Figure Legends

Figure S1, related to Figure 1. BCDIN3D is mainly localized in the cytoplasm. (A) Immunofluorescence staining of isogenic HELA-S3-Flp-In and HELA-S3-Flp-In-BCDIN3D-Flag stable cell lines with an anti Flag Antibody. **(B)** Whole cell extracts from MCF-7 cells transfected with siBCDIN3D and siNC were analyzed by Western Blot with an antibody against BCDIN3D. The membrane was stripped and analyzed with an antibody against GAPDH to control for equal loading of the samples. **(C)** Immunofluorescence staining of MCF-7 cells with the anti BCDIN3D antibody (same as in B).

Figure S2, related to Figure 2. siRNA mediated depletion of BCDIN3D suppresses tumorigenic phenotypes *in vitro*. (A-D) BCDIN3D depletion suppresses the invasiveness of the MDA-MB-231 cells, without affecting their growth and migration abilities. MDA-MB-231 cells were transfected with siBCDIN3D and siNC, and their growth (A, *MTT assay*), ability to migrate through a 8 μM pore size membrane (B, *Migration assay*) or to invade a 8 μM pore size membrane coated with dried basement membrane matrix solution (C&D, *Invasion assay*) were tested in parallel (see Extended Experimental Procedures for further detail). Error bars represent SEM values.

Figure S3, related to Figure 3. Depletion of BCDIN3D increases the levels of mature miR-145 in several cell lines. (A) Schematic view of the BCDIN3D gene locus. The BCDIN3D gene contains 2 exons. The sequence of the siRNA targeting the first exon of BCDIN3D is shown. The shRNA as well as the smart pool of siRNAs against BCDIN3D targets the second exon. The second exon contains 596 bp complementarity to two non

coding RNAs that are transcribed in the opposite direction to the BCDIN3D gene. The siRNAs were ordered as ON-TARGETplus® siRNAs from Dharmacon, where only the antisense oligonugleotide is 5' mono-phosphorylated in order to minimize the effect of these siRNAs on the non-coding RNAs. **(B)** MCF-7 cells were transfected with the siRNA targeted against the first exon of BCDIN3D (siBCDIN3D) or the pool of siRNAs targeted against the second exon of BCDIN3D (siBCDIN3Dsmartpool) or with non targeting siRNAs as a negative control (siNC). BCDIN3D mRNA levels from these cells were analyzed by quantitative Reverse Transcription and PCR analysis (qRT-PCR) and normalized to the ALAS1 and B2M mRNAs and to siNC cells. Error bars represent SEM values. **(C)** MCF-7 cells were transfected with siBCDIN3Dsmartpool or with siNC and the levels of the indicated mature miRNA were analyzed. The data are normalized to 5S and the U6 RNA and to miRNA levels in the siNC treated cells. Error bars represent SEM values. **(D)** MDA-MB-231 cells were transfected with siBCDIN3D and siNC and the levels of BCDIN3D, miR-21, miR-145 were analyzed by qRT-PCR. The BCDIN3D mRNA levels were normalized to the ALAS1 and B2M mRNAs and to siNC cells, while the miR-145 levels were normalized to the miR-21 RNA and to miRNA levels in the siNC treated cells. Error bars represent SEM values. **NB.** In depth analysis of several other miRNAs, as well as small RNAs broadly used to normalize levels of miRNAs, such as 5S, U6 and RNU6B, revealed that using miR-21 to normalize miR-145 levels is a more appropriate method even though it underestimates the apparent increase in the levels of miR-145 in siBCDIN3D versus siNC cells. **(E)** RNA from MDA-MB-231 [shBCDIN3D+BCDIN3DshR(esistant)-GFP], MDA-MB-231 [shBCDIN3D+GFP] and MDA-MB-231 [shNC+GFP] cell lines was analyzed as in (D). **(F)** BJ+hTERT cells

express significantly higher levels of miR-145 than MCF-7 cells. The levels of miR-21 and miR-145 from MCF-7 and immortalized human embryonic fibroblasts BJ+hTERT were analyzed by qRT-PCR and normalized to the RNU6B RNA and to BJ+hTERT cells. Error bars represent SEM values. **(G)** RNA from BJ-TERT cells transfected with siBCDIN3D and siNC was analyzed as in (D), as well as by Northern Blot with a probe against miR-145.

Figure S4, related to Figure 5. miRNA motifs are significantly enriched in the 3'UTR of genes down-regulated upon BCDIN3D depletion. Total RNA from MCF-7 cells transfected with siBCDIN3D and siNC was amplified, sequenced and analyzed as indicated in the Extended Experimental Procedures. **(A)** Volcano plot representation of the fold change of normalized mRNA read counts between siBCDIN3D- and siNC-treated samples (X axis, log2) and the p-value allocated to the fold change (Y axis, log10). Red points indicate significantly downregulated or upregulated genes based on fold change greater than 2, p-value equal to or lower than 0.05, mRNA exonic length higher than 500 bases and normalized read count greater than 100 for at least one of the samples. **(B)** The 3'UTRs of significantly downregulated or upregulated genes between siBCDIN3D- and siNC- samples were analyzed for the presence of putative miRNA regulatory sequences as indicated in the Extended Experimental Procedures. A Fisher's exact test was performed to calculate the p-value (Y axis, log10) associated with the likelihood of a given number of miRNA (X axis) to be present more than expected in the 3' UTR of mRNAs that are either downregulated (green line), upregulated (red line) or changing (sum of dowregulated and upregulated, black line) in siBCDIN3D *versus* siNC.

The red dashed line corresponds to the p = 0.05 threshold.

Figure S5, related to Figure 5. BCDIN3D regulates other miRNAs. (A) Total RNA from MCF-7 cells treated with siBCDIN3D or siNC were submitted to miRNA profiling by miRCURY LNA^{TM} Universal RT miRNA PCR (Exiqon) (see Extended Experimental Procedures for further detail). The list represents miRNAs whose expression was found significantly increased (red), or decreased (green) by a log2 ratio superior to 0.5 or inferior to -0.5, respectively, by the miRNA PCR profiling procedure. To present, we have not been able to validate the decrease of several of the miRNAs shown in green (miR-28, Figure S5B and data not shown). This could be due to the fact that we normalized the data to the mean expression of all miRNAs in each sample and the overall expression of miRNAs was increased in siBCDIN3D compared to siNC samples (Extended Experimental Procedures). However, we were able to validate the over-expression of several miRNAs shown in red (miR-23b, Figure S5B and data not shown). **(B)** MCF-7 cells were transfected with siBCDIN3D and siNC and the levels of the precursor, mature and passenger strand of miR-23b and miR-28 were analyzed by quantitative Reverse Transcription and PCR (qRT-PCR) analysis. The data are normalized to the U6 RNA and to siNC cells. Error bars represent SEM values. Similar results were obtained in MDA-MB-231 cells thereby validating miR-23b as a good candidate for siBCDIN3D dependent regulation. **(C)** Pre-miR-23b and miR-23b* share the same 5'end. Schematic showing the position of miR-23b and miR-23b* within the pre-miR-23b hairpin. **(D)** pre-miR-23b, and to a lesser extent, miR-23b* are modified in a BCDIN3D dependent manner. RNA from MCF-7 cells transfected with siBCDIN3D and siNC were treated or not with Terminator^{TM} 5'-P Dependent exonuclease as in Figure

5, and further analyzed by Reverse Transcription and semi-quantitative PCR with the indicated primers (upper panels). All the indicated RNAs were also analyzed by quantitative RT-PCR (lower graph). The data are normalized to the 5S RNA and to the [-Terminator] sample. Error bars represent SEM values.

Figure S6, related to Figure 5. BCDIN3D methylates other pre-miRNAs *in vitro*. *In vitro* methyltransferase assay using mock (1-2), recombinant BCDIN3D (5-10) or recombinant BCDIN3D-D72G74A (3-4), ^{3}H-radioactive SAM as methyl group donor and the following synthetic RNA molecules as substrate (1, 3, 5) pre-miR-145 [5'-OH]; (2, 4, 6) pre-miR-145 [5'-P]; (7) pre-miR-10b [5'-OH]; (8) pre-miR-10b [5'-P]; (9) pre-miR-23b [5'-OH]; (10) pre-miR-23b [5'-P]. The nucleic acids were purified and analyzed by autoradiography (panels, the lower panel shows the Staining with ethidium bromide of the gel used for the autoradiography shown in the upper panel) and liquid scintillation (graph). Error bars represent SD values.

Figure S7, related to Figure 6. Phospho-di-methylated pre-miR-145 is poorly processed by human Dicer *in vitro*. Synthetic pre-miR-145 molecules that have [5'-OH], [5'-P], [5'-Pme1] or [5'-Pme2] 5' ends were incubated with human Dicer or Mock at the indicated concentrations of $MgCl_2$ (see Extended Experimental Procedures for further detail). The Dicer processing reactions were loaded on a 15% Urea-PAGE gel and stained with Ethidium Bromide. The gel was photographed with the Chemidoc XRS+ system from Biorad and the miR-145 product was quantified using the Image Lab Software.

Supplementary Table 1, related to the Experimental Procedures. List of siRNAs, premiR mimics and inhibitors.

siRNA	Target Sequence
ON-TARGETplus siBCDIN3D_exon1	GGAGGGAGUGUUAAGGAGA
ON-TARGETplus SMARTpool L-018768-02-0005, hBCDIN3D	CUGCAAGGCGUCUCCGAAA
	ACAAUCAGGCAGCCGCAAA
	ACGUGGAAAUCCCAUCAAA
	CUUCAAGAGAAUUCCGUCU
ON-TARGETplus Non-targeting Pool D-001810-10-05	Not provided by the supplier
Pre-miR™ miRNA Precursor- hsa-miR-145-5p	Ambion
Pre-miR™ miRNA Precursor-Negative Control #1	Ambion
*mir*Vana® miRNA inhibitor- hsa-miR-145-5p	Ambion
*mir*Vana® miRNA inhibitor- Negative Control #1	Ambion

Supplementary Table 2, related to the Experimental Procedures. List of oligonucleotides.

Primers	Code	Sequence /Supplier
hBCDIN3D_FWD	TK08234	GCCCCGTTCGGAAATTTTC
hBCDIN3D_REV	TK08235	ACACTCAGATCCCCGGAGTTAC
hIRS1_FWD	TK10907	AGAGGACCGTCAGTAGCTCAAC
hIRS1_REV	TK10908	TTTCGCTTGGCACAATATAGAA
hALAS1	Hs_ALAS1_1_SG	Qiagen QuantiTect® Primer Assay QT00073122
hB2M	Hs_B2M_1_SG	Qiagen QuantiTect® Primer Assay QT00088935
pri-miR-21	Hs03302625_pri	ABI TaqMan® Pri-miRNA Assay # 4427012
pri-miR-145_FWD		AGGGCCAGCAGCAGGC
pri-miR-145_FWD		TCAGGAAATGTCTCTGGCTGTG
pre-miR-21_FWD	TK10134	TAGCTTATCAGACTGATGTT
pre-miR-21_REV	TK10135	ACAGCCCATCGACTGGTGTT
pre-miR-145_FWD	TK10132	GTCCAGTTTTCCCAGGAATC
pre-miR-145_REV	TK10133	AGAACAGTATTTCCAGGAAT
miR-21	hsa-miR-21	ABI TaqMan® MicroRNA Assay # 4373090
miR-145	hsa-miR-145	ABI TaqMan® MicroRNA Assay # 4395389
RNU6B	RNU6B	ABI TaqMan® MicroRNA Assay # 4427975
miR-10b	AM30018	Ambion mirVana™ qRT-PCR Primer Set (miR-10b)
miR-21	AM30102	Ambion mirVana™ qRT-PCR Primer Set (miR-21)
miR-125b	AM30022	Ambion mirVana™ qRT-PCR Primer Set (miR-125b)
miR-145	AM30047	Ambion mirVana™ qRT-PCR Primer Set (miR-145)
miR-155	AM30059	Ambion mirVana™ qRT-PCR Primer Set (miR-155)
5S	AM30302	Ambion mirVana™ qRT-PCR Primer Set for Normalization (5S)
U6	AM30303	Ambion mirVana™ qRT-PCR Primer Set for Normalization (U6)
Pre-miR-23b_FWD	BX00009	TGGCATGCTGATTTGTGACT
Pre-miR-23b_REV	BX000010	GGTAATCCCTGGCAATGTGA
miR-23b_FWD	BX000011	ACACTCCAGCTGGGATCACATTGCCAG
miR-23b_RT	BX000012	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGTAATCC
miR-23b*_FWD	BX000013	ACACTCCAGCTGGGTGGGTTCCTGGCAT
miR-23b*_RT	BX000014	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAATCAGC
Pre-miR-28_FWD	BX000015	GCTCACAGTCTATTGAGTTACCTTTC
Pre-miR-28_REV	BX000016	CCAGGAGCTCACAATCTAGTGG
miR-28-5p_FWD	BX000017	ACACTCCAGCTGGGAAGGAGCTCACAGT
miR-28-5p _RT	BX000018	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTCAATAG

miR-28-3p _FWD	BX000019	ACACTCCAGCTGGGCACTAGATTGTGAG
miR-28-3p _RT	BX000020	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCCAGGAG
pre-miR-145-5'OH	Synthetic RNA	5'OH – GUCCAGUUUUCCCAGGAAUCCCUUAGAUGCUAAGAUGGGGAUUCCUGGAAAUACUGUUCU
pre-miR-145-5'P	Synthetic RNA	5'PO_4 – GUCCAGUUUUCCCAGGAAUCCCUUAGAUGCUAAGAUGGGGAUUCCUGGAAAUACUGUUCU
pre-miR-145-5'Pme1	Synthetic RNA	5'PO_4CH_3 – GUCCAGUUUUCCCAGGAAUCCCUUAGAUGCUAAGAUGGGGAUUCCUGGAAAUACUGUUCU
pre-miR-145-5'Pme2	Synthetic RNA	5'$PO_4C_2H_6$ – GUCCAGUUUUCCCAGGAAUCCCUUAGAUGCUAAGAUGGGGAUUCCUGGAAAUACUGUUCU
miR-145-P	Synthetic RNA	5'PO_4 –GUCCAGUUUUCCCAGGAAUCCCU
miR-145*-P	Synthetic RNA	5'PO_4 –GGAUUCCUGGAAAUACUGUUCU
pre-miR-10b-5'OH	Synthetic RNA	5'OH – UACCCUGUAGAACCGAAUUUGUGUGGUAUCCGUAUAGUCACAGAUUCGAUUCUAGGGGAAU
pre-miR-10b-5'P	Synthetic RNA	5'PO_4 – UACCCUGUAGAACCGAAUUUGUGUGGUAUCCGUAUAGUCACAGAUUCGAUUCUAGGGGAAU
pre-miR-23b-5'OH	Synthetic RNA	5'OH – UGGGUUCCUGGCAUGCUGAUUUGUGACUUAAGAUUAAAAUCACAUUGCCAGGGAUUACC
pre-miR-23b-5'P	Synthetic RNA	5'PO_4 – UGGGUUCCUGGCAUGCUGAUUUGUGACUUAAGAUUAAAAUCACAUUGCCAGGGAUUACC

Supplementary Table 3, related to the Experimental Procedures. List of antibodies.

Antibody	Provider	Catalog number	Lot number
Anti-BCDIN3D	Sigma	HPA039911	R36480
Anti-IRS1	Thermo Scientific	# 1861242	# IK118947
Anti-p53	Active Motif	# 39553	# 00909001
Anti-GAPDH	Abcam	ab9485	954292
Anti-Drosha	Abcam	ab12286	552659
Anti-Dicer	Cell Signaling	# 3363	# 1
Anti-Ago2	Cell Signaling	# 2897	# 2
Anti-TRBP	Abcam	ab42018	754952
Anti-Tubulinα	Abcam	ab7291	525683
Anti-Tubulinβ	Abcam	ab6046	86729
GFP	Molecular Probes	A11122	779558
Flag	Sigma	F7425	026K4848
Flag	Sigma	F1804	124K6106
3xFlag Peptide	Sigma	F4799	

Extended Experimental procedures

BCDIN3D expression and purification: BCDIN3D ORF was cloned into pGEX-5X-1 in frame with a GST tag or into pQE32 in frame with a 6xHis tag. The BCDIN3D D72A, G74A and D72G74A mutations were introduced by site directed mutagenesis. The resulting GST and His tag fusion recombinant proteins were used in *in vitro* methyltransferase assays. Only the D72G74A mutation abolished the methyltransferase activity of BCDIN3D.

Cell lines: MCF-7 and MDA-MB-231 cells were purchased from ATCC and 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). The Hela-S3-Flp-In parental cells were constructed using the Flp-In™ system from Invitrogen. Isogenic Hela-S3-Flp-In cell lines containing a C-terminally Flag Tagged BCDIN3D construct inserted to a single site were constructed according to the protocol provided with the Flp-In™ system. The Hela-S3-Flp-In cells were grown in spinner flasks at 75 rpm in RPMI+10%FBS+PSQ supplemented with 200 µg/ml of Zeocin (parental) or 400 µg/ml hygromycin (BCDIN3D-Flag).

Immunofluorescence: Cells were grown in slide flasks, washed twice with 1 ml of PBS, fixed with 5% Formalin solution in PBS for 10 min at RT, washed 3 times with 1 ml of PBS and permeabilized with 1 ml of PBS+3%BSA+0.6% Triton for 10 min at RT. The fixed cells were incubated O/N at 4°C with 1 ml of PBS+3%BSA and the primary antibodies and for 1 hr at RT with DAPI and the appropriate secondary antibodies. Images were acquired with an Olympus FV1000 Upright confocal microscope and processed using Adobe Photoshop CS software.

siRNA transfection: MCF-7 and MDA-MB-231 cells were reverse transfected in two rounds with 10 or 20 nM of Dharmacon ON-TARGETplus siRNAs against BCDIN3D and control using Lipofectamine^{TM} RNAiMAX from Invitrogen or HiPerfect from Qiagen according to the manufacturers' instructions. The siRNAs sequences are listed in Supplementary Table 1.

Cellular assays: For Soft Agar Colony assays, $3x10^4$ cells from two independent clones of MDA-MB-231[shBCDIN3D] and MDA-MB-231[shNC] cell lines were imbed in 4 ml of 0.35% agar in DMEM+10%FBS+PSQ+Puromycin and plated over a 4 ml layer of 0.7 % agar in the same media in a well of a 6-well plate in duplicates. Colonies were analyzed 4-5 weeks after the initial plating. For growth assays, $0.5x10^3$ and $1x10^4$ cells were plated in 96-well plates immediately after the second round of siRNA transfection in 6 replicates. Growth was analyzed 72 hours later with the Vybrant® MTT Cell Proliferation Assay Kit (V13154) from Invitrogen according to the manufacturer's instructions. For migration and invasion assays, cells were reverse transfected in two rounds with siBCDIN3D and siNC. 48h after the second transfection, cells were trypsinised, counted and used in triplicates in the CytoSelect^{TM} 24- well Cell Migration and Invasion assay (8µm, Colorometric Format, CBA-100-C from Cell Biolabs, Inc) according to the manufacturer's instructions. The migration and invasion were assessed after 24h incubation. The real-time growth, migration and invasion assays were performed using the xCELLigence system from Roche (http://www.nature.com/nmeth/journal/v6/n8/full/nmeth.f.263.html). $1x10^4$ cells in DMEM+10%FBS+PSQ were plated per well in the E-plate for the growth assays. $6x10^4$ cells in DMEM+PSQ were plated per well in the upper chamber and

DMEM+10%FBS+PSQ was the attractant in the lower chamber of the CIM-plate for the migration and invasion assays. 1/40 dilution of Matrigel was used to coat the upper chamber of the CIM plate for the invasion assays. All assays were performed in quadruplets and followed for 48h.

RNA/Protein extraction: Total RNA and protein extraction was typically performed on ~ $5x10^5$ cells using the RNA/Protein purification kit from Norgen (Product # 23000). Cells grown on 6 cm diameter dishes were washed with 5 ml of PBS and lysed with 350 µl of Lysis Buffer supplemented with 10 µl of β-mercaptoethanol per ml for 5 min on a rocking table. RNA extraction, including DNase treatment, was performed according to the manufacturer's instructions. The whole flow-through after the RNA binding step was used for protein purification. The RNA and protein concentrations were measured with a NanoDrop (ND-1000) Spectrophotometer.

RNA Clean-up: After various *in vitro* enzymatic treatments, the resulting RNAs were purified using the Qiagen RNeasy MinElute Cleanup Kit with a modified protocol that allows recovery of RNAs of all sizes, as per the Illumina Directional mRNA-Seq Library prep. V1.0 kit Pre-Release Protocol. Briefly, each reaction was toped to 100µl with water and mixed with 350 µl of RLT buffer and 625 µl of 100% Molecular grade Ethanol. The mixture was passed through the Qiagen RNeasy MinElute column. The increased ratio of ethanol allows low molecular weight RNA to bind to the column. The column was successively washed with 500 µl of RPE buffer and 750 µl of 80% Ethanol, dried by centrifugation and the RNA was eluted with 20 µl of water. The validity of this method was confirmed by analysis of the RNAs on an Agilent Total Eukaryotic RNA Pico Chip

and qRT-PCR.

RNA Analysis: 500 ng of total RNA was used for cDNA synthesis with the Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) with both oligo$(dT)_{20}$ and random hexamers. Subsequently, $1/20^{th}$ of each reaction was used for PCR with gene specific primers. 20-200 ng of total RNA was used for detection of miRNAs with the *mir*Vana™ qRT-PCR miRNA detection kit and/or the Taqman® MicroRNA Reverse Transcription Kit from Applied Biosystems. In the –RT samples, water was used instead of the reverse transcription enzymes. Real-time PCR analysis was performed on a StepOne Plus system or Vii7A from Applied Biosystems. The sequences of the primers used for PCR analysis are listed in Supplementary Table 2. The Northern Blots were performed as previously described (Xhemalce and Kouzarides, 2010).

RNA library preparation and sequencing: 1 μg of total RNA was amplified with the use of the Illumina Directional mRNA-Seq Library prep. V1.0 kit, without prior poly-A enrichment, according to the manufacturer's instructions (Pre-Release Protocol). The libraries were sequenced with Small RNA sequencing primer (part # 1001375) and the Single Read Sequencing Protocol.

***in vitro* RNA Methyltransferase assay:** These assays were performed in a total volume of 100 μl in 25 mM Tris-HCl, pH8, 150 mM NaCl, 2 mM KCl, 10% glycerol, 1 mM EDTA, 1mM DTT supplemented with EDTA-free Complete Protease Inhibitor cocktail from Roche and 80 U of RNaseOUT from Invitrogen with 1 μg of recombinant BCDIN3D, 4 μl of 20 μM RNAs and 4 μl of ^{3}H-SAM (PerkinElmer NET155250UC) for 2h at 37°C. The RNAs were purified using the Qiagen RNeasy MinElute Cleanup Kit

with a modified protocol that allows recovery of RNAs of all sizes (see above). Where indicated, the samples were separated on SDS-PAGE or Urea-PAGE gels, fixed in 45 % Methanol, 10 % Acetic Acid, treated for 30 min with Enlightning (PerkinElmer), dried and exposed on film at -80°C.

Terminator 5'-Phosphate-Dependent Exonuclease treatment coupled to qRT-PCR: 2 µg of total RNA was used per reaction. The Terminator treatment was performed in a total volume of 20 µl in 1X Terminator Reaction A buffer with 20 U of RNaseOUT from Invitrogen and 1 U of Terminator^{TM} 5'-Phosphate-Dependent Exonuclease from Epicentre for 2 h at 30°C. In the no Terminator treatment, water was used instead of the enzyme. The RNA was purified using the Qiagen RNeasy MinElute Cleanup Kit with a modified protocol that allows recovery of RNAs of all sizes (see above). After elution with 20 µl of water, 1 to 4 µl of the resulting RNAs were used for the subsequent reverse transcription and real time or semi-quantitative PCR steps.

BCDIN3D co-immunoprecipitation: 2.10^7 Hela-S3-FlpIn and Hela-S3-FlpIn-BCDIN3D-Flag cells grown at a density of $4\text{-}5.10^5$ cells per ml were used per Co-IP. The cells were washed twice with 25 ml of cold PBS, extracted with 0.6 ml of cold co-IP buffer (20mM HEPES pH7.5, 150mM NaCl, 20% glycerol, 0.1% NP40, 1mM EDTA, 0.1mM PMSF supplemented with EDTA-free Complete Protease Inhibitor cocktail from Roche) for 1 h at 4°C and cleared by centrifugation for 5 min at 13,000 rpm at 4°C. The supernatant was incubated for 4h with 40 µl of pre-washed anti Flag M2 conjugated beads (Sigma) at 4°C. The beads were washed 3 times with 0.5 ml of co-IP buffer, once with 0.5 ml of TBS, and eluted with 100 µl of TBS containing 150 ng/µl of 3xFlag peptide for 30 min at 4°C. The proteins were precipitated with TCA, resuspended in 20 µl

of 1x Laemmli Buffer and analyzed by Western Blot or Coomassie as indicated. For the treatments with RNase A, the co-IPs were performed in the presence of 1 of 5 µl of RNase A solution (R6148) from Sigma.

Western Blot: Proteins were separated in SDS-PAGE gel and transferred onto 0.45 µM nitrocellulose membrane in 1X Towbin Buffer with 20% Methanol and 0.02% SDS for 90 min at 300 mA. The membranes were blocked for 30-60 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 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 and analyzed on film or with the Chemidoc XRS+ system from Biorad. The Western Blots against BCDIN3D were performed according to the BCDIN3D antibody supplier's (Sigma) instructions. The catalogue and lot numbers of the commercially available antibodies used are listed in Supplementary Table 3.

RNA Immunoprecipitation. Cells grown in 10 cm diameter dishes were washed with 10 ml of PBS, fixed with 10 ml of 1% formaldehyde (Sigma) in PBS for 10 min and quenched with 20 mM Glycine for 5 min. Cells were washed 2 times with 10 ml of cold PBS, scraped with 2.5 ml of cold PBS supplemented with Complete Protease Inhibitor cocktail from Roche and pelleted by centrifugation. The pellet was suspended in 0.4 ml of cold RIPA Buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Sodium Deoxycholate, 0.05% SDS) supplemented with EDTA-free Complete Protease Inhibitor cocktail from Roche and 400 U/ml of RNaseOUT from Invitrogen.

The suspension was sonicated in 2 ml tubes for 5 minutes at High, 30 sec ON/OFF cycles in a cooled Bioruptor® (Diagenode) and cleared by centrifugation for 15 min at 13,000 rpm. The supernatant was incubated with the appropriate antibodies O/N at 4°C, then with 25 µl of pre-washed Protein G Dynabeads® (Invitrogen) for 3h at 4°C. The beads were washed 5 times on ice with 1 ml of High Stringency RIPA buffer (25 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM EDTA, 1 M Urea, 1% NP-40, 1% Sodium Deoxycholate, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride). The immuno-complexes were eluted from the beads with 100 µl of elution buffer (50 mM Tris–Cl, pH 7.0, 5 mM EDTA, 10 mM dithiothreitol (DTT), 1% SDS) for 1h at 70°C. The eluates were purified and DNase-treated with the miRNeasy Purification kit from Qiagen and the RNA was eluted from the columns with 50 µl of water.

in vitro **Dicer processing assays** 100 pmol of synthetic pre-miR-145 (5'OH, 5'P, 5'Pme1 or 5'Pme2) were incubated with 500 ng of recombinant human Dicer in a total volume of 15 µl in 100mM KCl, 10mM Tris-HCl, pH8, 0.1mM EDTA, 0.5 or 1 mM $MgCl_2$, 0.5 mM dTT supplemented with 0.5 U/µl RNaseOUT for the indicated times at 37°C. The samples were mixed with 15 µl of Gel Loading Buffer II (Ambion), heated for 15 min at 70°C and separated on a 15% Urea-PAGE. The gels were stained for 15 min with 10 µg/ml ethidium bromide and the signal was detected and analyzed with the Chemidoc XRS+ system from Biorad.

Bioinformatic Analysis:

(i) Mapping Total RNA from siBCDIN3D- and siNC-treated MCF7 cells were sequenced using an Illumina Genome Analyzer II, resulting in 14,558,834 and 13,512,809 reads respectively, each having a read-length of 41 bp. Reads were mapped to

the human reference genome (build 37) using TopHat (Trapnell et al., 2009), which incorporates mapping across exon-exon boundaries for RNA-seq data. The resulting genome mappings were filtered to remove non-unique mappings and reads mapped with 3 or more mismatches or gaps. This resulted in 1,163,459 and 1,276,255 unique mappings for the siBCDIN3D- and siNC-treated samples respectively. The relatively low proportion of reads with corresponding mappings is likely due to the presence of large quantities of ribosomal RNA present in the total RNA samples used for the library generation (see above). Mapped regions were extended to 200bp to more closely represent the fragment lengths.

(ii) Differential Expression The h19 knownGene table from the UCSC genome browser (http://genome.ucsc.edu/) contains genome coordinates for 77,614 predicted genes from a variety of resources (RefSeq, GenBank, CCDS and Uniprot). For each predicted gene, the number of reads per kilobase exon model per million mapped reads (RPKM) was calculated by dividing the total number of overlapping reads in the coding portion by the total number of coding bases (in kilobases), and then normalizing this by the total number of millions of reads generated for each sample. The RPKM value was used as a proxy for the level of transcription. Differential expression between siBCDIN3D- and siNC-treated samples was calculated using the DESeq package in R (Anders and Huber). Genes showing a significant change in expression were selected based on a fold-change greater than 2 between the two samples and a p-value from the negative binomial model fitting less than 0.05, an mRNA length greater than 500 bp and a normalised read count greater than 100 for at least one of the samples.

(iii) Identification of miRNA binding to the 3'-UTR of known genes. Potential miRNA target predictions were taken from the microRNA.org repository (http://www.microRNA.org), which used an updated version of the miRanda algorithm to find miRNA binding sites in known genes (Betel et al., 2008). The August 2010 release of human target site predictions (human_predictions_S_C_aug2010.txt) was used. All entries with an alignment score less than 150 and a mirSvr score greater than -0.5 were removed to avoid potentially false binding sites. Genes were partitioned into classes based on whether or not the fold change was significant, and whether or not at least one miRNA was detected with a potential binding site in the 3' UTR of the gene. The significance of whether genes with significant fold change (up, down, or either) were enriched for potential regulation by miRNAs was calculated by performing a Fisher's Exact Test on the 2x2 contingency table. miRNAs significantly enriched within the 3' UTR of significantly changing genes (up, down, or either) were identified by estimating an expected count based on the proportion of occurrences across all non-significant genes and performing a chi-squared test on the observed counts.

microRNA profiling: Total RNA from MCF-7 cells treated with siBCDIN3D or siNC from two fully independent experiments were submitted to miRNA profiling by miRCURY LNA™ Universal RT miRNA PCR Human panel I and II v2 (742 assays total) from Exiqon. The integrity of RNA was verified by electrophoresis on a Agilent Total Eukaryotic RNA Nano Chip. Furthermore, the RNA quality control by qPCR did not detect presence of inhibitors in the samples. All microRNAs were polyadenylated and reverse transcribed into cDNA in a single reaction step. cDNA and SYBR Green

mastermix were transferred to qPCR panels preloaded with primers, using a pipetting robot. Amplification was performed in a Roche Lightcycler480. Raw crossing point (Cp) values and melting points, as detected by the Light cycler software, were exported. The datasets were cleared from: (i) reactions with several melting points; (ii) reactions with melting points that were not within assay specifications; (iii) reactions with amplification efficiency below 1.6; (iv) reactions giving Cp values that were within 5 Cp values of the negative controls reaction; (v) reactions with Cp>37. In addition, only the assays that showed good quality signal in all samples were further analyzed. Each assay within a sample was then normalized to the average of the assays within the sample with the formula:

Normalized Cp = average(n=110) – assay Cp

The averages and variances for siBCDIN3D and siNC groups were calculated, and in order to eliminate false positives, the assays with a variance higher than 50% of the absolute average were discarded. Then, the average Cp of the siNC group was subtracted from the average Cp of the BCDIN3D group with the formula:

ddCp= Normalized Cp[siNC]- Normalized Cp[siBCDIN3D]

The resulting values represent the difference in miRNA expression (log2 scale), with positive values representing up-regulation in siBCDIN3D compared to siNC and negative values down-regulation.

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Betel, D., Wilson, M., Gabow, A., Marks, D.S., and Sander, C. (2008). The microRNA.org resource: targets and expression. Nucleic Acids Res *36*, D149-153.

Trapnell, C., Pachter, L., and Salzberg, S.L. (2009). TopHat: discovering splice junctions with RNA-Seq. Bioinformatics *25*, 1105-1111.

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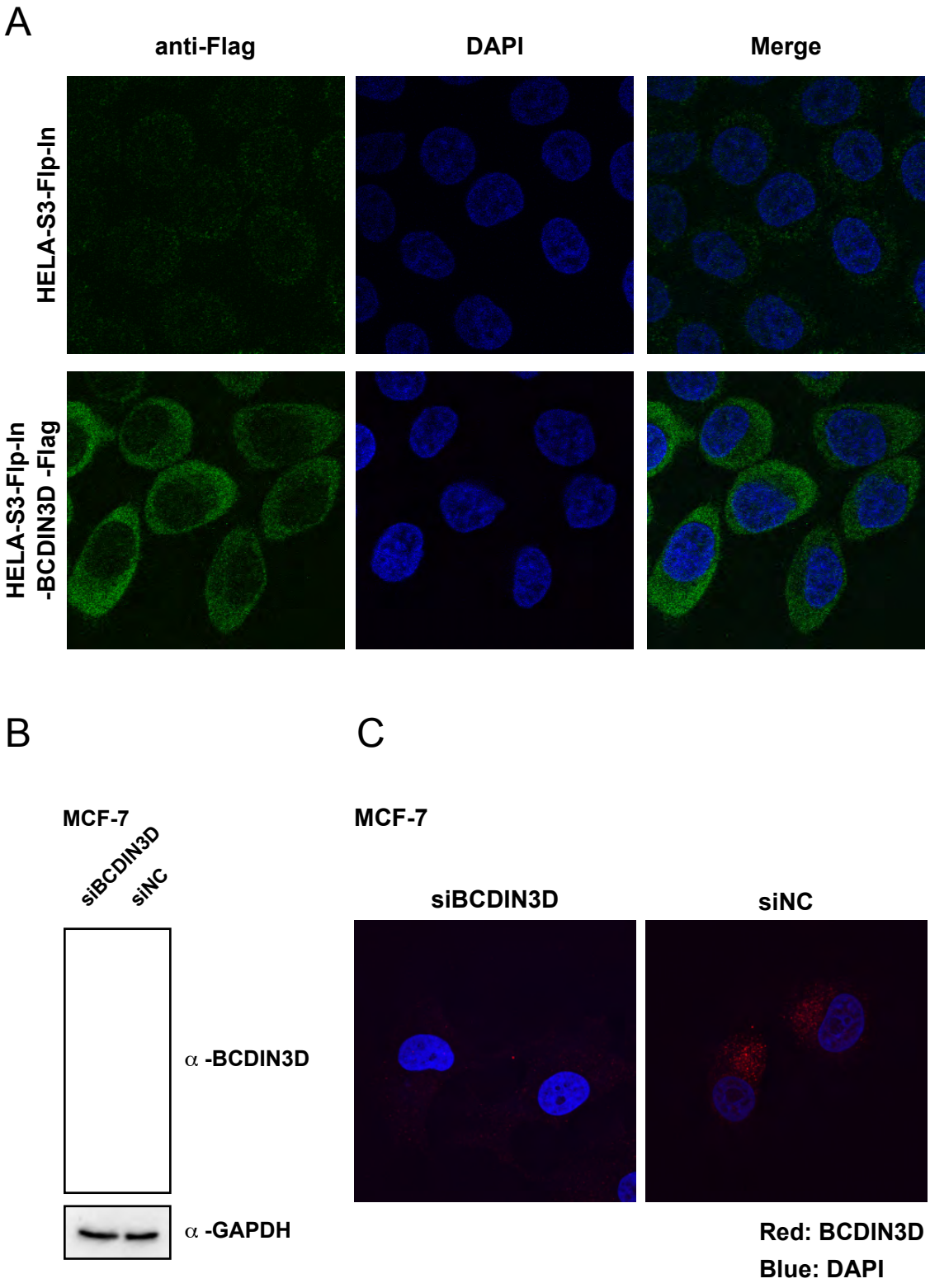


Figure S1, Xhemalce et al.

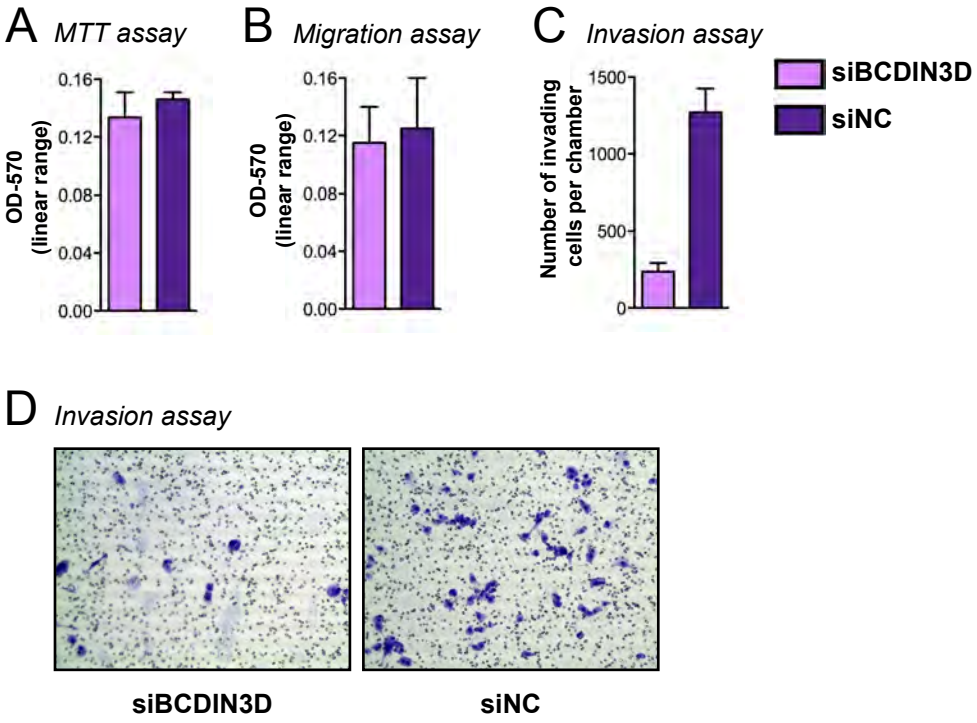


Figure S2, Xhemalce et al.

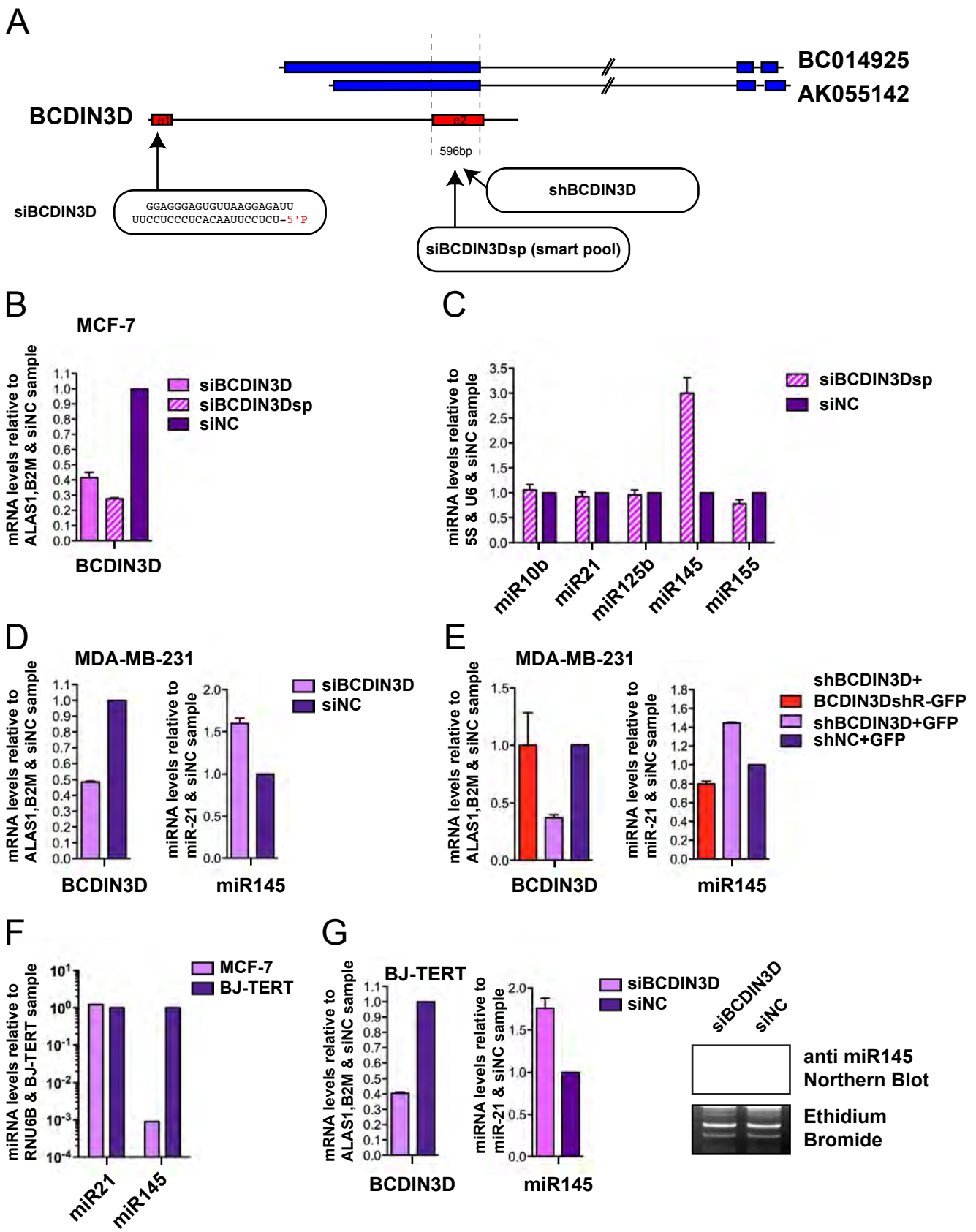
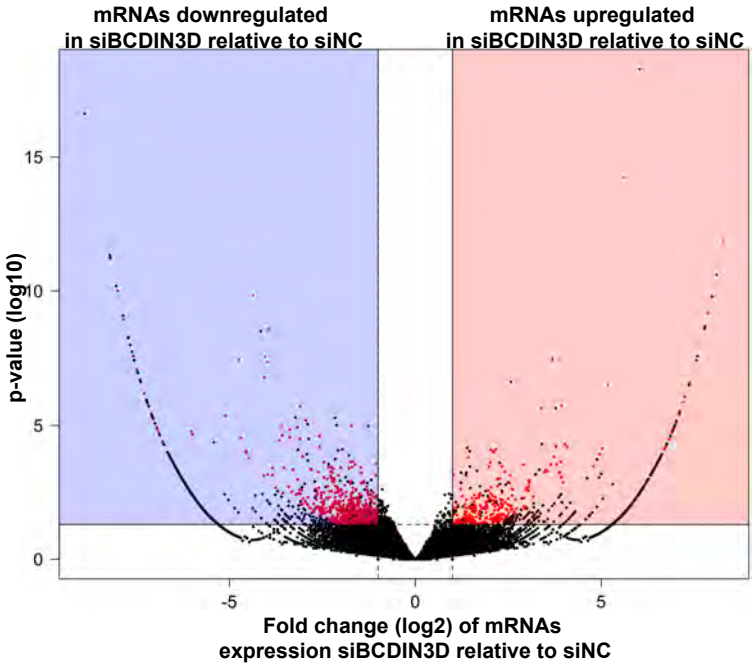


Figure S3, Xhemalce et al.

A



B

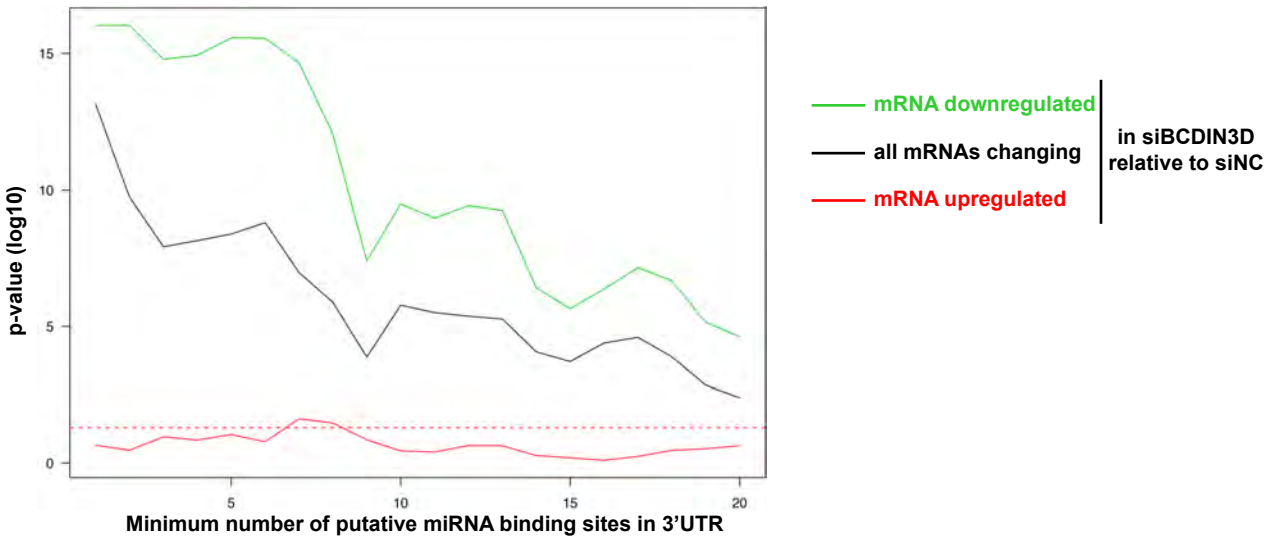


Figure S4, Xhemalce et al.

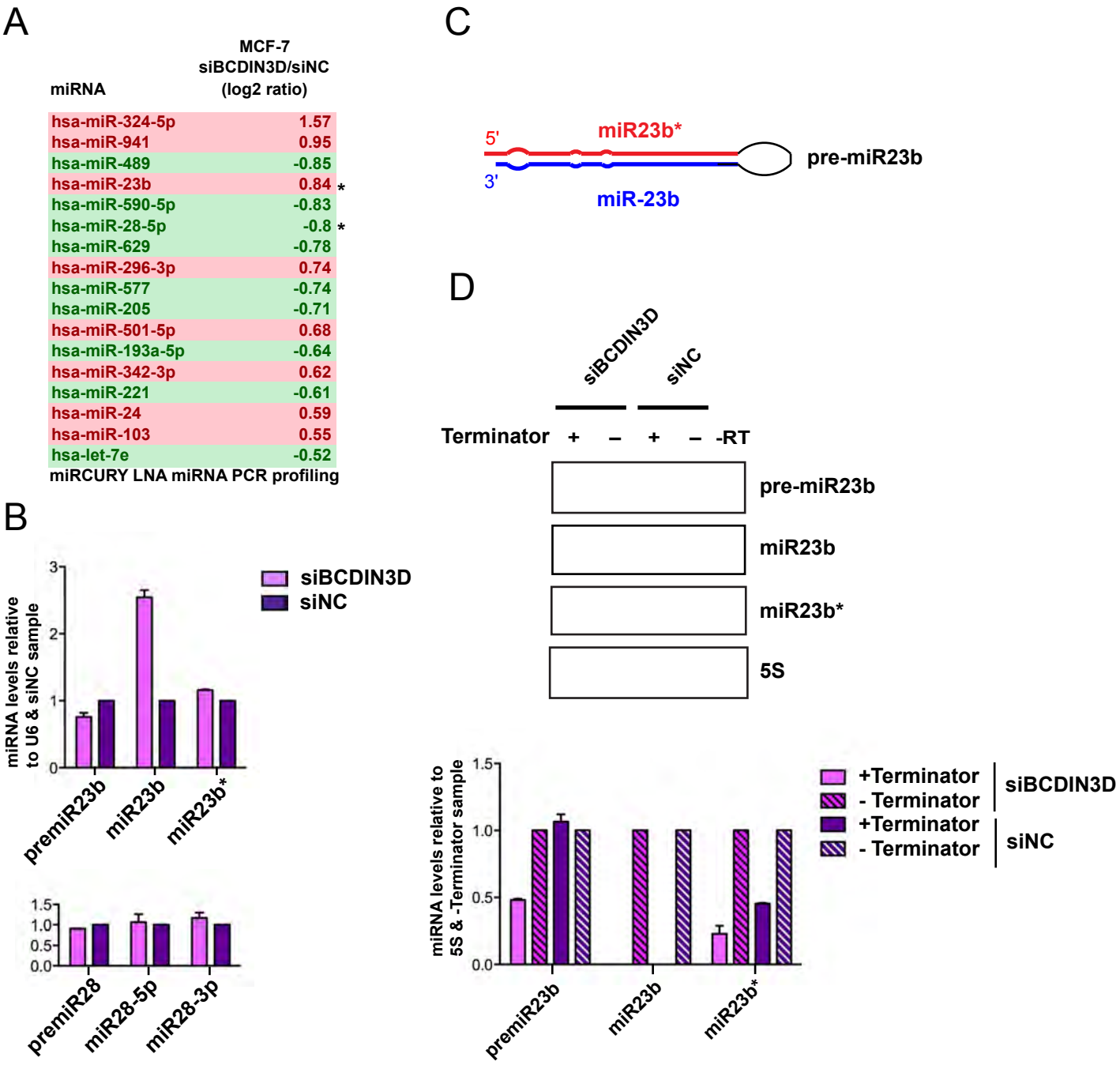


Figure S5, Xhemalce et al.

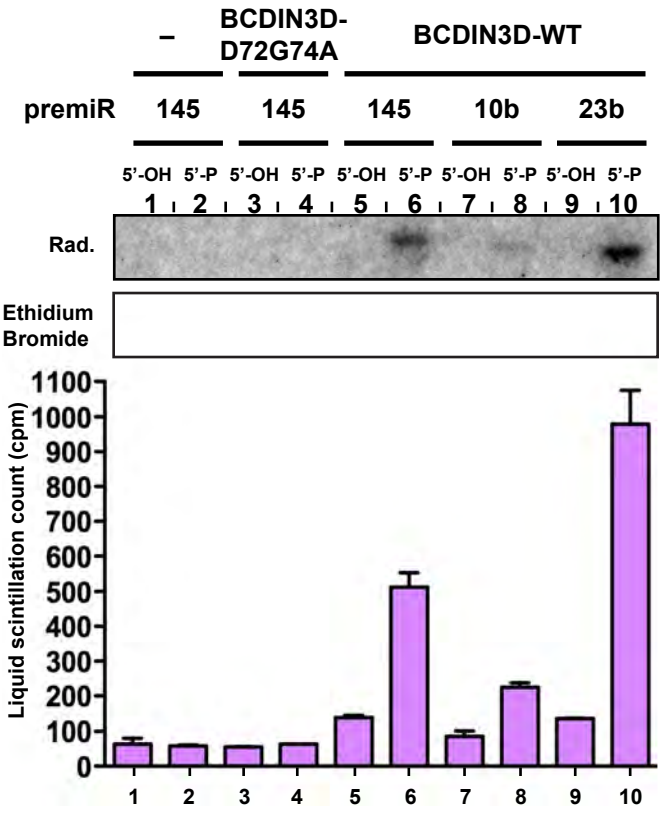


Figure S6, Xhemalce et al.

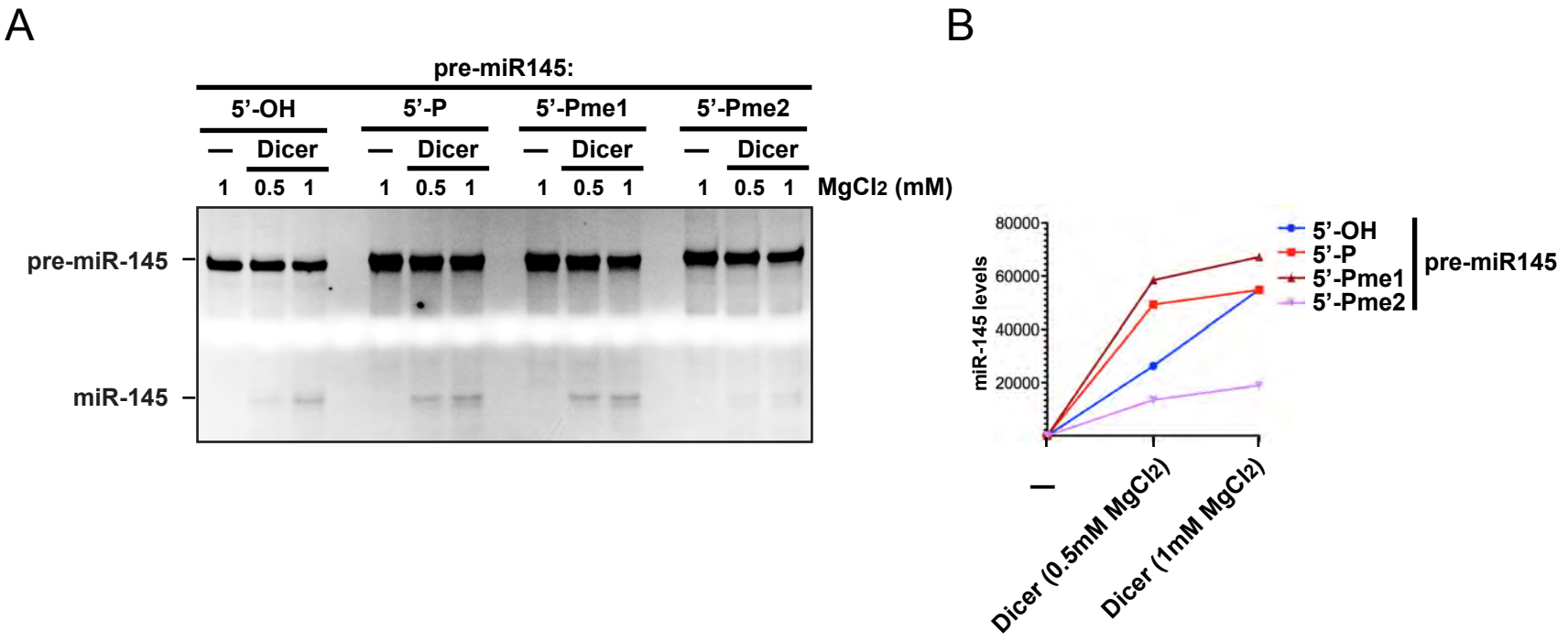


Figure S7, Xhemalce et al.