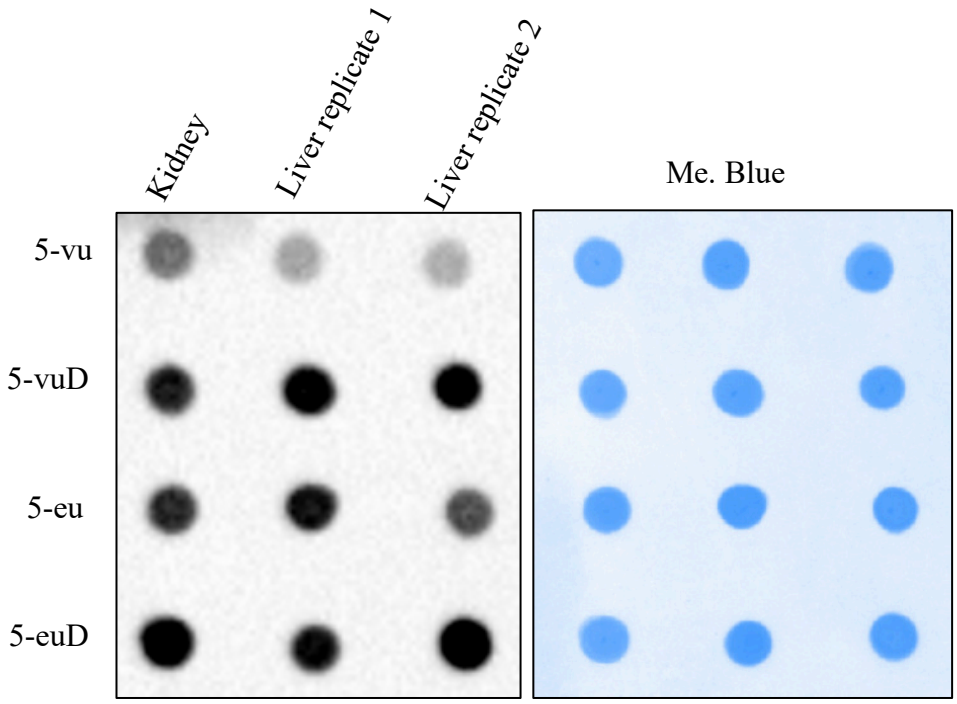


Figure S2: IP-injection with uracil and uridine analogs into WT mice. WT C57BL/6 mice were treated with 500 mM per injection over 24 hours. Organ RNA was isolated and reacted with biotin-tetrazine for dot blot analysis. 1 and 2 indicate technical replicates from the same mouse liver.

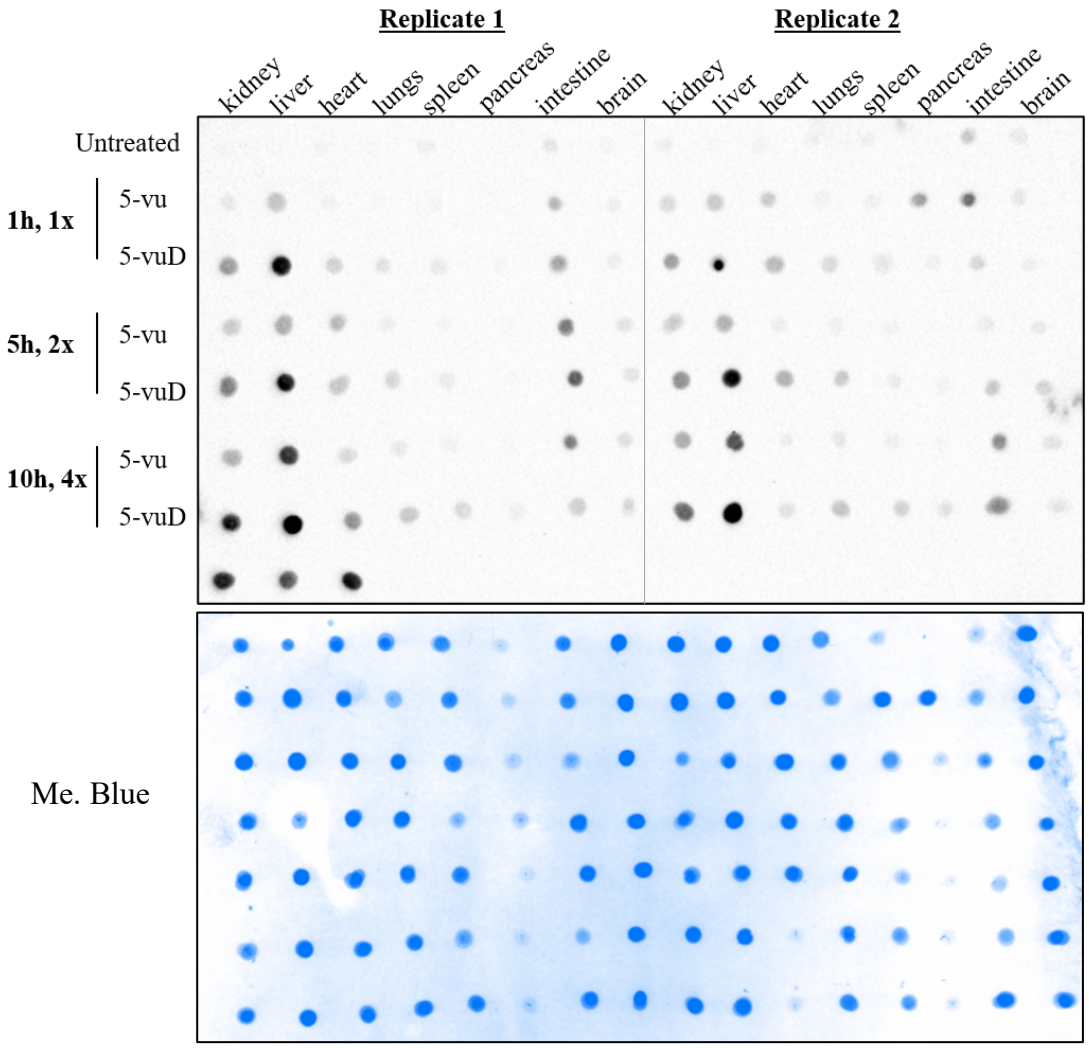


Figure S3: Comparison of 5-vu background labeling over increasing time and injections. Two biological replicates of mice were treated with 500 mM of 5-vu or 5-vuD (positive control) with increasing injections over time at 1h, 5h, and 10h. Double and quadruple injected mice were treated at 2.5-hour time increments between injections. Organs were isolated at the indicated time points. RNA was extracted and reacted with tetrazine-biotin for dot blot analysis. Untreated mouse RNA was used as negative control.

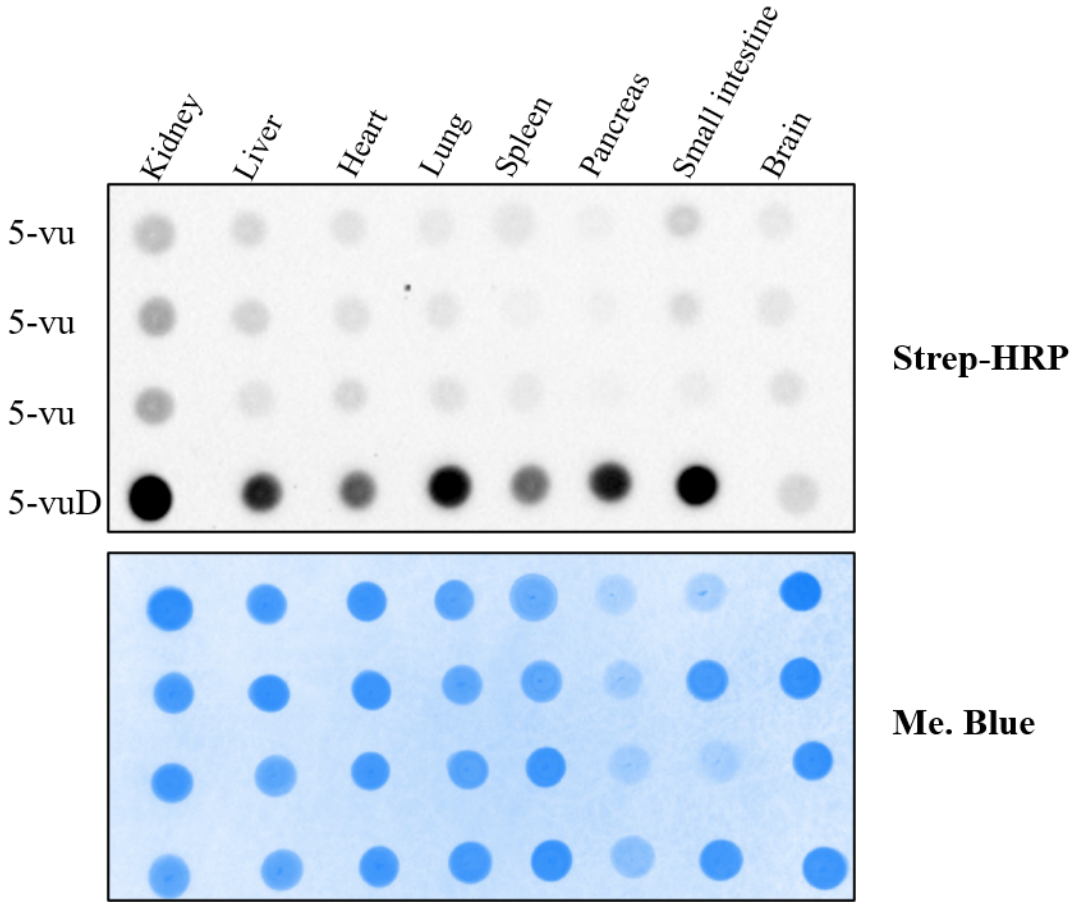


Figure S4: Organs from xenografts (Figure 2A) show background RNA labeling. Dot blot analysis depicts the signal of RNA labeling from tissues that do not express 3xUPRT. Low levels of metastasized 3xUPRT cells may be expressed in the lung below the *ex vivo* mCherry fluorescence detection limit.

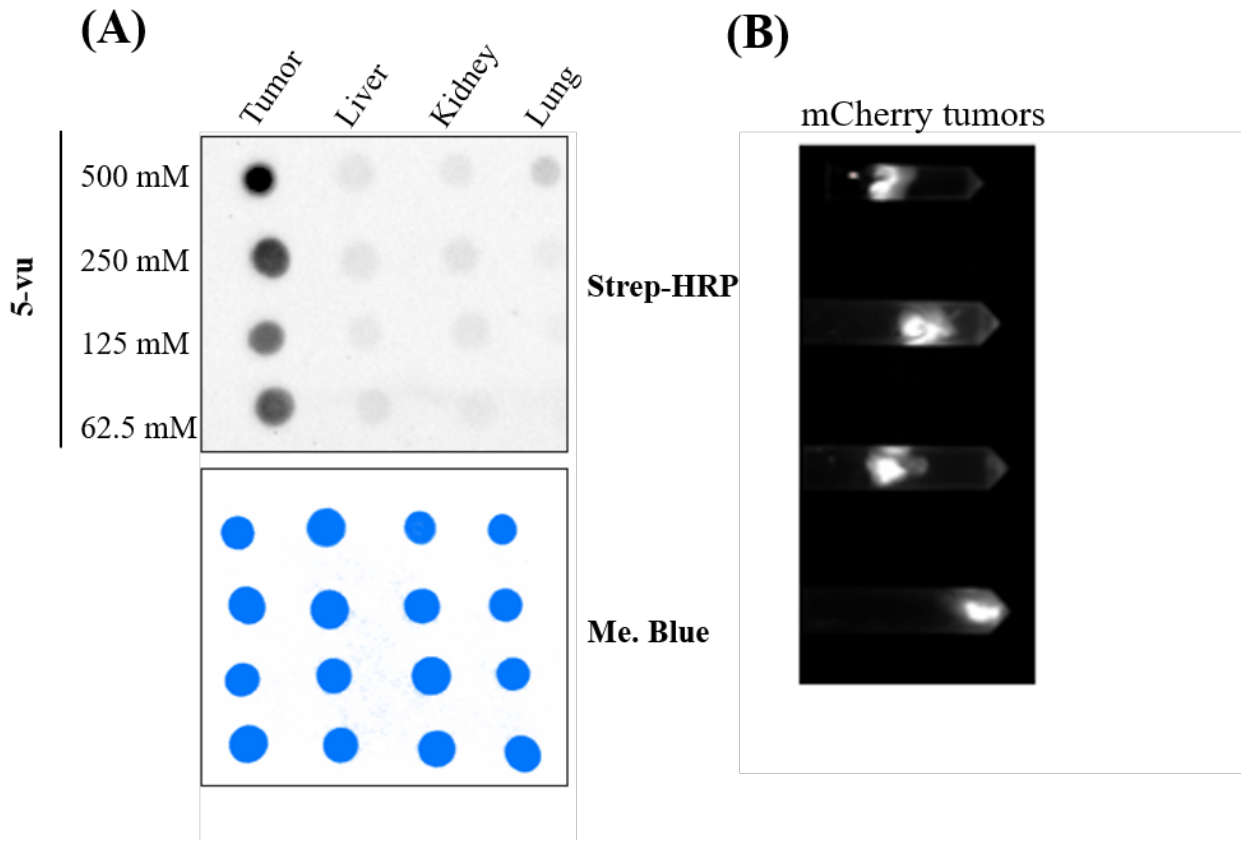
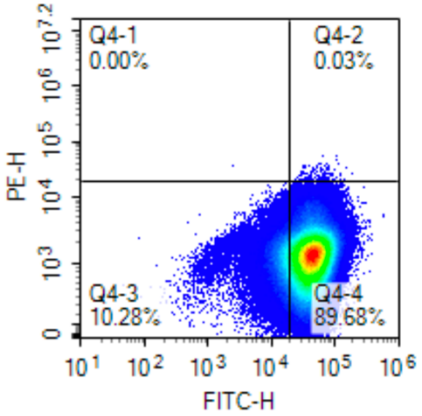


Figure S5: IP-injection of standard dose and 2-fold reduced dose in (+)-3xUPRT xenograft mice. A) Dot blot analysis of 5-vu treatment titration in mice with 3x-UPRT expressing tumors. Liver and kidney tissue do not have 3xUPRT expression. No or low levels of 3xUPRT tumors were detected in matching lung samples. B) *Ex vivo* mCherry fluorescence depicts co-expressed 3xUPRT in tumors.

WT MDA-MB-231 LM2 cells



(+)-3xUPRT MDA-MB-231 LM2 cells

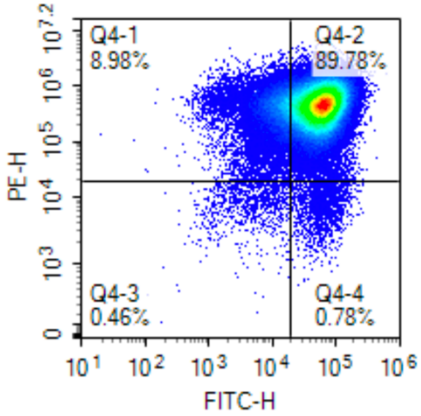


Figure S6: Flow cytometry analysis of WT and (+)-3xUPRT MDA-MB-231 LM2 cells prior to xenotransplantation. WT and (+)-3xUPRT cells both express GFP indicated by FITC detection. (+)-3xUPRT cells only express mCherry indicated by PE detection.

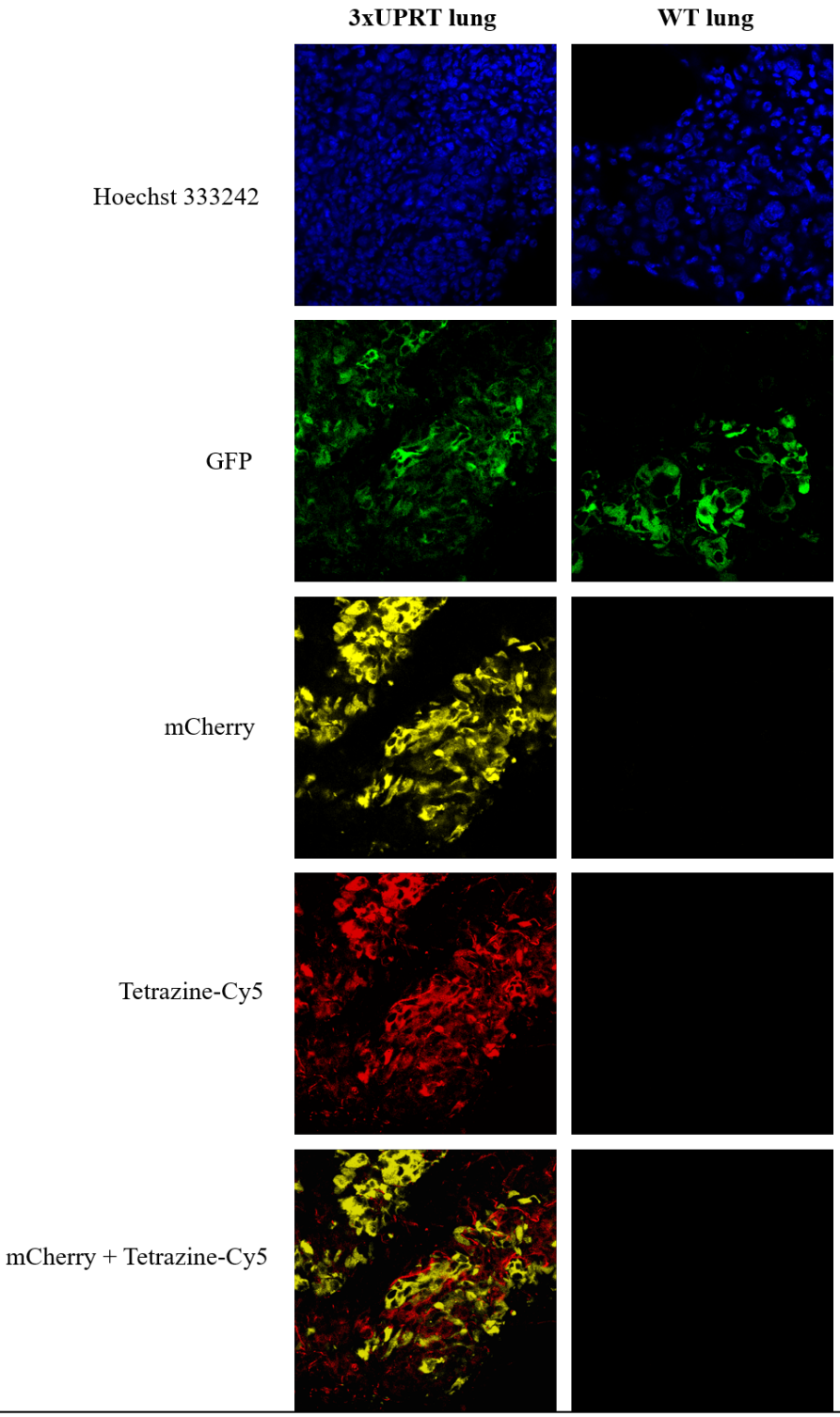


Figure S7: Metabolic labeling in (+)-3xUPRT-mCherry MDA-MB-231 LM2 metastatic cells in lung tissues. Vinyl-containing RNA are fluorophore-conjugated with tetrazine-Cy5. GFP signal is present in WT MDA-MB-231 LM2 cells (parental line).

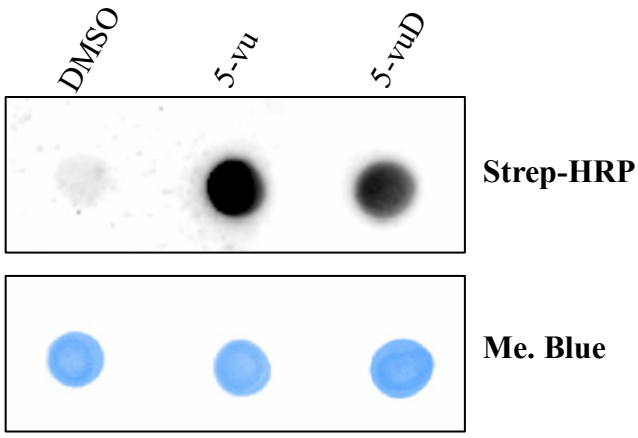


Figure S8: RNA dot blot with vimentin-3xUPRT stable MDA-MB-231 cells. Cells were treated for 3h with 1 mM of DMSO, 5-vu or 5-vuD, biotinylated with tetrazine-biotin and metabolically labeled RNA were detected with streptavidin-HRP chemiluminescent signal.

II. Experimental Methods

Cell lines used in the study: MDA-MB-231 LM2 4175 cells[11] were cultured in DMEM containing high glucose (4.5 g/L) and L-glutamine (Corning Ref# 10-017-CM) supplemented with 10% FBS, 1% penicillin-streptomycin (complete DMEM). These were purchased from Memorial Sloan Kettering Cancer Center (MSKCC) and developed in the lab of Joan Massagué. Cells were grown at 37 °C, 5% CO_2. Stable 3xUPRT-mCherry cell lines were selected in 1-10 μg/ml puromycin for selection in complete DMEM.

IP-injection experiments in mice. NSG or C57BL/6l mice were treated with nucleoside or nucleobase analogs intraperitoneally by injection into the lower left quadrant of the abdomen (peritoneum), lateral to the animal's midline at a <45° angle. Injections did not exceed 70 μl. For uridine analogs, 300 mg/kg were used per injection. For uracil analogs, 150 mg/kg were used per injection. DMSO was used as a solvent to avoid precipitation of analogs after injection. Optionally, researchers may use 95% corn oil and 5% DMSO only for 5-vu injections. WT mice were treated for 1-24 hours for optimizing these studies. Xenograft mice were treated for 3 hours before sacrificing with CO_2 and cervical dislocation. Animal use protocols were reviewed and approved by the Institutional Animal Care and Use Committee of University of California, Irvine.

Mouse orthotopic xenografts for metastatic breast cancer and organ harvesting (followed by RNA preservation): NSG female mice were shaved at the injection site and sterilized with ethanol wipes. Prior to injection, isoflurane levels of 1.5-2% were was used for anesthesia prior to cell transplantation. Mammary fat pad L4 was injected with $1x10^6$ MDA-MB-231 LM2 cells in (WT or with 3xUPRT expression) 100 μl PBS into the at 14-weeks of age. These mice grew

tumors for 3-4 weeks to develop until 1000 mm^3 volumes maximum. Orthotopic injections into the fat pad were performed without incision by direct injection into the site of the mammary fat pad, below the nipple and into the fatty layer[17]. Post-operative care included heat pad for anesthesia recovery and acetaminophen (1.6 ml oral suspension into 150 ml drinking water). Xenograft mice were IP-injected for 3 hours with analogs (as previously indicated) before sacrificing with CO_2 and cervical dislocation for harvesting organs and tumors. After dissection, organs were immediately placed into RNAlater (Thermofisher AM7021) solution and cut into 0.5 cm segments, submerged in solution for 24-48 hours at 4°C. Following this, tissue was minced avoiding RNAse contamination with RNAse Zap (Thermofisher AM9780) and directly homogenized with 0.9-2mm RNAse-free stainless-steel beads (Fisher NC0586331) in 1 mL TriZol (Thermofisher #15596026) for 1-minute increments. Between 1-minute increments, tubes were placed on ice, and homogenized for 2-3 more times. Homogenization tubes were centrifuged for 5 minutes prior to lysate collection and processed following manufacturer's instruction for RNA extraction. RNA was reconstituted in nuclease-free water and the concentration analyzed with Nanodrop 2000 spectrophotometer.

RNA biotinylation using biotin-tetrazine click reaction: 5-15 μg of total cellular, tumor or organ derived DNAse-treated RNA was reacted in solutions with 1 mM tetrazine-biotin (Sigma #793329) or 1 mM DMSO (negative controls), all of which were 10% DMSO final reactions in nuclease-free water and incubated in dark conditions. Tetrazine-biotin stocks were all dissolved and prepared in DMSO as 10 mM solution stocks stored in -20°C using amber tubes. RNA was purified after biotinylation using RNA Clean and Concentrator Kit #5 (Zymo Research #R1013) and eluted in 20 μl of nuclease-free water prior to dot blot analysis.

RNA dot blot analysis with chemiluminescence detection[1]: To detected metabolically labeled RNA, biotinylated RNA was crosslinked to a Hybond-N+ (GE Healthcare) membrane with 254 nm light using a UV stratalinker (Stratagene). The membrane was first equilibrated in 2x SSC buffer before spotting RNA and fixing with UV-light. After UV-crosslinking the RNA, membranes were blocked in blocking buffer[15] (0.12 M NaCl, 0.016 M Na2HPO4, 0.008 M NaH2PO4, 0.17 M SDS) for 30 minutes and followed by incubation with high sensitivity streptavidin-HRP (Fisher Scientific, Cat#: PI21130) at 1:5000 dilution in blocking buffer for 5 minutes. The membrane was washed twice in a Wash A buffer (1:10 dilution of blocking buffer in D.I. water) for 30 minutes and twice in Wash B buffer (0.1 M Tris-base, 0.1 M NaCl, 0.02M MgCl2, pH 9.5) for 5-10 minutes. Membranes were then incubated for 1-5 minutes in ECL Chemiluminescent Substrate (Fisher Scientific, Cat#: PI32106) and imaged on a ChemiDoc MP imaging system (Bio-Rad) to detect chemiluminescence on the membrane. After imaging is completed, methylene blue (0.04% methylene blue and 0.3 M sodium acetate) was used to stain onto membranes overnight, rinsed briefly with methanol, then D.I. water to remove excess stain, followed by colorimetric imaging with the ChemiDoc MP imaging systems to detect consistent loading of RNA onto the membranes.

Enrichment of metabolically labeled RNA:cDNA for qPCR analysis[3]: RNA was purified after biotinylation as described before dot blot analysis and treated with 2U DNase TURBO (Invitrogen, RNAse free) for 30 min at 37°C followed by column purification using Zymo iic columns and eluted in nuclease-free water. Purified RNA was quantified by Nanodrop 2000 and 500 ng or 1ug of RNA included in for reverse transcription using SuperScript IV (ThermoFisher), following the manufacturer's protocol except for the RNAse-H step. The resulting RNA:cDNA hybrids were gently mixed and separated into equal volumes. One half

was used as input and the other half for streptavidin magnetic bead separation. 10 μl of MyOne C1 streptavidin beads (ThermoFisher) were aliquoted per enrichment and washed 4x with binding buffer (1 M NaCl, 100 mM Tris-HCl, pH 7.0, 10 mM EDTA and 0.2% (vol/vol) Tween-20), then blocked in 10 μl of RNAse-free glycogen (Fisher FERR0551) for 1 hour at room temperature with adequate mixing (end-over-end). After blocking, beads were washed 2x in binding buffer, resuspended in 10 μl with equal volume of RNA:cDNA sample. To ensure improved mixing, samples were pipetted every 3-5 minutes over a 20-minute period at room temperature. Then, beads were immobilized by a Dynabeads 1.5 mL tube magnetic rack. Supernatant was removed and beads were washed 4x in high salt wash buffer ((4 M NaCl, 100 mM Tris-HCl, pH 7.0, 10 mM EDTA and 0.2% (vol/vol) Tween-20) followed by 2x washed in nuclease-free water. Beads were centrifuged and resuspended in 50 elution buffer (10 U RNase H, 1× RNase H buffer (New England Biolabs), 4 μg RNase A/T1 mix (Thermo Fisher Scientific) and 12.5 mM D-biotin) and incubated at 37 °C for 30 min at 750 rpm. Then 1 μl of DMSO was added and beads were heated at 95 °C for 8 minutes. The eluted cDNA and the input cDNA were equally purified at the same time using Zymo DNA Clean and Concentrator-5 kit, with avoidance of carryover and guanidine contamination through extra ethanol washes, and eluted in 40 μl of nuclease-free water. The resulting cDNA was quantified with using Luna qPCR master mix (New England Bioland) with primers specific for human vimentin and GFP using a CFX Connect Real-Time PCR Detection System (Bio-Rad). Raw Cq were quantified for fold enrichment using 2^{-dcT} normalized to the negative control (untreated mouse).

LC-MS analysis of vinyl-U modification: RNA samples were digested to nucleosides using Nucleoside Digestion Mix (New England Biolabs). LC-MS/MS analysis was performed by injecting digested RNAs on an Agilent 1290 Infinity II UHPLC equipped with a G7117A diode array detector and a 6495C triple quadrupole mass detector operating in the positive electrospray ionization mode (+ESI). UHPLC was carried out on a Waters XSelect HSS T3 XP column (2.1 × 100 mm, 2.5 μm) with a gradient mobile phase consisting of methanol and 10 mM aqueous ammonium acetate (pH 4.5). MS data acquisition was performed in the dynamic multiple reaction monitoring (DMRM) mode. Each nucleoside was identified in the extracted chromatogram associated with its specific MS/MS transition: rC $[M+H]^+$ at m/z 244.1→112.1, rU $[M+H]^+$ at m/z 245.1→113, rG $[M+H]^+$ at m/z 284.1→152.1, rA $[M+H]^+$ at m/z 268.1→136.1, Y $[M+H]^+$ at m/z 245.1→209, and 5-VU $[M+H]^+$ at m/z 271.1→139.1. External calibration curves with known amounts of the nucleosides were used to calculate their ratios within the samples analyzed.

Tumor and lung cryopreservation for tissue sectioning[4]: After mice were sacrificed, and the tumors and organs were collected and placed in a solution of 4% paraformaldehyde. The tumors and organs were fixed for 1 h at 4 °C with gentle shaking. After 1 hour, the PFA was removed and the samples were washed in PBS buffer for 10 minutes at 4 °C with gentle shaking. The wash was repeated twice. After the third wash, the PBS was removed and the samples were place in a 30% sucrose solution and left at 4 °C with gentle shaking overnight. The samples were then removed from the sucrose solution and placed in Tissue Tek-OCT (VWR 25608-930) and frozen. The frozen blocks were sectioned on a ThermoScientific Cryostar NX50 Cryostat. Tumor

and lung sections were cut at 10 μM thickness and placed onto VWR Superfrost Plus Micro slides (VWR 48311-703). Slides were placed at - 80 °C until further needed.

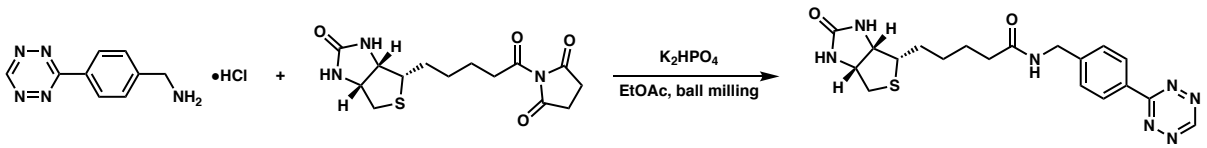
Fluorescent imaging of vinyl-RNA in mouse tissues: Cryo-preserved tumor and lung slices were washed in 2x PBS gently to remove all of the visible OCT off samples. A PAP pen (Immedge, Vector Labs) was used to create a hydrophobic barrier around the samples. 1 mg/ml BSA was used to block the tissue at room temperature without shaking, ensuring complete contact with tissue. Slides were rinsed 2x in PBS. 2 mM tetrazine-Cy5 (Lumiprobe) was dissolved in DMSO and kept in amber tubes and 1:1000 was dissolved in nuclease-free water, added to the slide, and incubated for 3h at 37°C in the dark. Slides were washed with 0.1% Triton-X100 (Sigma-Aldrich) in PBS 5x to ensure excess dye is removed and then washed 3x in PBS to remove detergent. PBS was removed and 1:1000 Hoechst (Cat#H3570, Invitrogen/LifeTech) was added to the tissue for a 10-minute incubation to stain nuclei, then washed 3x in PBS. Excess solution was removed from the slide and Vectasheild antifade mounting medium used to add a coverslip to the slide. A Leica Stellaris was used to capture nuclear signal, GFP, mCherry and Cy5 signals at 40x magnification with a water-immersion lens.

III. Synthesis[5]

Materials and Methods

Ball-milling/liquid assisted grinding[1] was performed in 2 mL microcentrifuge tubes containing 4 steel beads (5 mm) using a Benchmark BeadBug 6 Microtube Homogenizer set to 2500 rpm for the indicated length of time. Chemicals were purchased from Chem-Impex, AmBeed, Fisher Scientific, or VWR Scientific and used as received. Reactions were monitored by thin-layer chromatography (TLC) on glass-backed silica gel plates (Supelco 1.05175.001, 250 μM). Automated column chromatography was performed with the indicated solvents on a Teledyne ISCO CombiflashRf+ with Luknova's SuperSep HP silica (spherical, 25 μm, 70 Å). Mass spectra were acquired at UC Irvine's Department of Chemistry Mass Spectrometry Facility on a Waters Micromass LCT Premier high resolution mass spectrometer with electrospray ionization (ESI). NMR spectra were taken at ambient temperature at UC Irvine's Department of Pharmaceutical Sciences NMR Facility on a Bruker AvanceNeo Ascend 400. ^{1}H-NMR data was obtained in the specified solvent at 400 MHz. ^{13}C-NMR data was obtained in the specified solvent at 101 MHz. Spectra were calibrated to the residual solvent peak. Chemical shifts are reported in ppm. Coupling constants (*J*) are reported in Hertz (Hz) and rounded to the nearest 0.1 Hz. Multiplicities are defined as: s = singlet, d = doublet, t = triplet, dd = doublet of doublets, ddd = doublet of doublet of doublets, dt = doublet of triplets, td = triplet of doublets, dtd = doublet of triplet of doublets, q = quartet, m = multiplet.

***N*-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (biotin-tetrazine)**



In a 2 mL plastic microcentrifuge tube, the (4-(1,2,4,5-Tetrazin-3-yl)phenyl)methanamine hydrochloride (85% purity, 0.0481 g, 0.215 mmol, 1.0 eqiv) was combined with biotin-NHS ester (0.0826 g, 0.242 mmol, 1.1 equiv). The K_2HPO_4 (0.1520 g, 0.873 mmol, 4.1 equiv) was added, followed by 4 steel beads (5 mm) and the EtOAc (0.054 mL, 0.2 * total mass). The tube was shaken for two rounds (90 s cycles, 10 cycles per round), then additional EtOAc (0.054 mL) was added. The tube was shaken for an additional two rounds. The contents of the tube were suspended in 20% MeOH/CH_2Cl_2 and the supernatant transferred to a round bottom flask. This was repeated with varying amounts of MeOH until the residual material in the tube was only faintly colored. The resulting pink solution was dry loaded on celite and purified by automated column chromatography with a 4 g column. The column was pre-equilibrated with 20% MeOH (containing 10% $NH4OH_{(aq)}$)/CH_2Cl_2 and the gradient used the same solvents. 5 CV 0.1%, 20 CV 0.1 → 5%, 20 CV 5%, 10 CV 20%. The product was isolated as a pink solid (0.0463 g, 61%).

Initial NMR data was taken in DMSO-d_6, however we noticed that the compound started to precipitate during the experiment. Therefore, we also took NMR data in a mixture of $CDCl_3$ and CD_3OD (~70/30), where the compound remained soluble throughout the course of the experiment. The CD_3OD residual was used for calibration in that case.

^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.57 (s, 1H), 8.48 (s, 1H), 8.45 (d, *J* = 7.8 Hz, 2H), 7.53 (d, *J* = 8.0 Hz, 2H), 6.44 (s, 1H), 6.37 (s, 1H), 4.40 (d, *J* = 5.8 Hz, 2H), 4.32-4.29 (m, 1H), 4.14-4.12 (m, 1H), 3.13-3.08 (m, 1H), 2.83 (dd, *J* = 12.4, 5.0 Hz, 1H), 2.59 (d, *J* = 12.4 Hz, 1H), 2.19 (t, *J* = 7.3 Hz, 2H), 1.68-1.54 (m, 3H), 1.48 (dd, *J* = 18.0, 10.1 Hz, 1H), 1.35 (dt, *J* = 13.5, 6.7 Hz, 2H).

^{13}C-NMR (101 MHz, DMSO-d_6): δ 172.31, 165.42, 162.74, 158.10, 145.10, 130.30, 128.06, 127.80, 61.08, 59.23, 55.47, 41.85, 39.89, 35.18, 28.28, 28.07, 25.31.

^{1}H-NMR (400 MHz, $CDCl_3$/CD_3OD): δ 10.21 (s, 1H), 8.52 (d, *J* = 8.1 Hz, 2H), 7.49 (d, *J* = 8.1 Hz, 2H), 4.46 (m, 3H), 4.25 (dd, *J* = 7.6, 4.7 Hz, 1H), 3.13 (m, 1H), 2.90-2.86 (m, 1H), 2.71-2.67 (m, 1H), 2.28-2.21 (m, 2H), 1.74-1.66 (m, 3H), 1.59 (m, 2H), 1.42 (dt, *J* = 15.2, 7.5 Hz, 3H).

^{13}C-NMR (101 MHz, $CDCl_3$/CD_3OD): δ 174.57, 166.56, 157.95, 144.56, 132.60, 130.71, 128.71, 128.65, 62.22, 60.44, 55.82, 43.16, 40.52, 35.86, 28.64, 28.32, 25.76.

ESI-HRMS calculated for: $C_{19}H_{23}N_7O_2SNa$ [$(M+Na)^+$]: 436.1532; observed 436.1546.

IV. References

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