Supplemental Information

The Secretion of miR-200s by a PKCζ/ADAR2 Signaling Axis Promotes Liver Metastasis in Colorectal Cancer

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Supplemental Information

Figure S1. miR-200s positively correlate with PKCζ in CRC, Related to Figure 1.
(A and B) GSEA plots of miR200b/c/429 signature enrichment using C3-MSigDB database in shNT vs. shPKCζ SW480 cells (A), and low vs. high PRKCZ expressing-tumours from TCGA-COAD dataset (B). (C) Scheme of miR-200 family. (D) qPCR of miRNA expression levels (left) and immunoblot for PKCζ (right) in RKO shNT and shPKCζ cells; n = 3 biological replicates. (E) qPCR of miRNAs (left) and immunoblot for PKCζ (right) in HCT116 shNT and shPKCζ cells; n = 3 biological replicates. (F) Kaplan-Meier recurrence free survival analysis of TCGA colon adenocarcinoma patients. Results are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure S2. PKCζ regulates the steady-state levels of the miR-200 family, Related to Figure 3.

(A) Scheme of the miR-200b/a/429 transcript and targets of qPCR primers amplifying the primary miRNA. (B) qPCR of the miR-200b/a/429 primary miRNA in SW480 shNT and shPKCζ cells; n = 3 biological replicates. (C) Expression of a luciferase-reporter construct under the control of the endogenous miR-200b/a/429 promoter in SW480 shNT and shPKCζ cells; n = 3 biological replicates. (D-F) qPCR of the miRNA biogenesis machinery in SW480 shNT and shPKCζ cells; n = 3 biological replicates. (G) Immunoblot of individual components of the miRNA biogenesis machinery in SW480 shNT and shPKCζ cells; n = 3 biological replicates. Results are presented as mean ± SD. *p < 0.05.
Figure S3. PKCζ regulates secretion of miR-200s, Related to Figure 4.

(A) Cell viability in SW480 shNT or shPKCζ, and HCT116 shNT or shPKCζ cells treated with the indicated concentrations of GW4869 for 72 hours. Viability was determined by WST-1 assay; n = 8 technical replicates. (B) Cell proliferation of SW480 shNT or shPKCζ, and HCT116 shNT or shPKCζ cells treated with DMSO or GW4869 (10µM). Number of cells were determined by trypan blue exclusion assay; n = 3 technical replicates. (C) NTA size and concentration measurements of EVs from HCT116 cells prepared by ExoQuick; n = 3 biological replicates. (D) Immunoblot of intracellular and EV markers in whole cell lysates (WCL) and EVs from SW480 shNT and shPKCζ#1 cells; n = 2 biological replicates. (E) qPCR of miRNAs in CD63+ EVs from HCT116 shNT, and shPKCζ cells; n = 3 biological replicates. (F) Immunoblot of CD63 after CD63 pull-down. Supernatant (Sup) is 10% of the volume on top of the beads after immunoprecipitation and before washing. IP-CD63 is 10% of the bead slurry after washing. Results are presented as mean ± SD. *p < 0.05.
Figure S4. PKCζ phosphorylates and regulates ADAR2 activity, Related to Figure 6.

(A) Scheme of the analogue sensitive (AS)-PKCζ screening assay. (B) Size and concentration measurements of CD63+ EVs and EVs prepared by ultracentrifugation (UC) or ExoQuick from SW480 shADAR2 cells; n = 3 biological replicates. shNT samples are the same as the ones in Figure 4F and 4I. (C) Immunofluorescence (left), and quantification of Zeb1 intensity (right) in SW480 shNT and shADAR2 cells; n = 3 biological experiments. Scale bars, 20 µm. (D) qPCR of epithelial and mesenchymal genes in SW480 shNT, shADAR2, and shADAR2 cells rescued with miR-200a/b; n = 3 biological replicates. (E) qPCR of ADAR2 mRNA in SW480 shNT and shPKCζ cells; n = 3 biological replicates. (F) Immunoblot of ADAR2 in SW480 and HCT116 shNT and shPKCζ cells; n = 3 biological replicates. (G) Immunofluorescence of ADAR2 levels in tumors from Lgr5;APCf/f and Lgr5;PKCζf/fAPCf/f mice; n = 3 mice per group. Scale bars, 50 µm. (H) LC-MS/MS analysis spectrum for ADAR2 phosphopeptide. (I) The Ser211 and Ser216 phosphorylation sites in ADAR2 are highly conserved among different species. (J) qPCR of miRNAs in SW480 cells stably-expressing ADAR2WT, ADAR2SS211/216AA, or ADAR2E396A after 10 µg/mL Actinomycin D treatment; n = 3 biological replicates. (K) COPA164V editing frequency in human tumor tissues and adjacent normal tissue from TCGA-COAD patients; n = 18 patients. Results are presented as mean ± SD. *p < 0.05; **p < 0.01, ***p < 0.001, ****p < 0.0001.
Table S2. Primer sets, Related to Experimental Procedures.

| Gene                  | Primer Sequence                      |
|-----------------------|--------------------------------------|
| miR-200a-3p           | TAACACTGTCTGGTAACGATGT               |
| miR-200c-3p           | TAATACTGCCGGTAATGATGGA               |
| miR-200b-3p           | TAATACTGCCGGTAATGATGGA               |
| miR-429               | TAATACTGCCGGTAATGATGGA               |
| miR-141-3p            | TAACACTGTCTGGTAACGATGT               |
| miR-16-5p             | TAGCAGCAGCTAAATTTGGCG                |
| miR-26a-5p            | TTCAAGTAAATCCAGGATAGGCT              |
| hsa-pri-miR-200b-Fwd  | TACTGAGCTTCCCAGCGAGT                 |
| hsa-pri-miR-200b-Rev  | CTGTGTGGGAGGGGAGTGT                  |
| hsa-pri-miR-200a-Fwd  | TCCCCTGGGCTTCACAG                    |
| hsa-pri-miR-200a-Rev  | GAGTAGAGCTCCGGATGTT                  |
| hsa-pri-miR-429-Fwd   | CAGACACCAGCCAGGAC                    |
| hsa-pri-miR-429-Rev   | GCAGGCAGCTCTCTCTCTTACA               |
| pri-miR-200 cluster tail Fwd | AGTGGGGCTCActCCTCCAC               |
| pri-miR-200 cluster tail Rev | AGGAGGAGGAGGAGGAGAAGA               |
| 18S                   | GTGGAGCGATTTGTCTGGTT                 |
| 18S                   | CGCTGACGAGTCATGTAG                   |
| RPL13                 | GTTCCGTGACACACCGAAGGT                |
| RPL13                 | TGGGGAAGGAGATGAGTTTG                 |
| 18s                   | CCATCCAATCGTGAATGAGCG                |
| 18s                   | GAAACCGGTGAACCCCATTT                 |
| PRKCZ-Fwd             | ATCTTCAATCCAGCGTGGA                  |
| PRKCZ-Rev             | AGCTCCTCGAATCTGACTGG                 |
| DROSHA-Fwd            | TAGCTGTGGGAAAGGACCAAG                |
| DROSHA-Rev            | GTCGATGAAACCGCTCAGTG                 |
| DICER1-Fwd            | CAATGCTAGCTGCTAGTCAGTC              |
| DICER1-Rev            | CAATCCACCAATCTCCTCAGATC             |
| DGCR8-Fwd             | CAAGCAGGACAGATCAGGACAGA             |
| DGCR8-Rev             | CACATGGACATCGAGCTCCTC               |
| TRBP-Fwd              | GCCAGCCACCGCAAAGAAT                  |
| TRBP-Rev              | TGCCACTCCCAATCTCAATG                 |
| AGO1-Fwd              | AAGTCCGCAAGGACGACGAAGAA             |
| AGO1-Rev              | GAAACTGCTACCTCGCATCA                 |
| AGO2-Fwd              | TCCATCGTGGTGAGAGAGAG                |
| AGO2-Rev              | ATGCCAGGTCGACTACAG                   |
| AGO3-Fwd              | AGCTCATTGAAATGGAATC                 |
| AGO3-Rev              | CAACCACCTCCTGCTCCTC                 |
| AGO4-Fwd              | TATATGGCCCGGCTGGTGAC                |
| AGO4-Rev              | ACATCGTGTCCTGCTGATCA                |
| ADARB1-Fwd            | CGTGTTAATCGTGCTGAGT                 |
| ADARB1-Rev            | GGGTGGCATATCTTGGAGCG                |
| CDH1-Fwd              | GCCTGGAGTTAATCCGGGAACA              |
| CDH1-Rev              | TTAGGGCTGTCGACTGCTG                 |
| CLDNI-Fwd             | CGTGTGGCATGAAAGTGATG                |
| CLDNI-Rev             | CCACTGCTGCAACTAAAAA                 |
| EPCAM-Fwd             | CTCCACGTGCTGGTGTCGTC                |
| EPCAM-Rev             | TGTGTTGATTCATGAGTGATCCAGTA          |
| TGFβ2-Fwd             | GTGCCGCTCAGCCTGTCT                  |
| Gene   | Primer Sequence 1 | Primer Sequence 2 |
|--------|------------------|------------------|
| TGFB2  | CCTGATCCTCTTGCGCAT |                  |
| ZEB1   | GCACCTGAAGAGACCAGAG |                  |
| ZEB1   | TGATCTGATTGGCTCTTTT |                  |
| FN1    | TCCACAAGCGTCATGAAGAG |                  |
| FN1    | CTCTGATCCTGGCATTTGT |                  |
| CDH11  | GGACTCTCAGGGACAACTCA |                  |
| CDH11  | GGACCTCAGGGACAACTCA |                  |
| ITGB3  | CCGTGACGAGATTGAGTCA |                  |
| ITGB3  | AGGATGGAACCCTATTTTCTAGAA |                  |
| MMP2   | TCTCTGACATGGACCTTGCC |                  |
| MMP2   | CAAGGTGCTGGCAGTAGATC |                  |
| MMP9   | TCTCTGACATGGACCTTGCC |                  |
| MMP9   | GACACCAAACTGATGCAGATG |                  |
| BMI1   | CATTCCTGCTGGGCATCGTAAG |                  |
| BMI1   | GAGACCCATTGCCGATTGCTCTTC |                  |
| CD133  | GAGACCCATTGCCGATTGCTCTTC |                  |
| CD133  | CAGGACACACGCAATGAGAATC |                  |
| CD44   | TGGCAACCGGTATTGCTGAG |                  |
| CD44   | GTAGCAGGAGATTGCTGTCG |                  |
| EPHB2  | AGTCCGGCAGAATTGCTCAAC |                  |
| EPHB2  | TCTCTGATCTGGCCTCATTC |                  |
| VIM    | CCCTCACCTTGAGACTGAGAT |                  |
| VIM    | TCCACGACGCTCTCTGTAGGT |                  |
| ALU    | CACCTGTAATCCAGCACTTTT |                  |
| ALU    | CACCTGTAATCCAGCACTTTT |                  |
| Gapdh  | GCACAGTCAAGGCCGAGAAT |                  |
| Gapdh  | GCCTTCTCCATGGTGGTAA |                  |
| ADAR2editing | CATAGAGATCTGGCAACGAG |                  |
| ADAR2editing | GTGTTCAGATGCGGGTTTT |                  |
| T7-pre-miR-200b | AATTAATAATACGACTCATTAGATGGATCTTACTGGCAGC |                  |
| T7-pre-miR-200b | GCTCCGCCGTCATCATTA |                  |
| ADAR2 S211A mutagenesis-Fwd | CTTGTCTGCTgCCCCGGTGCTGCC |                  |
| ADAR2 S211A mutagenesis-Rev | GGCACCGGGGCAGCAGACAAGCTGAGG |                  |
| ADAR2 SS211/216AA mutagenesis-Fwd | GGTGCCGCTGCCgCCTAGCCCAGGCCTCCTCCTCCTGCG |                  |
| ADAR2 SS211/216AA mutagenesis-Rev | GCTGGGCTAGGgcGGCAGGCACCGGGGcAGCAGACAAGC |                  |
| ADAR2 E396A mutagenesis-Fwd | CTGGCGTCTGCgGCTGAGGCGGGCGTGCCTGCC |                  |
| ADAR2 E396A mutagenesis-Rev | GAGATATTATTgCTCATGCGGACGTACTTACTATGCGAGAGTGGAGAGTGGAGAGTGAGGAG |                  |
| PKC-AS mutagenesis-Fwd | GGTTGTTTCTGCTGGCTGAGTACGATCGCAACGGCG |                  |
| PKC-AS mutagenesis-Rev | CGCCGGTACGTACTCACCAGCAGGAAACACCAC |                  |
| COPA I164V PCR – Fwd | AGCAGCCACTGTTCGCTCCTCTCT |                  |
| COPA I164V PCR-Rev | CTCCCATGCCTTATTGACCATTCCTCTCTCT |                  |
| COPA I164V sequencing-Fwd | AGACCATCCTCAGTGGGAAC |                  |
| COPA I164V sequencing-Rev | AAGGCAGCCCAGTTACTCC |                  |
| COPA EcoRI cloning   | ATTGAAGAATTCTTTAACCAAATTCGAGACCAAGAGCGCGCGGGTCAA |
|----------------------|--------------------------------------------------|
| COPA SalI cloning    | TATATTGTCGACATCATTAGCGAAACTGCAGAGGACTGATCCTTA   |
| COPA I164V mutagenesis-Fwd | CGTTTGGGATgTTTCTGGTCTGAGGAAAAAAAACC           |
| COPA I164V mutagenesis-Rev | AGACCAGAAAcATCCCCAACGCGCACAGTC             |

**Supplemental Experimental Procedures**

**Mice**
For tumor induction experiments, 8 weeks-old Lgr5-PKCζ^WT/WT/ APC^WT/Wt or Lgr5-PKCζ^WT/Wt/APC^WT/Wt mice were injected with 2 mg/day of tamoxifen for 6 consecutive days. Intestinal tumor samples for staining and organoids were collected 15 days after the first injection, and samples were subjected to histological analysis. NSG mice were purchased from the Sanford-Burnham-Prebys Animal Facility core. All mice were born and maintained under pathogen-free conditions. All genotyping was done by PCR. The WD (D12079B; OpenSource Diets) was available ad libitum. For all animal studies, the investigators were not blinded to the group allocation.

**Cell culture**
SW480, SW620, RKO, HCT116, HEK293T, and Phoenix-GP cells were purchased from ATCC and tested for mycoplasma. HCT116 cells were cultured in RPMI-1640. All other cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM). Media was supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin, in an atmosphere of 95% air and 5% CO2. Lentiviruses and retroviruses were prepared and used as previously described (Ma et al., 2013). For transient miRNA transfection experiments, 100 nM of the miRNA mimics for hsa-miR-200b-3p, hsa-miR-200a-3p, and the negative miRNA mimic transfection control with Dy547 were obtained from (Dharmacon) and were reverse transfected at the time of seeding using Lipofectamine RNAmax (Life Technologies). For transient siRab27a transfection experiments 50nM of pooled siRNA (Santa Cruz Biotech.) were reverse transfected at the time of seeding using Lipofectamine RNAmax (Life Technologies). Over-expression of ADAR2 constructs were performed by seeding SW480 cells into a 60mm dish, and transfecting with 5 μg of the respective plasmid. Cells were analyzed 72 h after transfection. For measuring the steady state levels of the miRNAs, 10 μg/ml of Actinomycin D (Sigma-Aldrich) was added 24 h after seeding.

**Crypt and intestinal epithelial cell isolation and intestinal organoid culture**
Crypt and IEC isolation were performed as described previously (Llado et al., 2015; Sato et al., 2009). For small intestine organoid culture, crypt number was counted after isolation and a total of 300 crypts were mixed with 50 μl of Matrigel and plated in 48-well plates. After polymerization of Matrigel, 300 μl of crypt culture medium (Advanced DMEM/F12 containing 10 mM HEPES, 1X Glutamax, 1X N2 supplement, 1X B27 supplement, 50 ng/ml EGF, 1000 ng/ml R-spondin1, 100 ng/ml Noggin, and 10 μM Y-27632) was added. Tumor organoids were maintained in similar conditions, but without EGF, R-spondin1, Noggin, or Y-27632.

**Cell lysis, immunoprecipitation and immunoblotting**
Cells for protein analysis were lysed in RIPA buffer (20 mM Tris-HCl, 37 mM NaCl, 2 mM EDTA, 1% Triton-X, 10% glycerol, 0.1% SDS, and 0.5% sodium deoxycholate, with phosphatase and protease inhibitors). For immunoprecipitations, cells were lysed in σ3 buffer (100 mM NaCl, 25 mM Tris, 1% Triton X, 10% glycerol, with phosphatase and protease inhibitors) or nuclear extracts were isolated as described previously (Duran et al., 2016). Cell lysates were pre-cleared with 30 μl of 50% slurry of protein A sepharose (GE healthcare) for 30 min. Then 25 μl of Glutathione-Sepharose 4B beads (BioWorld), 25 μl of FLAG-M2 affinity gel (Sigma), 1 μg of primary antibody, or control immunoglobulin (Santa Cruz Biotech) was added to the lysates and incubated with rotation overnight at 4°C. The next day, 30 μl of 50% slurry of protein A sepharose was added to non-bead samples, and the incubation was continued for 1 h. After a 15 min centrifugation at 2,500 rpm at 4°C, the immunoprecipitates were washed several times with lysis buffer. Cell extracts and immunoprecipitated proteins were denatured, subjected to SDS-PAGE, transferred to PVDF membranes (GE Healthcare), and immunoblotted with the specific antibodies. Band intensities were quantified using ImageJ (NIH). The following antibodies were used: PKCζ (Cell Signaling, #9368, Santa Cruz, #sc-216), Drosha (Abcam, #ab12286), Dicer (Santa Cruz, #sc-30226), TRBP (Santa Cruz, #sc-292550), β-actin (Sigma, #A1978), HA-probe (Santa Cruz, #sc-805), GST (Santa Cruz, #sc-138), Thiophosphate ester (Abcam, #ab92570), FLAG (Sigma, #F3165), ADAR2 (Santa Cruz, #sc-393068, #sc-393069), and RAB27A (Cell Signaling, #2107).
Cohesion Biosciences, #CPA1017), CD63 (Santa Cruz, #sc-5275), TSG101 (Abcam, #Ab30871), Rab27a (Proteintech, #17817-1-AP), Flotillin (BD Bioscience, #610820), Alix (Abcam, #Ab117600), Peroxiredoxin 3 (Abcam, #Ab16751), Histone 3 (Abcam, #Ab1791), AGO2 (Cell Signaling, # 2897), E-cadherin (BD Bioscience, #610181), ZEB1 (Bethyl, #A301-992A-T), TGFβ (Cell Signaling, #3711), and CD44 (Abcam, #Ab24504).

**Gene-expression analyses**

RNA, including miRNA, from cells was isolated using the mirVana miRNA Isolation Kit (Ambion) and treated with TURBO DNase (Ambion). Nuclear fractionation for RNA analysis was performed as previously described (Wang et al., 2006) and RNA was extracted using TRIZOL (Invitrogen). 1 µg of RNA was reverse-transcribed using the Mir-X miRNA First-Strand Synthesis kit (Clontech). Quantitative real-time PCR was performed using iTaq Universal SYBR green supermix (BioRad) on a BioRad CFX96 detection system. qPCR primers used in the study are listed in Table S2. U6 was used as a housekeeping gene for normalization of the miRNA reactions, and RPL13 and 18S were used as the housekeeping gene for normalization.

**Invasion and migration assays**

Cells (2.5 x 10^4) were seeded without FBS onto 8 µm-pore Matrigel Invasion chambers and control inserts (Corning) in 24-well plates. Media containing FBS was added to the lower chamber as a chemoattractant. Cells were allowed to migrate/invade for 48 h at 37°C. Non-invading cells in the upper surface were removed and those on the lower surface were fixed in methanol and stained with crystal violet. The entire field of the stained cells was quantified using ImagePro Plus Version 6.0.

**Immunofluorescence assays**

SW480 shNT, shPKCζ, and shADAR2 cells were plated on coverslips, fixed in 4% paraformaldehyde, blocked and permeabilized (0.3% BSA and 0.1% Triton X-100). Fixed cells on cover slips, or deparaffinized sections from mouse tumor samples, were blocked for one hour in blocking buffer (0.3% BSA in PBS), and then incubated with primary antibody and corresponding Alexa-conjugated secondary antibodies (Life Technologies), as described previously (Llado et al., 2015). Slides were mounted on Mowiol or VectaShield and examined with a Zeiss LSM 710 NLO Confocal Microscope. For intensity quantification, corrected total cell fluorescence was measured as integrated density minus the background mean using ImageJ. The following antibodies were used for immunofluorescence: ZEB1 (Santa Cruz, # sc-25388), and E-cadherin (BD Bioscience, #610181).

**Sanger-sequencing**

Sanger sequencing of mutagenesis reactions and COPA editing were performed by Eton Bioscience Inc. Sequencing of COPA editing levels was performed by direct sequencing method to estimate the editing frequency and using a calibration curve as described previously (Kawahara, 2012). Briefly, cDNA from three independent biological replicates was PCR amplified using Phusion High-Fidelity DNA Polymerase (New England BioLabs Inc.) and the primers listed in Table S2. The PCR product was purified using the Qiagen MinElute PCR Purification Kit (Qiagen) and sequenced using a different set of primers listed in Table S2. In parallel, the PCR product was subcloned into a pGEM-T Easy Vector (Promega) to generate plasmids with a single COPA transcript that contains the A (unedited) or G (edited) site at COPA164. Mixtures (10:0, 8:2, 5:5, 2:8, 0:10) of the edited and unedited versions of the plasmid DNA were submitted for sequencing in both directions and a calibration curve from the combined ratio was generated. A linear regression model was used to calculate the known ratio and the observed ratio in order to determine the editing frequency. Sequencing images were made using 4Peaksv1.8. A-to-I editing was measured by calculating the area under the curve (AUC) levels for guanine relative to adenine using ImageJ software.

**EV isolation and characterization**

Conditioned cell-culture medium (CCM) was collected after 48 h from cells cultured in DMEM + 10% exosome-depleted FBS (Systems Bioscience, SBI), and differential centrifugation was used to discard dead cells and debris. Briefly, for all extracellular analysis the CCM was clarified by centrifuging 300g for 10 min to remove live cells, and again by centrifuging 2,000g for 10 min. The clarified CCM was then either subjected to 50-kD molecular weight cut off (MWCO) ultrafiltration (UF; Amicon Ultra-15) to analyze the total conditioned medium or subjected to ExoQuick (EQ; SBI) to analyze EVs. Alternatively, exosomes were enriched by performing an additional centrifugation at 10,000g for 30 min to remove microvesicles before the clarified media was subjected to ExoCap CD63 magnetic beads (JSR Life Sciences), or ultracentrifugation (UC) at 100,000g for 1 h. For UC, EVs were washed with PBS and ultracentrifuged again. For CD63 pull-down, 1 ml of clarified sample was diluted with 1 ml of dilution buffer and 200 µl of beads were incubated with rotation at 4°C for 20 hours. The supernatant was recovered and analyzed for pull-down efficiency before the beads were washed three times, and EVs were eluted or RNA extraction was performed directly on the beads. Purified EVs were then
characterized by electron microscopy, immunoblot, and Nanoparticle Tracking Analysis (NTA; Malvern). For RNA analysis, 20 pm of 5′phos-cel-miR-39-3p was spiked into the sample at the time of RNA extraction with the Total Exosome RNA and Protein Isolation Kit, according to the manufacturer’s instructions (Life Technologies). miRNA was then amplified using the TaqMan Advanced miRNA assay (Applied Biosystems). TaqMan Advanced Master Mix and TaqMan Advanced miRNA probes for hsa-miR-200b-3p, hsa-miR-200c-3p, and hsa-miR-26a-5p were used for qPCR and normalized to cel-miR-39-3p. For the in vitro experiments, GW4869 (Santa Cruz Biotechnology Inc.) was dissolved in DMSO as a 1.5mM stock suspension. Immediately prior to treatment, 5% methane sulfonic acid (MSA) was added to the GW4869 stock suspension, and cells were treated with 10 µM GW4869 or DMSO/MSA as a vehicle control.

Transition Electron Microscopy (TEM)  
For the transmission electron microscopy, samples were fixed and mounted on copper slot grids coated with parlodion and stained with uranyl acetate and lead citrate for examination on a Philips CM100 electron microscope (FEI) at 80 kV. The images are taken using Radius 1.3 software with a Megaview G2 CCD camera manufactured by EMSIS GmbH, Germany.

Exosomes size distribution measurement  
Exosomes size and particles density were measured by NTA (NanoSight NS300, software version 3.1). Exosomes prepared by UC, EQ, or eluted exosomes from ExoCap CD63 pull-down were resuspended in 0.2 µm filtered PBS and added to the measurement system. Tracking was performed by taking at least three 40 sec videos of each sample with a camera level of 15, and a syringe pump speed of 50.

Northern blotting  
A non-radioactive method for northern was employed, as previously described (Kim et al., 2010). Briefly, 10 µg of RNA from SW480 shNT and shPKCζ cells were run on a 15% denaturing gel and transferred onto a positively charged nylon membrane (Roche). RNA-membrane cross-linking was performed at 60°C for 2 h using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma). Membranes were hybridized using ULTRAhyb hybridization buffer (Ambion) and a 5′-DIG and 3′-DIG labeled miRCURY LNA detection probe (Exiqon) for hsa-miR-200b-3p at 37°C overnight. The membrane was washed and blocked using the DIG Wash and Block Buffer Set (Roche), and DIG was detected by chemiluminescence using the DIG Luminescent Detection Kit (Roche). Afterwards, the membrane was stripped using northern stripping buffer (1% SDS, 0.1X SSC) and hybridized, as described above, with a 5′-DIG labeled miRCURY LNA detection probe (Exiqon) for U6 as a loading control. Band intensities were quantified using ImageJ (NIH).

In vitro precursor miRNA processing assay  
The processing of pre-miRNA-200b was performed using SW480 shNT and shPKCζ cellular extracts. Briefly, T7-pre-miR-200b was PCR amplified from SW480 genomic DNA and purified using Qiagen Minielute PCR Cleanup kit. DNA was biotin labeled by in vitro transcription using T7 RNA polymerase (Roche) and biotin RNA labeling mix (Roche). RNA was treated with TURBO-DNase to remove template DNA and precipitated with ethanol. The pre-miRNA was refolded using RNAi buffer [30 mM Hepes (pH 7.4), 100 mM KCl, 5 mM MgCl2, 10% glycerol] and heated for 10 min at 60°C before slowly cooling to room temperature. SW480 shNT and shPKCζ cells were lysed in 500 µl of Buffer D [20 mM Tris-HCl (pH 8.0), 100 mM KCl, 1.5mM MgCl2, 0.2 mM EDTA, 5% glycerol, 0.5 mM DTT and protease inhibitors], briefly sonicated, and clarified by centrifugation. The folded pre-miRNA was added to 700 µg of protein lysate, in a reaction containing a final concentration of 50% (v/v) total extract, 20 mM creatine phosphate, 0.5 mM ATP, 3.2 mM MgCl2, and RNase inhibitor (Roche). The reaction was incubated for 30 min at 37°C. RNA was extracted by phenol chloroform and run on a 15% denaturing gel. Cross-linking, and blocking were performed as described earlier. Finally, biotin was detected by chemiluminescence using streptavidin-HRP (Roche).

Metastasis studies  
Experimental liver metastases were performed by intrasplenic injection of 2 x 10^6 SW480 cells expressing shNT or shPKCζ, and pLenti-PGK-Blast V5-LUC (Addgene #19166) in 6 weeks old NSG mice. For experiments with the exosome inhibitor, 1.25 mg/kg/day of GW4869, or DMSO as a vehicle control, was injected intraperitoneally (IP) starting one day before splenic injection, and for five days thereafter. After 28 days, luciferase imaging was performed using a Xenogen IVIS 200 imager. Animals were euthanized, and livers were excised, and portions were embedded in paraffin for hematoxylin and eosin (H&E) staining. The H&E stainings were used to measure the metastatic area in five random fields using ImagePro Plus Version 6.0 and divided by the total area of their respective field. The average measurement was calculated for each mouse and presented as a biological replicate of metastases area/liver area. The metastases were also quantified using the detection of human ALU sequences present in mouse liver genomic DNA preparations.
Dual-Luciferase reporter assay
SW480 shNT and shPKCΣ cells were transfected with a pGL3-miR-200b/a/429 promoter plasmid provided as a gift from Dr. Jin-Tang Dong (Zhang et al., 2013). Luciferase activity was measured by using the Promega Dual-Luciferase reporter assay system.

Cell proliferation and viability assays
Cell proliferation was determined by trypan blue exclusion assay at the indicated time after treatment with 10 μM GW4869 or equal volume of DMSO. Cell viability was measured using WST-1 (Roche) according to the manufactures instructions after cells were treated with the indicated concentrations of GW4869 for 72 hours.

Cloning and Plasmids
The following shRNA clones were used in this study: pLKO.1-shPKCΣ#1 (TRCN0000001219), pLKO.1-shPKCΣ#2 (TRCN000010121), pLKO.1-shADAR2#1 (TRCN000050939), and pLKO.1-shADAR2#2 (TRCN0000050942). pWZL-HA-PKCΣWT, and pWZL-HA-PKCΣKD clones were previously described (Galvez et al., 2009). GST-ADAR2 plasmids were generated by cloning the pENTR223-ADARB1 (DNASU, HsCD00516207) into the pDEST-27 vectors using the Gateway LR Clonase II Enzyme mix (Invitrogen). Alternatively, the ADARB1 was subcloned into the pWZL-FLAG. The ADAR2ΣS211/216AA, and ADAR2ΣE396A mutants were generated by site directed mutagenesis reactions using the primers listed in Table S2. The analog-sensitive PKCΣ (AS-PKCΣ) construct was generated by site directed mutagenesis of isoleucine 330 to glycine (I330G) from human WT-PKCΣ using the primers listed in Table S2.

Kinase-assay and MS/MS phosphopeptide identification
For in vitro phosphorylation assays, SW480 cells were transfected with GST-empty vector, GST-ADAR2, or GST-ADAR2ΣS211/216AA and lysed with RIPA buffer. GST-ADAR2 was pulled down from 1 mg of lysate using Glutathione-Sepharose 4B beads (Bioworld). The beads were washed five times with RIPA and incubated at 30°C for 60 min in kinase assay buffer [25 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 0.5 mM EGTA,1 mM DTT and 400 μM ATP] in the presence of recombinant PKCΣ. For in vitro ATP analog-based phosphorylation detection, the protocol described previously (Allen et al., 2007) was followed with minor modifications. Briefly, after the phosphorylation reaction, p-nitrobenzyl mesylate (PNBM; Abcam) and EDTA were added to a final concentration of 2.5 mM and 20 mM, respectively, and incubated for 1 h at room temperature. Immunoblotting detection was performed with α-thiophosphate ester antibody (Abcam, # ab92570). For in vivo phosphorylation assays, the retroviral construct for analog-sensitive PKCΣ (AS-PKCΣ) was generated by site directed mutagenesis of isoleucine 330 to glycine (I330G) from human WT-PKCΣ using the primers listed in Table S2. These constructs were used to generate SW480 cell lines stably expressing WT-PKCΣ or AS-PKCΣ SW480 cells were subjected to in-vivo phosphorylation assay in the presence of bulky ATP analog (benzyl-ATP, Biolsg). Briefly, cells were incubated 20 min at room temperature in the presence of 25 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 0.5 mM EGTA, 1 mM DTT, 400 μM Bn-ATP, 30 µg/ml digitonin, 5 mM GTP and 1X protease and phosphatase inhibitor cocktail. After adding 20 mM EDTA, cells were sonicated and clarified by centrifugation. PNBM was added to a final concentration of 2.5 mM, and the samples were then incubated for 1 h at room temperature. Specific thiophosphorylation sites were detected by LC-MS/MS.

Array and Gene Set Enrichment Analysis
Microarray studies were performed in the Genomics Core at SBP Medical Discovery Institute. The accession number for this microarray is GEO: GSE78760. Briefly, total miRNA was extracted from SW480 shNT and shPKCΣ cells and quantified using the TaqMan Human MicroRNA Array v3.0 (Applied Biosystems). Similarly, mRNA was extracted and quantified using Affymetrix Human Gene 1.0 ST GeneChip Arrays as per the manufacturer's instructions. The data were normalized to endogenous controls, and the ΔΔCt was calculated using Integomics StatMiner software. False discovery rate (FDR) was measured using the Benjamini and Hochberg approach. The Cancer Genome Atlas (TCGA) Colon Adenocarcinoma (COAD) dataset was obtained from The UCSC Cancer Genomics Browser. Gene set enrichment analysis was performed using GSEA v5.1 software (http://www.broadinstitute.org/gsea/index.jsp) with 5000 gene-set permutations using the Pearson metric for ranking genes and the geneset compilations C2.all.v5.1.symbols, C3.mir.v5.1.symbols, and C5.bp.v5.1.symbols(Subramanian et al., 2005). We acknowledge our use of the gene set enrichment analysis, GSEA software, and Molecular Signature Database (MSigDB) (Subramanian et al., 2005), http://www.broad.mit.edu/gsea.

Bioinformatic Analysis of Clinical Data
Data for TCGA-COAD was accessed through the UCSC Cancer Genomics Browser and TCGA Data Portal. “Low” and “High” groups for TCGA-COAD were stratified based on the bottom and top quartile, respectively. Correlation analyses of
PKCζ gene neighbors and the colorectal molecular subtypes were generated by first generating a ranked ordered gene list of PKCζ gene neighbors using GSEA for the TCGA-COAD dataset. The values for the specific genes in each of the molecular subtypes were then analyzed for Pearson’s correlation against the ranked order gene list. To analyze the PKCζ levels in the CCMS molecular subtypes (Marisa et al., 2013) we used data from Isella et al. (Isella et al., 2015) to access the specific patients from TCGA-COAD in each of the subtypes, and normalized and plotted PKCζ mRNA expression levels for each of the groups. RNA editing data for TCGA-COAD patients was accessed directly from Paz-Yaacov et al. (Han et al., 2015; Paz-Yaacov et al., 2015).

Statistical Analysis
Statistical analyses for figures were performed using GraphPad Prism software (San Diego, CA). Data are presented as independent biological replicates, with the mean. For qPCR experiments, Gaussian distribution was assumed and a Student’s t-test (two-tailed unpaired) was used to determine statistical significance. For human and mouse studies, significant differences between groups were determined using a Student’s t-test (two-tailed unpaired) when the data met the normal distribution tested by D’Agostino test. If the data did not meet this test, a Mann-Whitney test was used. Outlier analysis was performed using the ROUT method in GraphPad Prism. Gene expression correlation analyses were performed using Pearson’s correlation coefficients, with a two-tailed test. A log-rank (Mantel-Cox) test was used to evaluate statistical significance for the Kaplan-Meier survival plots. Immunofluorescence colocalization analyses were performed using Pearson’s correlation coefficients in ImageJ software. Data display normal variance.

Supplemental References
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