

BCDIN3D RNA methyltransferase stimulates Aldolase C expression and glycolysis through let-7 microRNA in breast cancer cells

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Supplemental Material

The Supplemental Material contains:

- Author Contributions
- Detailed Materials and Methods
- Supplemental Tables S1-S12
- Supplemental Figures S1-S10
- Supplemental References

Author Contributions

CWR and BX performed the mouse xenograft assays with help from CVDB; HI collected the samples for the metabolomic, RNA-Seq, small RNA-Seq and proteomic analyses, and prepared the small RNA-Seq and ribosome profiling libraries; MM performed the mass spectrometry analysis; NSA and BX analyzed proteomic, metabolomic, RNA-Seq, small RNA-Seq, ribosome profiling and public data; PJW performed luciferase assays, CWR, HI, EBG, and BX performed all other experiments, BX wrote the paper, with contributions from other authors.

Detailed Materials and Methods

1) Cell Lines: MDA-MB-231 shBCDIN3D cells were generated by transfection of MDA-MB-231 cells with TR317908C/TI368844 plasmid from Origene, while the matched MDA-MB-231 shNC cells were generated by transfection with pRS-Scrambled TR30012. These cell lines were previously validated [1]. The GFP and ALDOC_{DDK-Myc} expressing MDA-MB-231 cell lines were generated by transfection of eGFP-C1 and pCMV6-Entry-ALDOC_{DDK-Myc} (Origene, #RC200333) plasmids into the cells and selection with G418 at 1.5 mg/mL. MDA-MB-231-luc-D3H2LN generated by Jenkins *et al.* [2] were purchased from Caliper LifeSciences and the corresponding shNC and shBCDIN3D cell lines were generated as above. These cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (PSQ), as well as 1 µg/mL puromycin for shRNA plasmid selection. A Triple-Negative Breast Cancer Panel (ATCC(r) TCP-1003(tm)) containing 17 different cell lines was obtained from ATCC. The growth medium for each cell line is indicated in Table S12. siRNA and miRNA mimic reverse transfections were performed with Lipofectamine RNAiMax reagent.

2) Xenograft assays: Mice homozygous for Foxn1^{nu} (JAX stock #002019, 5-6 week old, female, weighing 18-20g) were purchased from Jackson laboratories, and maintained according to the University of Texas at Austin Institutional Animal Care and Use Committee guidelines (approved protocol #AUP-2017-00279). Cages of animals (4 animals per cage) were randomly separated in two groups for injection with MDA-MB-231-D3H2NL shNC or shBCDIN3D cells. For orthotopic injection, 5×10^5 of MDA-MB-231-luc-D3H2NLshNC or shBCDIN3D cells in 100 µL of PBS were injected into the #4 mammary fat pad. Mice were palpated three times weekly, monitored for signs of distress, and terminated 5 weeks post-injection. Tumor size was expressed

as tumor volume (mm³) and calculated by the formula: volume = (smaller dimension² × larger dimension)/2. Statistical significance of the resected tumor volumes between the shNC and shBCDIN3D conditions was evaluated using an unpaired, two-tailed t-test with Prism 8. For tail vein injections, 5 × 10⁵ of MDA-MB-231-luc-D3H2LNshNC or shBCDIN3D cells in 100 μL of PBS were injected. Mice were monitored in the same way. For imaging, mice were first sedated with 4% isoflurane, then injected with luciferin (150 mg/kg) intraperitoneally and imaged within 5 min of injection using IVIS.

Power calculations indicated that a minimum sample size of 10 mice in each group was required to achieve statistically significant data (>80% power, p<0.05), assuming a 50% difference in tumor growth or metastasis. We increased the group number to 12 to account for unforeseen issues during injection of cells.

3) RNA/Protein extraction: Total RNA and protein extraction was performed with the Norgen RNA/Protein purification kit from Norgen (#23000) or with the Norgen RNA & Protein purification Plus kit (#48200).

4) RNA-Seq: 1.5 μg of total RNA (RIN~10) was used per RNA-Seq library (TruSeq stranded Total RNA). 8 barcoded libraries were sequenced in one lane with a HiSeq2000 instrument to obtain paired-end, 75 bp reads. FastQC was used to assess adapter contamination and duplication rates. rRNA and tRNA sequences were filtered out, and the remaining sequences were aligned to hg38 using Tophat2/Bowtie2. Alignments marked secondary by Tophat2 or with a mapping quality below 5 were removed. Read counts per gene were calculated using HTSeq with the Gencode v21 comprehensive genome annotation. Differentially expressed genes were identified using edgeR with default parameters except for two modifications: first, a gene was required to have an expression value of at least 1 count per million reads in at least one sample to be tested and second,

a differentially expressed gene was required to have both an absolute fold change of 1.5 or greater and a statistically significant FDR adjusted P-value < 0.05. All final results were exported to Excel and all downstream plotting and analysis was performed with custom scripts in R (available on demand) with the ggplot2 graphics package, or Ingenuity Pathway Analysis as indicated.

5) Western blots: For the anti-BCDIN3D, anti-ALDOC, anti-SRSF3, and anti- β -Tubulin western blots, proteins were separated in a 12% SDS-PAGE gel and transferred onto a 0.45 μ M PVDF membrane in 1X Towbin Buffer with 15 % Methanol for 90 min at 400 mA. The membranes were blocked for 30 min at room temperature in TBS-TM (Tris-buffered saline, 0.1% Tween 20, 5% Nonfat Dry Milk from Cell Signaling #9999) and incubated overnight at 4°C with TBS-TM buffer containing the indicated antibodies. The membranes were washed 3 times for 10 minutes with TBS-T, incubated 1 h with TBS-T containing the appropriate secondary antibodies, washed and revealed with ECL (Amersham). The ECL signal was detected with the Syngene 05-GBOX-CHEMI-XR5 or by exposure to film. For the anti-mTOR-P/anti- α -Tubulin western blots, proteins were separated in a 8% SDS-PAGE gel and transferred onto a Immobilon®-FL PVDF membrane (LI-COR, PN 926-31099) in 1X Towbin Buffer with 20 % Methanol and 0.02% SDS for 90 min at 400 mA. The membrane was blocked for 30 min at room temperature in Intercept® Blocking Buffer (TBS) and incubated overnight at 4°C with Intercept® Blocking Buffer (TBS) buffer containing the mTOR-P antibody. The membrane was washed 3 times for 10 minutes with TBS-T, incubated 1 h with TBS-T containing 1/10,000 of the secondary IRDye® 680RD Goat anti-Rabbit IgG (H + L) antibody (LI-COR, PN 925-68071), washed again and scanned with an Odyssey Imaging System. The membrane was then incubated overnight at 4°C with Intercept® Blocking Buffer (TBS) buffer containing the α -Tubulin antibody. The membrane was washed 3 times for 10 minutes with TBS-T, incubated 1 h with TBS-T containing 1/10,000 of the secondary IRDye®

800RD Goat anti-Mouse IgG (H + L) antibody (LI-COR, PN 925-32210), washed again and scanned with an Odyssey Imaging System. Western blot quantification was performed with ImageJ. The list of antibodies is in Table S10.

6) Polysome fractionation: Cells from one 254x254 mm square plate were lysed as in steps 1 to 6 in the procedure described in the Nature Protocol article from Ingolia *et al.* [3] using the flash freezing option without cycloheximide or harringtonine pre-treatment. The supernatant from the centrifugation in step 6 was layered on top of a linear 7%-50% (w/v) sucrose gradient containing cycloheximide (200 µg/mL). The tubes were centrifuged in a Beckman SW41TI rotor at 36,000 rpm for 2h30 min at 4°C. Polysome profiles were monitored by absorbance at 254 nm and gradient fractions were collected on an ISCO density gradient fractionator.

7) Metabolic labeling: 30 min prior to harvesting the cells, the regular DMEM+10%FBS+PSQ+puromycin medium was substituted for a DMEM with no glutamine, no methionine, no cystine+10%FBS+PSQ+puromycin and supplemented with Methionine, L-[35S] (NET155V250UC). Protein and RNA were extracted with the Norgen RNA & Protein purification Plus kit (#48200). 5 µg of proteins were loaded on a NuPAGE 4-12% Bis-Tris gel (Novex) and run for 3h at 60V. The gel was stained with Coomassie, treated for 30 min with autoradiography enhancer Fluoro-Hance (RPI), dried at 80°C for 2 h, and exposed on an Amersham hyperfilmTMMP (GE-Healthcare) at -80°C.

8) Ribosome profiling

- **Library construction:** Cells from one 100 mm diameter plate were used to perform ribosome profiling as in Ingolia *et al.* [3] using the flash freezing option without cycloheximide or harringtonine pre-treatment.

- **Bioinformatic Analysis:** For all samples, adapters were removed using the fastx_trimmer tool from the Fastx Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html) following the procedure from Ingolia *et al.* [3]. Next, reads with remaining sequence greater than 16 bp were aligned against the hg38 build of the human reference genome using Tophat v2.0.8 with the “--b2-sensitive” set of options and the Genode v21 set of transcriptome annotations. Next, all BAM files were filtered to remove i) non-unique or ii) non-perfect alignments, again following the Ingolia *et al.* [3] procedure. Resulting files were used to compute and plot read densities relative to the start codon in order to determine the offset between the reads and the ribosome P-site and perform all down-stream analyses. Start codon analysis shown on Fig. 2g and Fig. S3d was performed using custom scripts. Codon usage analysis shown on Fig. S3e was performed using the standard workflow from the RiboProfiling R package [4]. Specifically, for each gene, the coding sequence with the highest read count was selected, and then read start position was used to assign each read to a specific codon. The resulting counts were log₂ transformed and plotted using custom R scripts. Visualization to verify expected read distributions across selected genes was performed using the genomecov utility in the Bedtools software suite to generate bedgraph intermediate files, which were converted to bigwig files using the UCSC bedGraphToBigWig tool.

9) Metabolomic Analysis: MDA-MB-231 cells grown on 10 cm diameter dishes were trypsinized, and washed 3 times with 1 mL of ice cold 1X PBS. Pellets of 4×10^6 cells were frozen at -80°C, and sent for analysis to the West Metabolomic Core of UC Davis, where an untargeted profiling of primary metabolism was performed.

10) Proteomic Analysis. Pellets of 4×10^6 cells were resuspended in 50 μ L of PBS. 50 μ L Trifluoroethanol (TFE) was added and the samples were vortexed thoroughly. 11 μ L of 100 mM DTT was added (10 mM DTT final concentration) and the samples were incubated for 45 minutes

at 55°C, followed by 5 min at room temperature. 6.46 µL of 1 M Iodoacetamide (IA) (55 mM IA final concentration) was added and the samples were incubated for 30 minutes at room temperature in the dark. 53.6 µL of 100 mM DTT (55 mM DTT final concentration) was added to quench IA. 798.94 µL of Digestion Buffer (50 mM Tris-HCl pH 8.0, 2 mM CaCl₂) was added to dilute TFE concentration to 5%, and Trypsin digestion was performed by adding 20 µL of 100 µg/mL Trypsin and incubating for 5 hours at 37°C. The digestion was stopped by addition of 10 µL of Formic Acid (1% (vol./vol.) Formic Acid final concentration). After reducing the volume to ~ 250 µL with a SpeedVac, the sample was loaded onto an Amicon Ultra 10kD spin-cap that was pre-washed twice with 500 µL of Buffer C (95% Water, 5% Acetonitrile, 0.1% Formic Acid). The column was centrifuged at 14,000 × g for 10 minutes. 250 µL of Buffer C was added to the Amicon Ultra 10kD spin-cap and centrifuged again for 10 min. The resulting 500 µL filtrate was collected, and the resulting peptides were cleaned with a C18 tip. Liquid chromatography was performed with an EASY-nLC™ 1000 Integrated Ultra High Pressure Nano-HPLC System and MS/MS with a Q-EXACTIVE System equipped with a Nanospray Flex Ion Source. The data was analyzed with MaxQuant using a label-free quantification method [5].

The LC-MS/MS raw files can be downloaded on the Xhemalce lab server:

<https://web.corral.tacc.utexas.edu/xhemalce/MS-1.raw>

<https://web.corral.tacc.utexas.edu/xhemalce/MS-2.raw>

<https://web.corral.tacc.utexas.edu/xhemalce/MS-3.raw>

<https://web.corral.tacc.utexas.edu/xhemalce/MS-4.raw>

11) Immunofluorescence: Immunofluorescence was performed as previously described [6].

12) Immunohistochemistry: 35 days after the orthotopic injections, the tumors were carefully excised, measured with a Caliper, cut in 2 halves, one of which was fixed for 24 h in 5% formalin,

before being stored in 70% ethanol. Then the fixed tumor samples were imbedded in paraffin. For IHC staining, tissue sections were deparaffinized and hydrated in xylene followed by graded alcohols (100%, 95% ETOH) to water. The endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 10 minutes. After washing, the antigen was retrieved with 10 mM Citrate Buffer pH 6.0 in a microwave for 10 minutes under standardized conditions, cooled down for 20 minutes, then washed with water. Non-specific antibody binding was blocked by incubating slides with Biocare Blocking Reagent # BS966M for 10 minutes. The slides were drained and incubated with primary BCDIN3D rabbit polyclonal antibody (Sigma #HPA039911) at a 1:50 dilution, or Aldolase C rabbit polyclonal antibody (abcam #87122) at a 1:250 dilution overnight at 4°C. After two buffer washes of five minutes, the slides were incubated with Envision plus labeled polymer, anti-rabbit-HRP (Dako) for 30 minutes at room temperature. After two buffer washes of five minutes, the slides were incubated with Dako DAB monitoring staining development. The slides were then washed, counterstained, dehydrated, cleared and covered with coverslip for viewing. The slides were scanned at 40x with an Aperio Scanscope.

13) RNA Analysis: 500 ng of total RNA was reverse transcribed into cDNA with either the Superscript®III First-Strand Synthesis System for RT-PCR kit (Invitrogen) using both oligo(dT)₂₀ and random hexamers, or with the qScript cDNA SuperMix kit (Quantabio). Subsequently, 1/20th of each reaction was used for real-time PCR with gene specific primers on a StepOne Plus system. The list of oligonucleotides is in Table S11. The northern blots were performed as previously described (Xhemalce and Kouzarides, 2010). Northern blot quantification was performed on ImageJ 1.51s. A rectangle was drawn around the area including all bands of interest, and the signal was measured with the “Analyze → Plot Profile” function.

14) Seahorse Analysis: Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Real Time ATP rate kit in the Seahorse XFp Flux Analyzer (Seahorse Bioscience–Agilent Technologies, Billerica, MA). MDA-MB-231 cells at a density of 2×10^5 cells/mL were seeded in 80 μ L DMEM+10%FBS+PSQ on XFp 8-well micro-plates and incubated for 24h at 37°C, 5% CO₂. Cells were washed two times with XF medium pH 7.4 freshly supplemented with 10 mM of XF Glucose and 2 mM of XF Glutamine. Cells were then incubated in that same media for 1h at 37°C in a CO₂-free incubator. After calibration the device first measures a baseline then levels of OCR and ECAR after sequential addition of Oligomycin (final concentration of 1.5 μ M), or Antimycin A/Rotenone (final concentration of 0.5 μ M). After each assay each well was dried by removing the remaining media and the plates were frozen at -80°C overnight. Cells were then lysed by adding 10 μ L of lysis buffer (10 mM Tris pH8, 0.1% Triton 100X) in each well. To obtain total protein levels, a Bradford assay was carried adding 200 μ L of 1X dye Bradford reagent in each well and using a bovine serum albumin (BSA) standards scale (Bio-Rad). Assays were analyzed using the Seahorse Data Analysis Software (Seahorse Bioscience–Agilent Technologies) and Prism 8.

15) Meta-analysis of public databases.

- **TCGA:** The data from The Cancer Genome Atlas (TCGA) was accessed on the <https://portal.gdc.cancer.gov/> website. Query of the Harmonized Cancer Datasets with the ALDOC gene ID resulted in 831 cases affected by 769 copy number variation (CNV) events across 29 projects. CNV is as defined in https://docs.gdc.cancer.gov/Data/Bioinformatics_Pipelines/CNV_Pipeline/.
- **GTEX:** The data from the Genotype-Tissue Expression (GTEX) was accessed on the <https://www.gtexportal.org/home/> website. The data shown for ALDOC and

BCDIN3D are from GTEx Analysis Release V8 (dbGaP Accession phs000424.v8.p2). Expression values are shown in TPM (Transcripts Per Million), calculated from a gene model with isoforms collapsed to a single gene. No other normalization steps were applied. Box plots are shown as median and 25th and 75th percentiles; points are displayed as outliers if they are above or below 1.5 times the interquartile range.

- **CBioPortal:** The data from the METABRIC cohort was accessed on the <https://www.cbioportal.org/> website. First, the data from 2173 patients with Copy Number Alterations (CNA) data was queried for putative copy-number alterations from DNACopy. Calls were made after normal contamination correction and CNV removal using thresholds. Values: -2 = homozygous deletion; -1 = hemizygous deletion; 0 = neutral / no change; 1 = gain; 2 = high level amplification. Only the patients with high level amplification (CNA=2; number at risk = 96) were shown in red in Fig 3f. Comparison of clinical attributes of ALDOC-amplified patients or samples to the rest is shown in Table S7. Second, the data from 1904 patients with mRNA data was queried for mRNA expression (Illumina Human v3 microarray) z-Scores relative to diploid samples ± 2 . No samples showed low mRNA levels (z-score < -2), while 80 samples showed high mRNA levels and are shown in red in Fig 3g. Comparison of clinical attributes of ALDOC-mRNA high patients or samples to the rest is shown in Table S8.

16) Small RNA-Seq: 10 μ g of total RNA was separated on a denaturing 15% polyacrylamide/urea gel and small RNAs < 34 nt were extracted from the gel. The rest of the library preparation was performed as in Ingolia *et al.* [3] with minor modifications. For miRNA-seq samples, reads were trimmed using cutadapt and filtered to require a minimum length of 15 bp. Then, reads were mapped against either the custom sRNA reference as previously described for 3' tRNA^{His}

fragment [1, 6] (including tRNA, piRNA, and miRNA among others), or against a microRNA reference composed only of mature human miRNAs defined by miRBase v22. Counts were obtained from the resulting alignments using samtools idxstats and processed as described for RNA-seq.

17) RNA methyltransferase assay: The RNA methyltransferase assay was performed as in [7] with 300 nM recombinant BCDIN3D and 1 μ M RNA for 2 h at 37°C.

Supplemental Tables

Table S1. Results of the untargeted profiling of primary metabolism in MDA-MB-231 cells where BCDIN3D is depleted via siRNA or shRNA.

Table S2. Results of the proteomic analysis in MDA-MB-231shNC and shBCDIN3D cells analyzed via MaxQuant. LFQ: label-free quantification.

Table S3. Results of the RNA-Seq analysis in MDA-MB-231shNC and shBCDIN3D cells.

Table S4. Ingenuity pathway analysis (IPA) of the RNA-Seq analysis in MDA-MB-231shNC and shBCDIN3D cells, showing Canonical Pathways ranked by their enrichment $-\log(\text{p-value})$ and showing the ratio of genes differentially regulated in each pathways, together with their z-scores (a negative z-score predicts overall inactivation, while a positive z-score predicts overall activation).

Table S5. Ingenuity pathway analysis (IPA) of the RNA-Seq analysis in MDA-MB-231shNC and shBCDIN3D cells, showing 47 protein coding genes belonging to the “Molecular Mechanisms of Cancer” category that were differentially regulated in shBCDIN3D compared to shNC cells.

Table S6. Ingenuity pathway analysis (IPA) of the RNA-Seq analysis in MDA-MB-231shNC and shBCDIN3D cells, showing 19 protein coding genes belonging to the “mTOR pathway” category that were differentially regulated in shBCDIN3D compared to shNC cells. Shown are also their proteomic results extracted from Table S2.

Table S7. Comparison of clinical attributes of ALDOC-amplified patients to samples from the rest of patients in the METABRIC cohort.

Table S8. Comparison of clinical attributes of ALDOC-mRNA high patients or samples to the rest in the METABRIC cohort.

Table S9. List of differentially expressed genes (DEG) in MDA-MB-231 shBCDIN3D vs shNC cells from Table S3 with a false discovery rate (FDR) of less than 0.05 separated in two categories, based on whether they had (YES) or not (NO) a predicted let-7 target site with a probability of conserved targeting (PCT) of more than 0.95 by TargetScan.

Table S10. List of antibodies.

Antibody	Provider	Catalog number	Lot number
BCDIN3D	Sigma	HPA039911	R36480
mTOR-phospho Ser 2448	Cell Signaling	#5536	2
ALDOC (IHC)	abcam	ab87122	GR3175216-2
ALDOC (western blot)	Novus Biologicals	NBP2-25144SS	
SRSF3	Santa Cruz	sc-13510	
TUT7	Novus Biologicals	NBP1-82257	
α Tubulin	Cell Signaling	#3873	8
β Tubulin	abcam	ab6046	GR86729

Table S11. List of oligonucleotides.

Oligonucleotide	Code	Sequence
ON-TARGETplus Non-targeting Pool	D-001810-10-05	
ON-TARGETplus SMARTpool, hBCDIN3D	L-018768-02-0005	
siSRSF3	RNA-BX00053	GAGUGGAACUGUCGAAUGG[dT][dT] (sense) [Phos]CCAUUCGACAGUCCACUC[dT][dT] (antisense)
siTUT4	RNA-BX00024-27	GGUUGCUUCAGACUUUAUA[dT][dT] (sense) 5'[Phos]UAUAAAGUCUGAAGCAACC[dT] [dT] (antisense) GGAAUGAAGAAGAGAAAGA[dT][dT] (sense) 5'[Phos]UCUUUCUCUUCUUCUUCUUC[dT] [dT] (antisense) GGAGAAACGACAUAAAGAAA[dT][dT] (sense) (antisense) 5'[Phos]UUUCUUAUGUCGUUUCUCC[dT] [dT] (antisense) GGUUAGAGCUGCUUAAAUU[dT][dT] (sense) 5'[Phos]AAUUUAAGCAGCUCUAACC[dT] [dT] (antisense)
siTUT7	RNA-BX00028-31	GAAAAGAGGCACAAGAAA[dT][dT] (sense) 5'[Phos]UUUUCUUGUGCCUCUUUUC[dT] [dT] (antisense) "GCAAAGAGGACAAAGAAAU[dT][dT] (sense) 5'[Phos]AUUUCUUUGUCCUCUUUGC[dT] [dT] (antisense) GAUAAGUAUUCGUGUCAAAA[dT][dT] (sense) 5'[Phos]UUUGACACGAAUACUUAUC[dT] [dT] (antisense) GAACAUGAGUACCUAUUUA[dT][dT] (sense) 5'[Phos]UAAAUAGGUACUCAUGUUC[dT] [dT] (antisense)
let-7f probe	BX00800	AACTATACAATCTACTACCTCA

pre-let-7f-1 probe	RNA-BX2	UUAUCUCCUGAACAGGGUAAAAUCAC UA-3'-Biotin
pre-let-7f-1-5'P	RNA-BX3	5'Phos- UGAGGUAGUAGAUUGUAUAGUUGUG GGGUAGUGAUUUUACCCUGUUCAGG AGAUAACUAUACAAUCUAUUGCCUUC CC
pre-let-7f-1+G-5'P	RNA-BX4	5'Phos- GUGAGGUAGUAGAUUGUAUAGUUGU GGGUAGUGAUUUUACCCUGUUCAG GAGAUAACUAUACAAUCUAUUGCCUU CCC
pre-let-7f-1-5'Pme2	RNA-BX42	5'Phos-me2- UGAGGUAGUAGAUUGUAUAGUUGUG GGGUAGUGAUUUUACCCUGUUCAGG AGAUAACUAUACAAUCUAUUGCCUUC CC
BCDIN3D_FWD	BX00070	GCCCCGTTTCGGAAATTTTC
BCDIN3D_REV	BX00071	ACACTCAGATCCCCGGAGTTAC
ALDOC_FWD	BX601	AGGGCAAGTATGAAGGCAGT
ALDOC_REV	BX602	GAAGACAAGTTGGTGCCAGG
ALAS1_FWD	BX00060	CCTTGGCCTTAGCAGTTTTG
ALAS1_REV	BX00061	CCAAGATGATGGAAGTTGGG
B2M_FWD	BX00062	AATGTCGGATGGATGAAACC
B2M_REV	BX00063	TCTCTCTTTCTGGCCTGGAG

Table S12. Growth medium of Triple-Negative Breast Cancer Panel (ATCC(r) TCP-1003(tm)).

CRL-2314	HCC38 Breast Ductal Carcinoma	RPMI +10% FBS +PSQ
CRL-2315	HCC70 Breast Carcinoma	RPMI +10% FBS +PSQ
CRL-2321	HCC1143 Breast Carcinoma	RPMI +10% FBS +PSQ
CRL-2322	HCC1187 Breast Ductal Carcinoma	RPMI +10% FBS +PSQ
CRL-2324	HCC1395 Breast Carcinoma	RPMI +10% FBS +PSQ
CRL-2331	HCC1599 Breast Carcinoma	RPMI +10% FBS +PSQ
CRL-2335	HCC1806 Breast Carcinoma	RPMI +10% FBS +PSQ
CRL-2336	HCC1937 Breast Carcinoma	RPMI +10% FBS +PSQ
HTB-19	BT-20 Breast carcinoma	DMEM +10% FBS +PSQ
HTB-24	MDA-MB-157 Breast Carcinoma	DMEM +10% FBS +PSQ
HTB-26	MDA-MB-231 Breast Carcinoma	DMEM +10% FBS +PSQ
HTB-122	BT-549 Breast Ductal Carcinoma	RPMI +10% FBS +PSQ
HTB-123	DU4475 Mammary Gland	RPMI +10% FBS +PSQ
HTB-126	Hs 578T Breast Carcinoma	DMEM +10% FBS +PSQ
HTB-130	MDA-MB-436 Breast Cancer	DMEM +10% FBS +PSQ
HTB-131	MDA-MB-453 Breast Carcinoma	DMEM +10% FBS +PSQ
HTB-132	MDA-MB-468 Breast Adenocarcinoma	DMEM +10% FBS +PSQ

Supplemental Figures

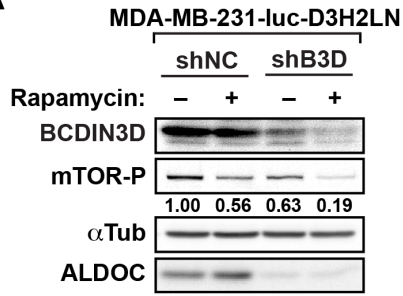
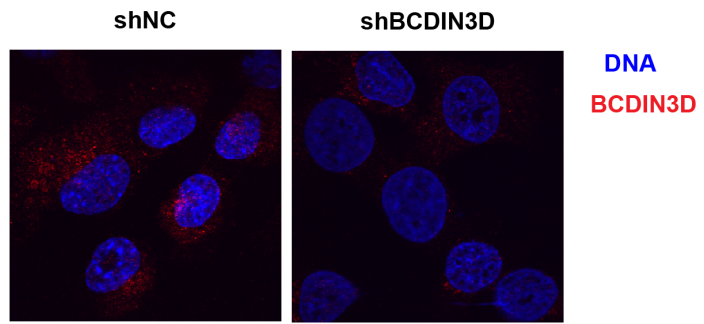
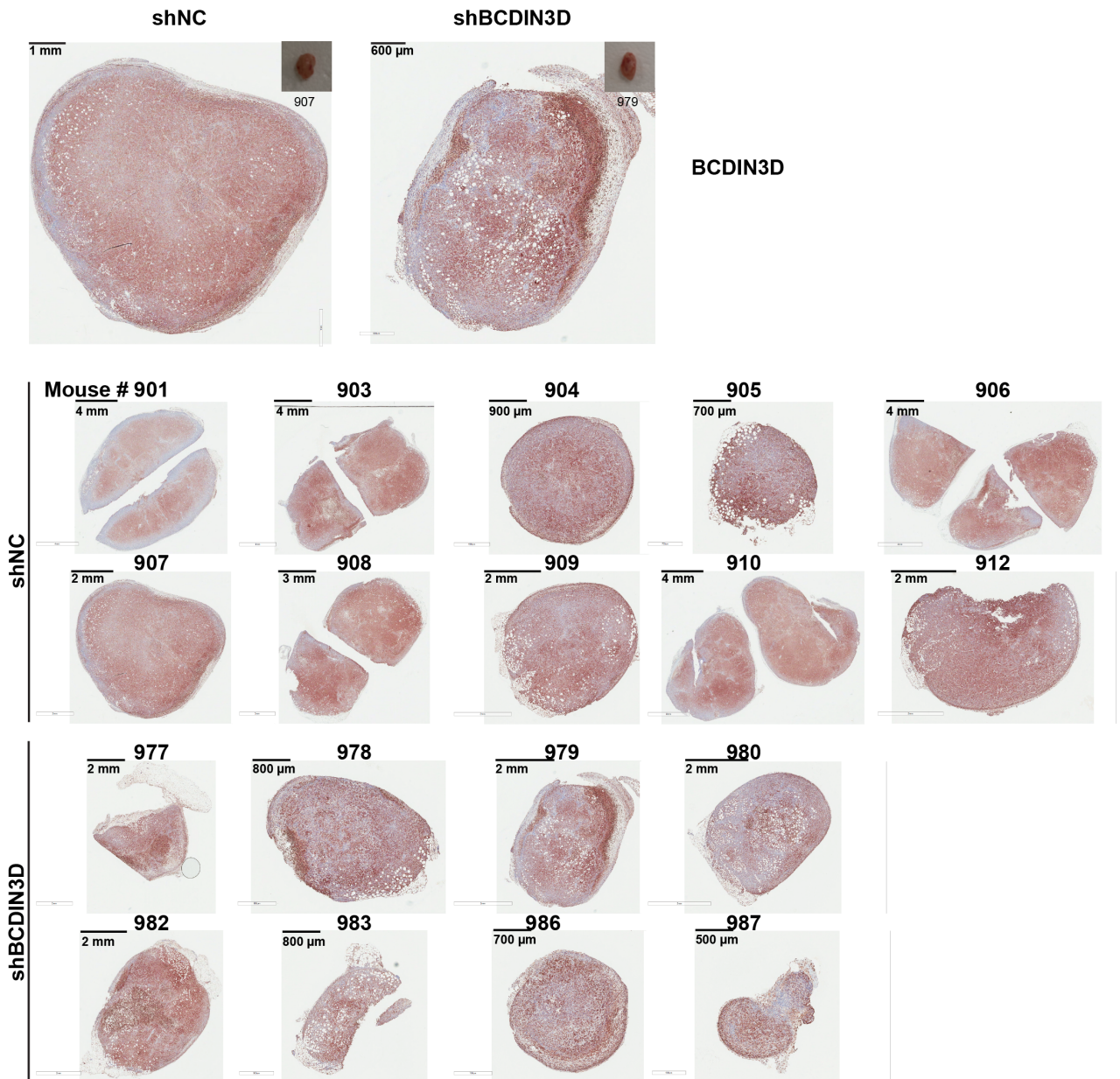
A**B** Immunofluorescence in MDA-MB-231-luc-D3H2LN cells**C** Immunohistochemistry of tumors formed after orthotopic injection of MDA-MB-231-luc-D3H2LN into nude mice

Figure S1.

- a** Western blot analysis of MDA-MB-231-luc-D3H2LN-shNC and shBCDIN3D cells untreated, or treated with 10 nM Rapamycin for 24h. The numbers beneath the anti-mTOR-P western blot corresponds to the mTOR-P signal normalized to α Tubulin signal from the same western blot, and to shNC.
- b** Immunofluorescence analysis of endogenous BCDIN3D localization in MDA-MB-Luc-D3H2LN-shNC or shBCDIN3D cells.
- c** Immunohistochemistry with BCDIN3D antibody of tumors formed after orthotopic injection of MDA-MB-231-Luc-D3H2LNshNC and shBCDIN3D into nude mice, with two representative tumors of approximately the same size shown in more detail at the top.

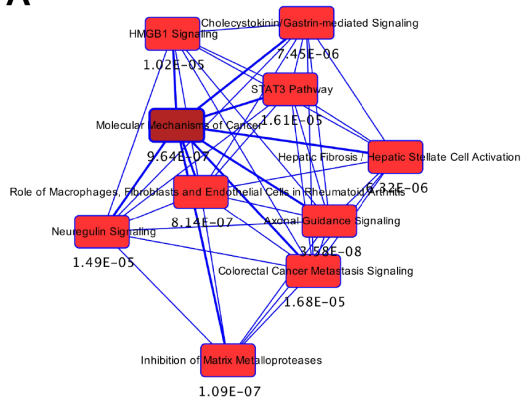
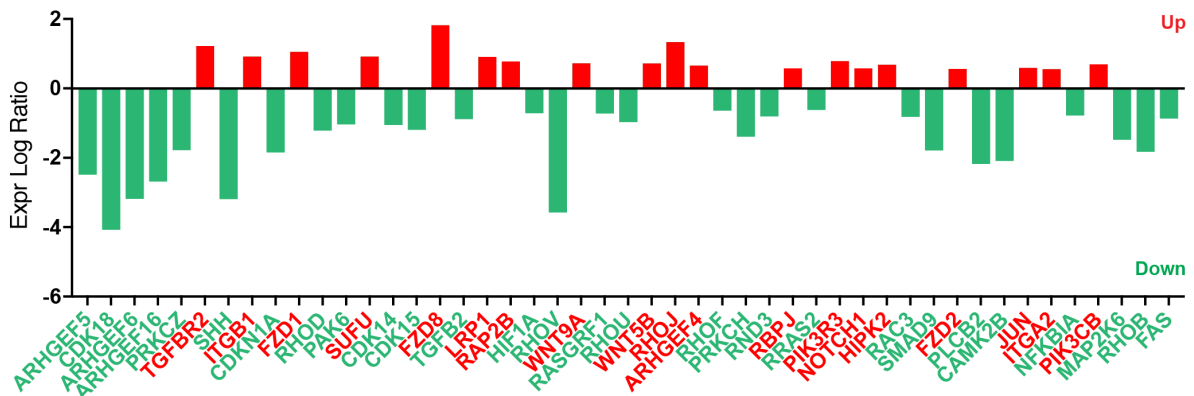
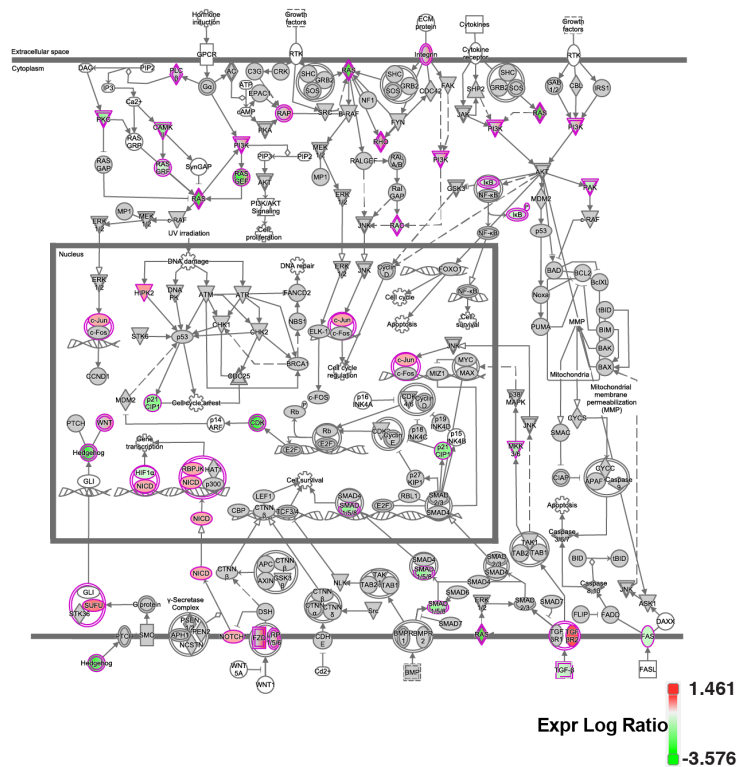
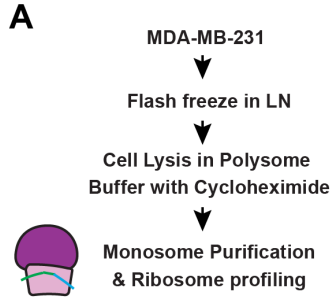
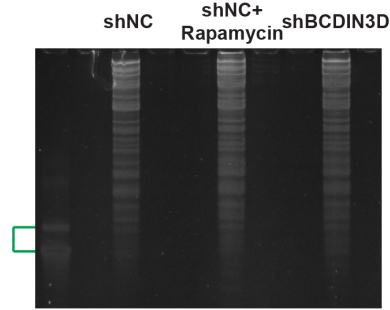
A**B** Differentially expressed genes involved in “Molecular Mechanisms of Cancer”**C** IPA analysis “Mechanisms of Cancer” of RNA-seq data from MDA-MB-231shBCDIN3D vs shNC

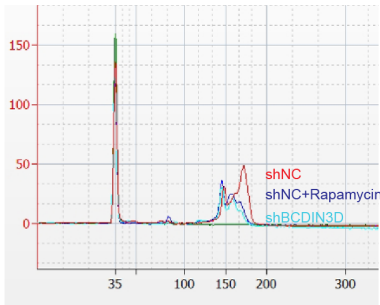
Figure S2. Ingenuity pathway analysis (IPA) of the RNA-Seq analysis in MDA-MB-231shNC and shBCDIN3D cells, showing overlapping pathways relevant to cancer in **(A)**, the log Fold Change of 47 protein coding genes belonging to the “Molecular Mechanisms of Cancer” category in **(B)**, and their position in signaling pathways in **(C)** (green: downregulated; red: upregulated, see legend for exact correspondence between expression log ratio and color).



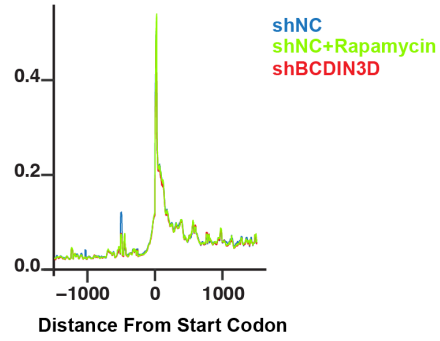
B Size selection of ribosome footprint fragments



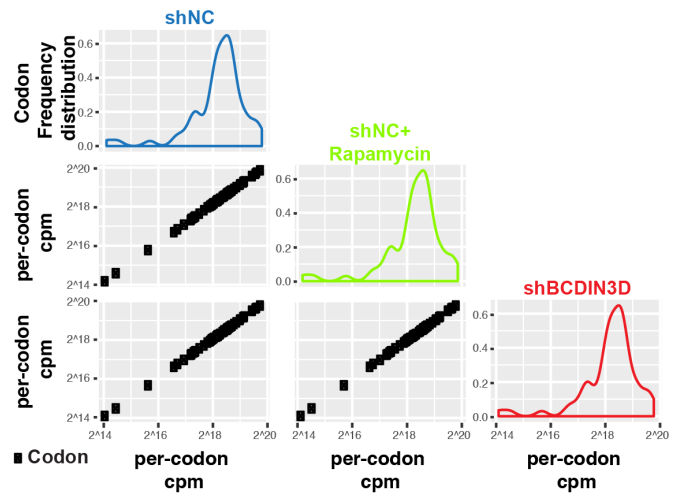
Ribosome profiling libraries



D Meta-analysis at start codon



E Codon usage analysis



C Quality control

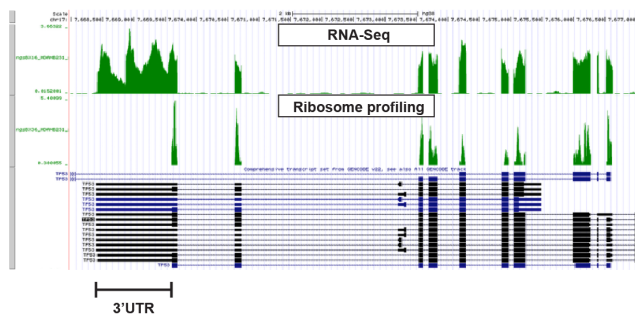


Figure S3. MDA-MB-231 cells treated with Rapamycin or shBCDIN3D display reduced levels of ribosome footprints.

- a** Experimental design for ribosome profiling experiments.
- b** Top: Representative gel used for the size selection of ribosome footprint fragments. Bottom: Bioanalyzer trace of the corresponding ribosome profiling libraries. The peak at ~175 bp corresponds to the ribosome protected fragments, the other peaks to primer auto-amplification characteristic of this method. Red line: shNC, Blue line: shBCDIN3D, Cyan line: shNC+Rapamycin.
- c** UCSC browser screen-shot of reads mapping to TP53 from RNA-Seq and ribosome profiling experiments: the RNA-Seq shows reads on the TP53 3'UTR that are not present on the ribosome profiling, thus showing absence of contamination of ribosome footprint fragments by free mRNA.
- d** Meta-analysis of ribosome profiling reads around the TSS (± 1.5 kb) of unique protein coding genes. Rapamycin treatment was included as a positive control and was for 24h at 10 nM.
- e** Codon frequencies in ribosome profiling data from MDA-MB-231 shNC, shNC+Rapamycin and shBCDIN3D cells. For all conditions, reads were assigned to a specific codon based on start position using the R package RiboProfiling, and per-codon counts per million were calculated and log₂ transformed for each sample. A strong correlation between codon counts was observed between conditions, with similar distributions. Additionally, within each dataset most codons had similar read counts, with the three low outlier points in all conditions corresponding to the three stop codons. This shows that no codons are under- or over-represented in the shNC+Rap or shBCDIN3D ribosome profiling data, indicating that the global translation defect in these conditions is not due to defects in specific tRNA levels or function.

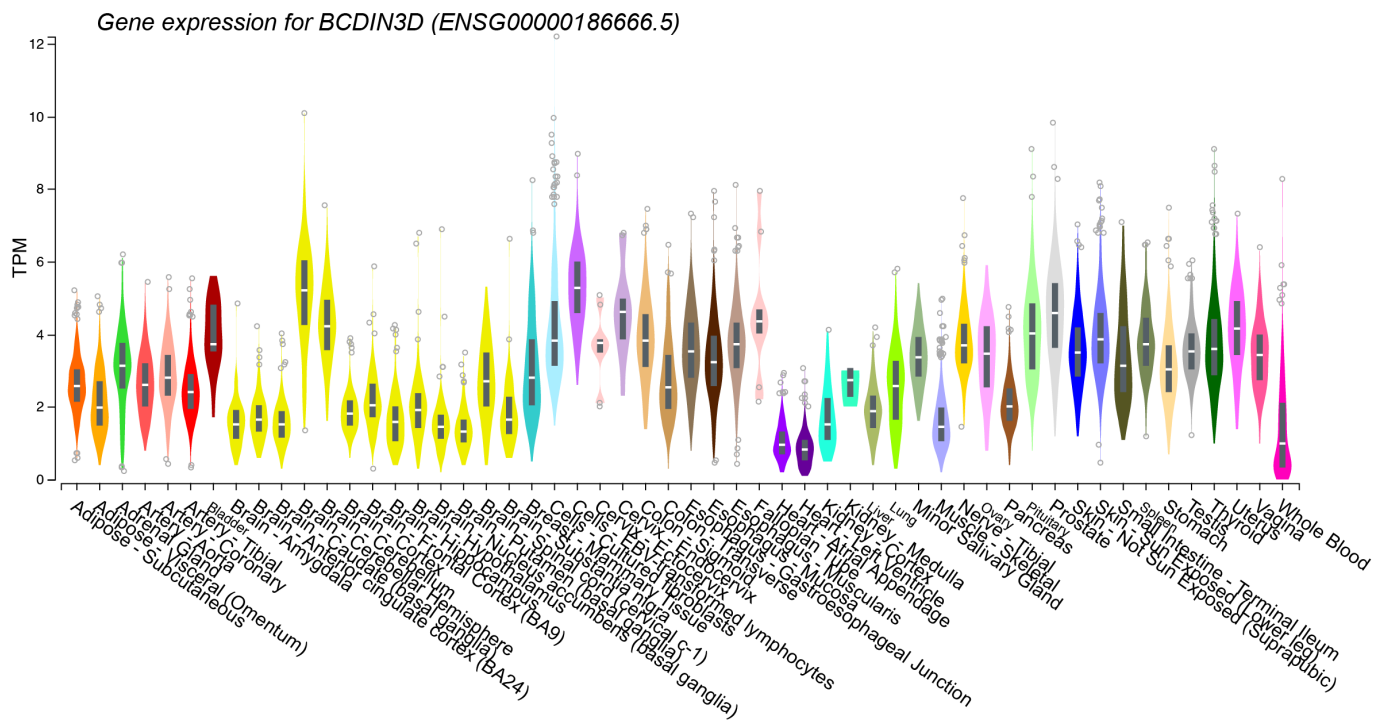
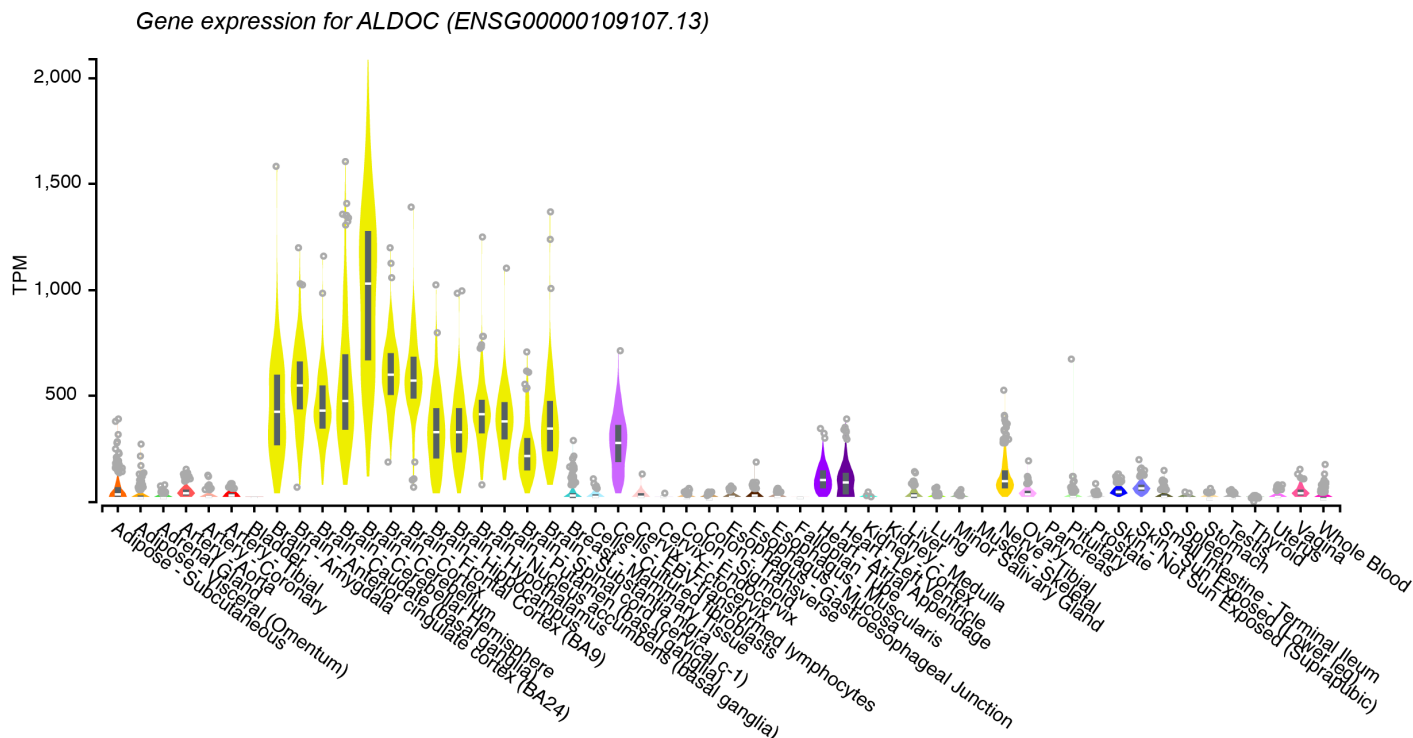


Figure S4. ALDOC and BCDIN3D mRNA expression in human tissues from GTEx.

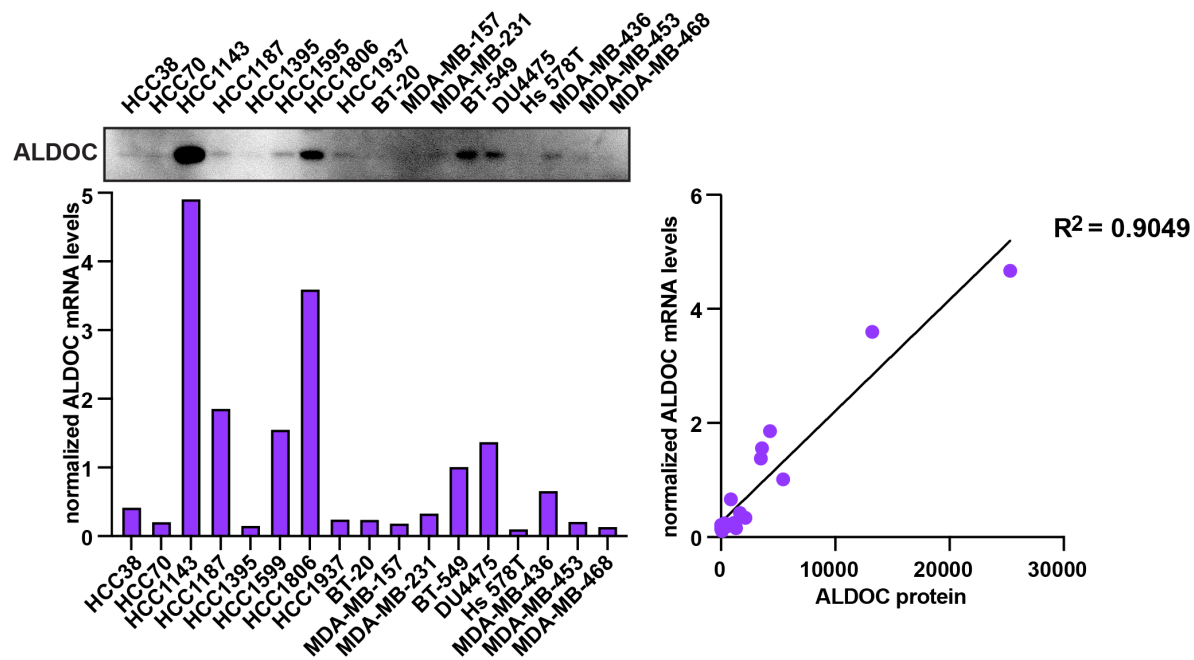


Figure S5. ALDOC mRNA and protein levels are highly correlated in breast cancer cell lines. Total RNA and proteins were purified from a panel of 17 Triple Negative Breast Cancer cell lines as previously described [8]. ALDOC protein levels were analyzed from 5 μ g of total proteins by western blot with an ALDOC antibody (top left panel) and quantified with ImageJ. ALDOC mRNA levels were analyzed by RTqPCR, and normalized to ALAS1, and to the resulting average value (bottom left graph). Correlation between ALDOC mRNA and protein levels was analyzed with Prism 8, using a least squares regression method with no special handling of outliers. The first order polynomial (straight line) and the R squared value of this analysis is shown in the bottom right graph.

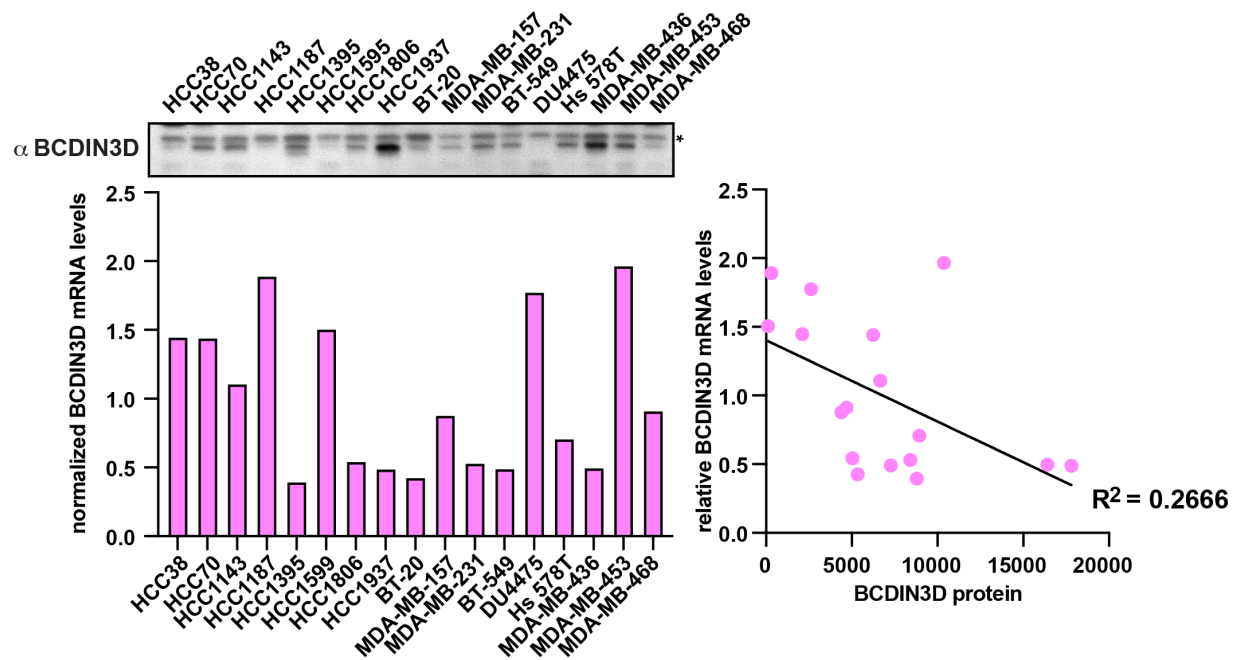


Figure S6. BCDIN3D mRNA and protein levels are not well-correlated in breast cancer cell lines. Total RNA and proteins were purified from a panel of 17 Triple Negative Breast Cancer cell lines. BCDIN3D protein levels were analyzed from 5 μ g of total proteins by western blot with a BCDIN3D antibody (top left panel) and quantified with ImageJ. The asterisk indicates a non-specific band. BCDIN3D mRNA levels were analyzed by RTqPCR, and normalized to ALAS1, and to the resulting average value (bottom left graph). Correlation between BCDIN3D mRNA and protein levels was analyzed with Prism 8, using a least squares regression method with no special handling of outliers. The first order polynomial (straight line) and the R squared value of this analysis is shown in the bottom right graph.

Primary *let-7f* microRNA expression in MDA-MB-231 cells

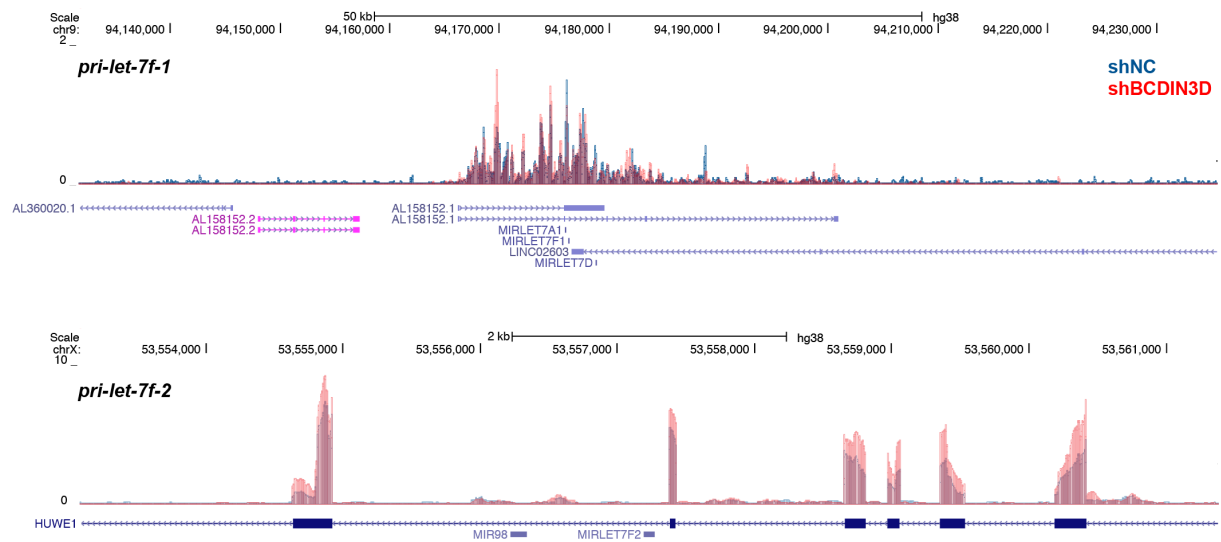


Figure S7. UCSC genome browser traces of RNA-Seq in MDA-MB-231shNC and shBCDIN3D cells at the *pri-let-7f-1* and *pri-let-7f-2* loci. The traces (50% transparent) are overlaid, with the shNC in blue, and the shBCDIN3D trace in red.

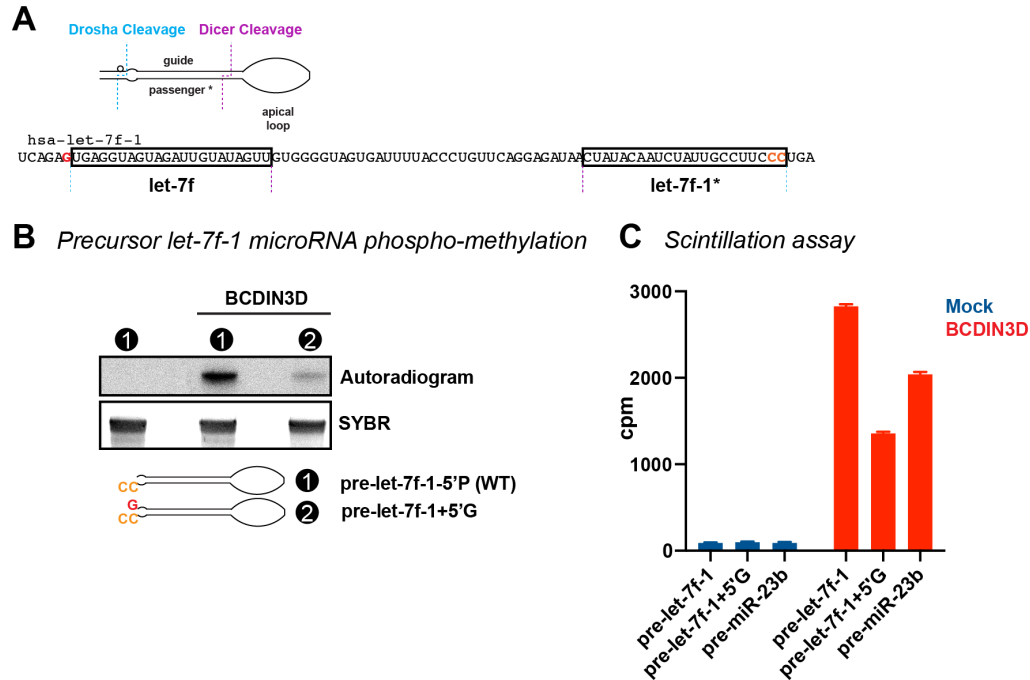


Figure S8. BCDIN3D methylates pre-let-7f-1 *in vitro*.

- a** Schematic of the pre-let-7f-1 stem-loop as defined in miRBase, with the predicted cuts performed by Drosha to generate the pre-miRNA, and by Dicer to generate the miRNA duplex (top), as well as the corresponding sequences (bottom).
- b** *in vitro* RNA methyltransferase assay with BCDIN3D using radioactive [³H]-SAM as methyl group donor, and pre-let-7f-1-5'P precursor with either the WT predicted sequence, or containing an extra G nucleotide at the 5' end. The bottom panel shows the SYBR stained gel that was used for the autoradiography in the top panel.
- c** Scintillation counts in counts per minute (cpm) of C[³H]₃ incorporated into the RNA from the RNA methyltransferase assay in (B). pre-miR-23b is included as a control [6].

Precursor *let-7f-1* microRNA processing

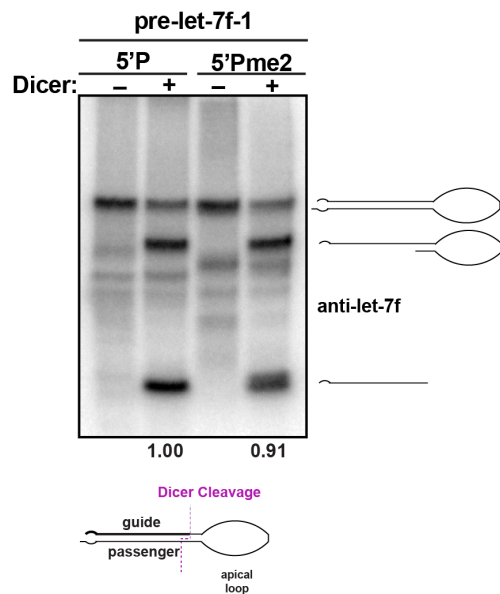


Figure S9. Pre-*let-7f-1* 5' phospho-methylation does not affect Dicer processing. *In vitro* Dicer assay with synthetic 5'P and 5'Pme2 pre-*let-7f-1* using mock or 2 μ L of human Dicer at 1 μ g/ μ L, corresponding to 50% precursor microRNA cleavage. Shown is a northern blot analysis of the Dicer assay with an anti-*let-7f* probe. The numbers at the bottom of the image correspond to the quantification of mature *let-7f* generated after Dicer cleavage, normalized to the 5'P sample.

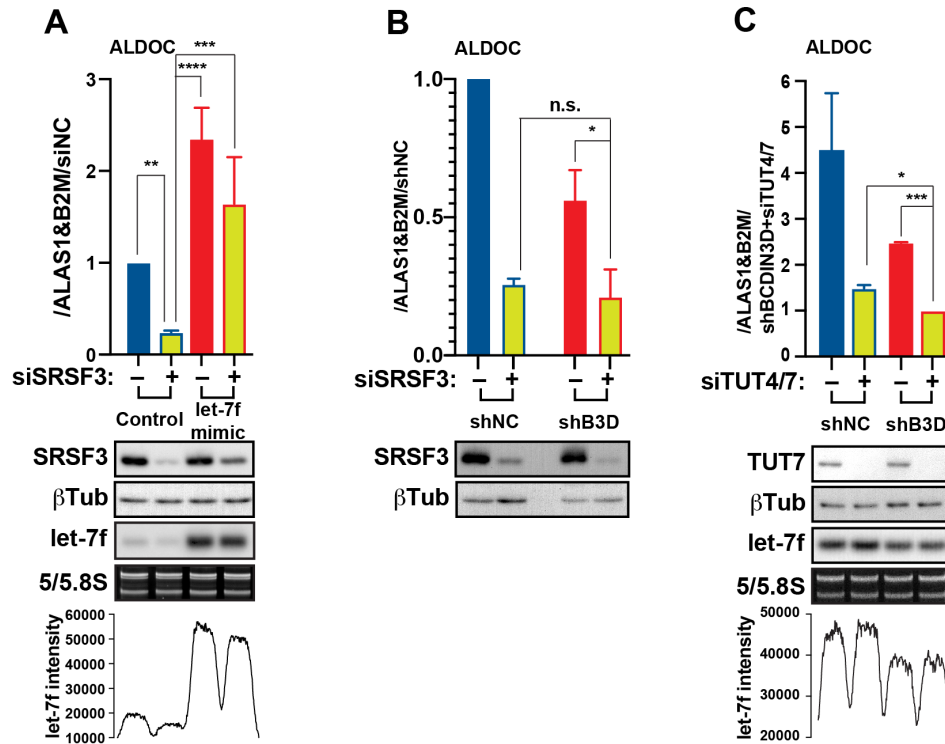


Figure S10. Other let-7f processing regulators affect ALDOC mRNA levels.

- a** ALDOC gene expression from MDA-MB-231 transfected with siNC or siSRSF3, and siNC (control) or let-7f mimics in all combinations. Data is normalized to ALAS1, B2M and control (mean \pm SD, n=3 biological replicates). The asterisks indicate significant p-values (1 way Anova with multiple comparisons). Protein levels of SRSF3 and β -Tubulin (loading control) were analyzed by western blot, while let-7f levels were analyzed by northern blot with an anti-let-7f probe. 5/5.8 S panel shows the SYBR Gold stained gel at the level of the 5/5.8S ribosomal RNA (loading control). The bottom graph shows the raw intensity of the let-7f signal quantified with ImageJ. Please note that the siSRSF3 siRNA was validated by [9].
- b** ALDOC gene expression from MDA-MB-231shNC and shBCDIN3D transfected with siNC or siSRSF3 analyzed as in (A).
- c** ALDOC gene expression from MDA-MB-231shNC and shBCDIN3D transfected with siNC or siTUT4+siTUT7 analyzed as in (A).

Supplemental information references.

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