Supplemental Materials

Molecular Biology of the Cell

Ghosh et al.

Supplemental Information

Polysome arrest restricts miRNA turnover by preventing their exosomal export in growth retarded mammalian cells

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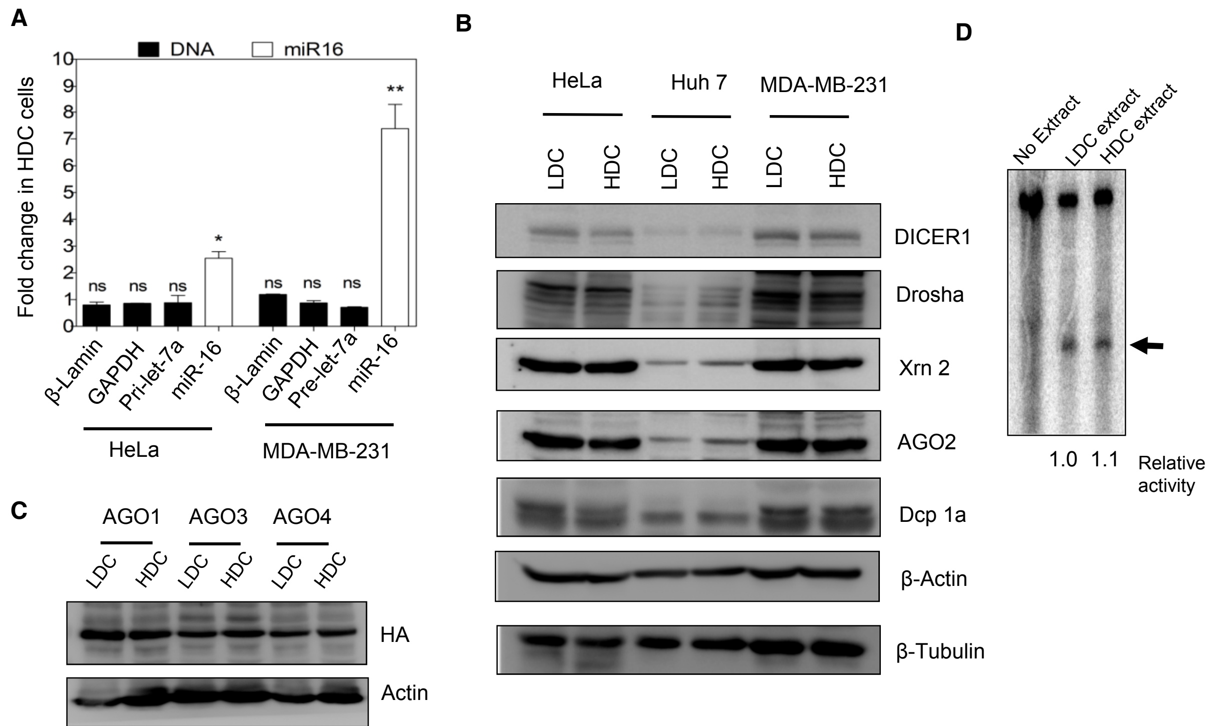

Figure S1. Effect of cell density on cellular content of DNA, AGO and related proteins in mammalian cells. (a) Real time based quantification of DNA content by estimation of DNA encoding β-Lamin, GAPDH and Precursor Let-7a from total DNA isolated from HeLa and MDA-MB-231 cells. Cells were grown to HDC and LDC states before total DNA was isolated from each cell. Graph represents the relative level of DNA in HDC states, considering LDC state DNA levels as 1. Change in miR-16 level, normalized to cell number, is used as positive control (B) Western blot detection for proteins involved in miRNA functional pathway. B-Tubulin, β-Actin levels serve as internal controls for cell number of each cell type and growth. (C) Western blot for N-HA tagged AGO1,AGO3 and AGO4 in LDC and HDC state MDA-MB-231 cells. (D) *In vitro* Dicer1 activity measurement in cleavage assay using pre-miR 122 as substrate. FLAG-DICER1 transfected HeLa cells were grown to HDC and LDC states and immunoprecipitated with Anti FLAG antibody coupled agarose beads. Equivalent amounts of DICER1 obtained from each growth state were used to cleave a precursor miR-122 in an *in vitro* cleavage assay. The cleaved product is indicated with an arrow (→). All experiments were performed minimum three times. ns; non-significant, *;P<0.01, **; P<0.001, ***; P<0.0001.

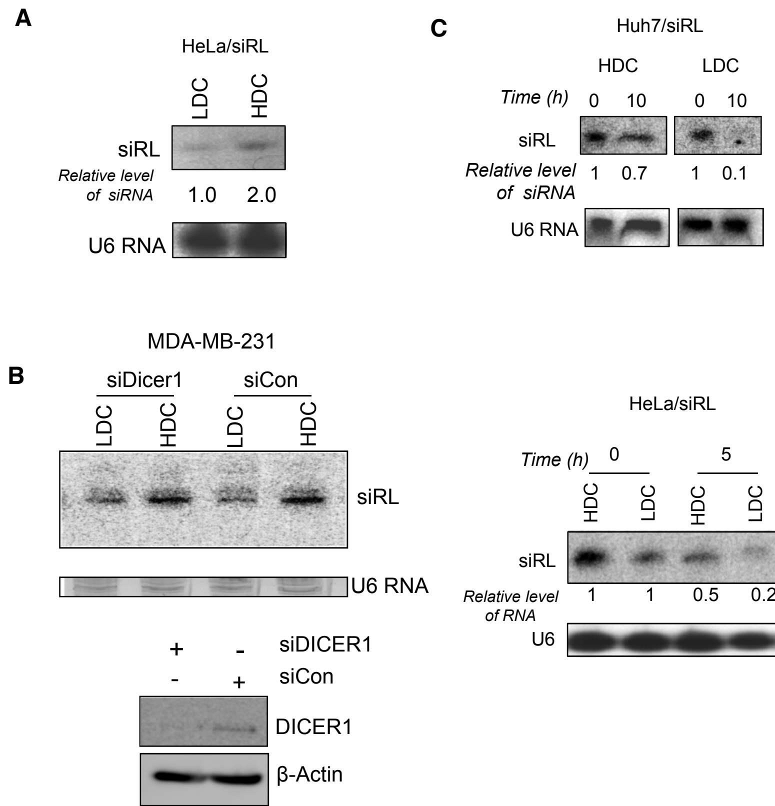

Figure S2. Exogenously administered siRNA has higher stability in HDC state cells. (A). Northern blot was done for siRL, a siRNA against RL mRNA, in transfected HDC or LDC HeLa cells after 48 h of transfection. U6 was used for normalization and relative amount of siRL are mentioned below the Northern blot picture. (B) Relative high siRL level in HDC stage cells is independent of DICER1. Level of siRL was measured in HDC and LDC stage control and DICER1 depleted MDA-MB-231 cells. U6 Rna as loading control. (C) Stability measurement of siRL in LDC states of Huh7 and HeLa cells. Time points indicated are intervals at which cells were lysed for extraction of RNA after a minimum 48 h of siRL transfection (set as start point; 0h). U6 snRNA levels serve as an endogenous control. Numbers indicate the relative levels of residual siRL at each time points.

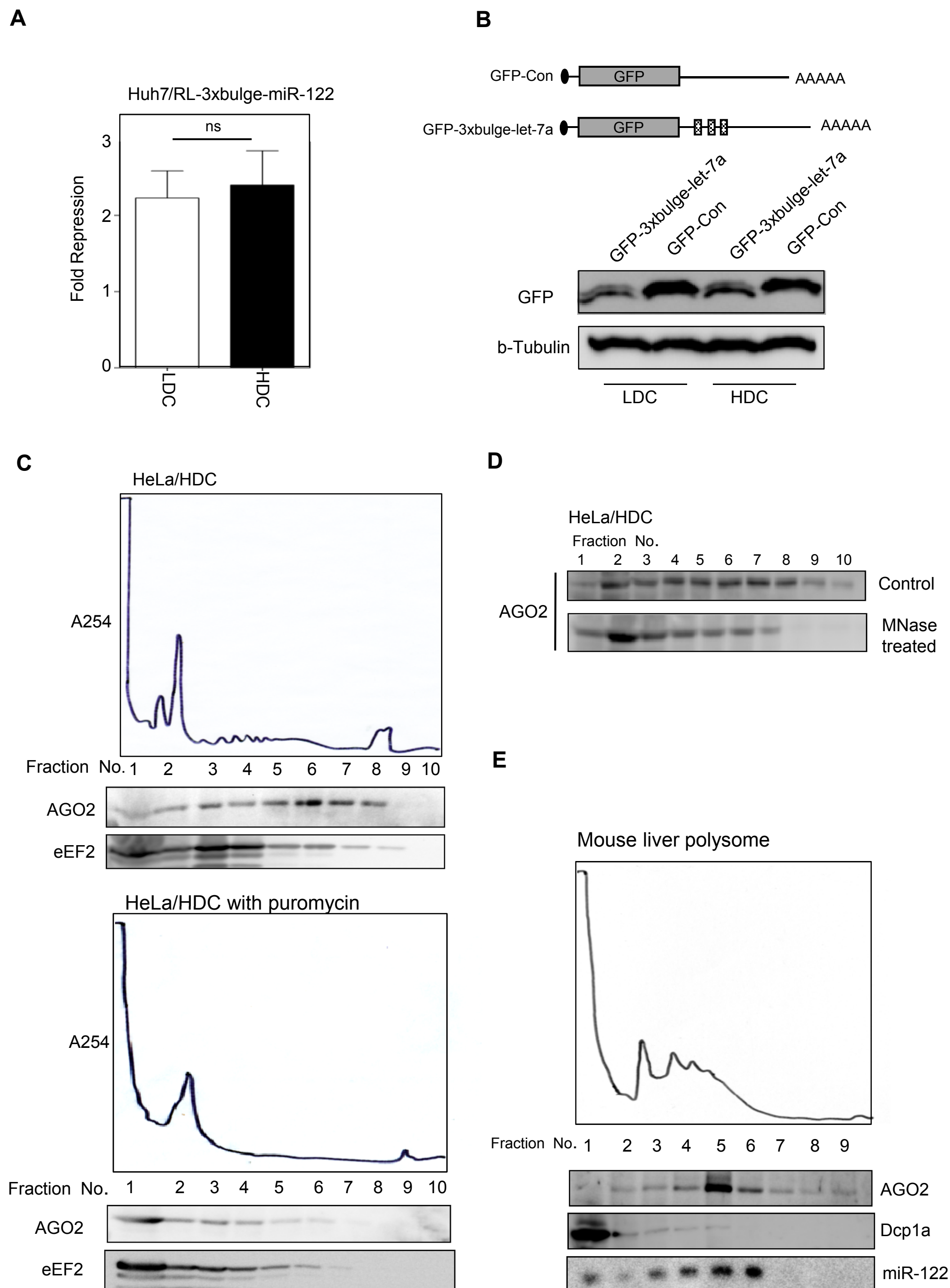

Figure S3. Comparable translation repression of miR-122 and let-7a reporters in cells grown to HDC and LDC states. (A) Fold repression of a miR-122 reporter in HDC and LDC state Huh7 cells (B) Western blot of GFP to detect expression of GFP from control and a let-7a reporter in HeLa HDC and LDC states. (C) Effect of puromycin on polysomal distribution of AGO2. HDC HeLa extracts were either untreated or treated with puromycin before it was run on a sucrose density gradient. The absorbance at 254 nm was plotted and the fractions were collected. Presence of AGO2 and eEF2 in individual fractions were detected by western blotting. (D) Effect of nuclease treatment on polysomal AGO2 distribution in HDC HeLa cells. Lysate of HDC HeLa cells treated with Micrococcal nuclease (MNase) was resolved on a 15-55% sucrose gradient and fractions western blotted for AGO2 levels. (E) Polysome profile and distribution of miR-122, Ago2 and Dcp1a in live lysate of adult Balb/C mouse .

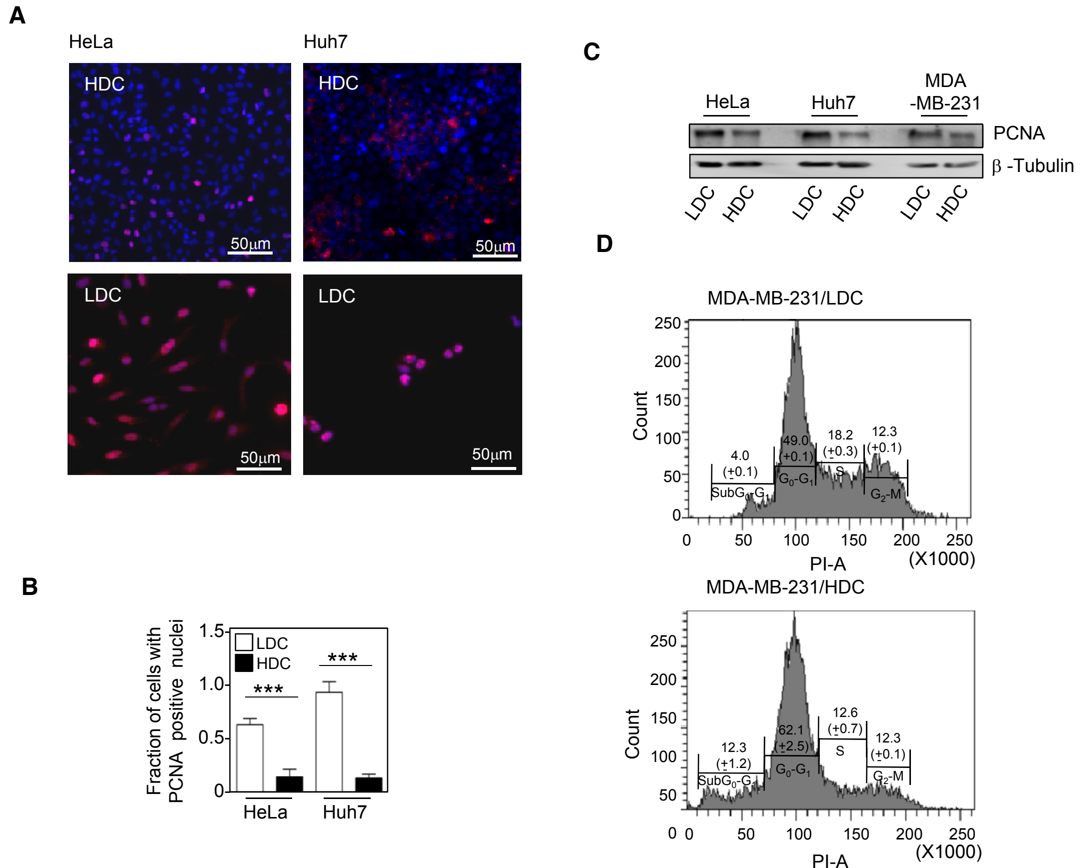

Figure S4. Lesser number of proliferative cells in HDC state human cells. (A-C) Loss of nuclear localization of proliferating cell nuclear antigen (PCNA/Mitosin) protein in HDC human cells. The HDC and LDC cells were stained for PCNA (Red) and DAPI (Blue) in HeLa (P=0.0008) and in Huh7 cells (P<0.0001) (A) and quantification of PCNA-positive nuclei were done (mean +/- s.e.m., n=3 independent experiments) (B). The protein extracts prepared from the cells were western blotted for PCNA. A low molecular weight cross reacting band of PCNA blot and β Tubulin were used as loading controls (C).(D) Accumulation of cells at sub G_0 stage in HDC culture of HeLa cells. Histogram analysis of propidium iodide (PI) stained LDC and HDC MDA-MB-231 cells. The estimated mean values are based on three independent experiments to note the number of cells in different phases of cell cycle.

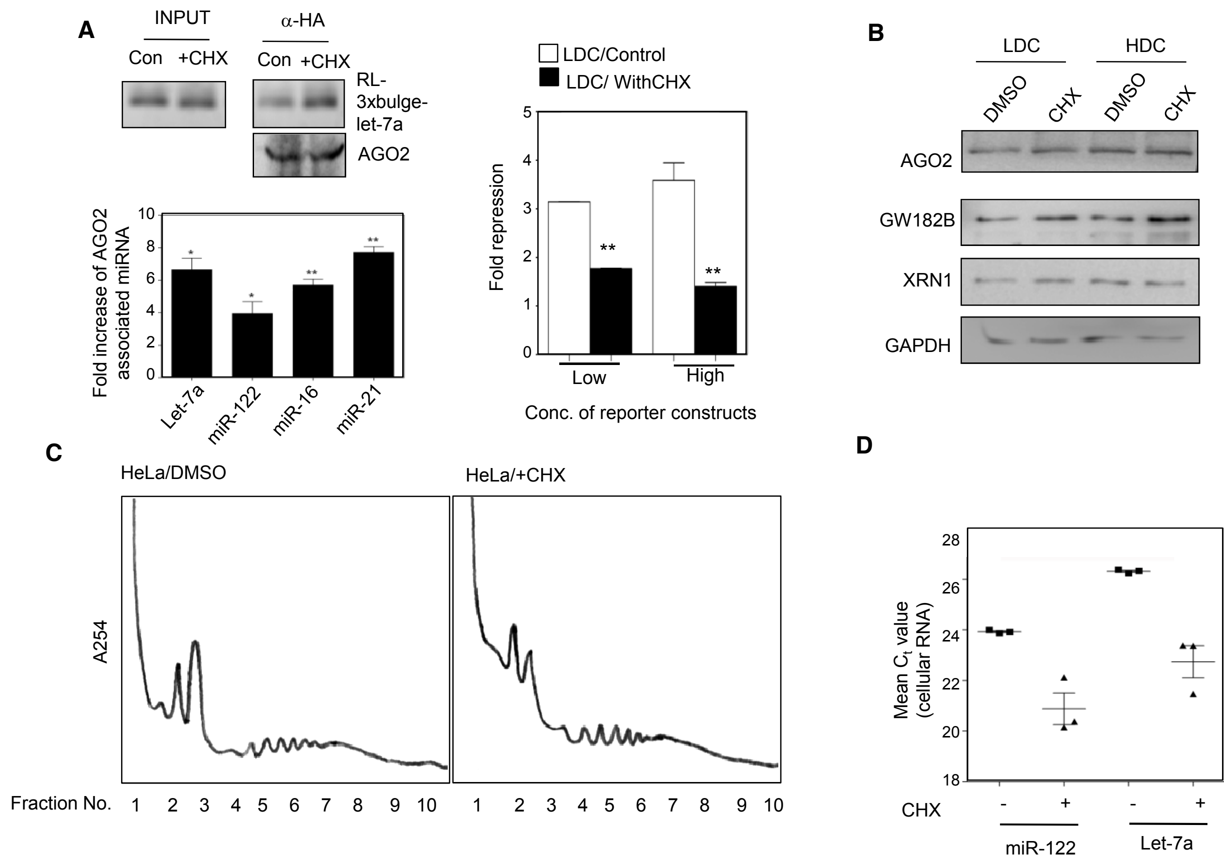

Figure S5. Retarded protein translation increases miRNA level in human cells. (A) Semiquantitive RT-PCR based detection of AGO2 associated let-7a target messages in control and CHX treated cells. Immunoprecipitation of FLAG-HA-AGO2 from DMSO or CHX-treated LDC HeLa cell extracts was verified by Western blot analysis (*left upper panels*). Level of AGO2 associated three endogenous and one exogenously expressed miR-122 in CHX treated HeLa cells (*left bottom panel*). Relative repression level of RL-3xbulge-let-7a reporter in DMSO and CHX treated LDC HeLa cells (*right panel)* (B) Western blots of the proteins associated with miRNA function in CHX-treated and untreated LDC and HDC HeLa cells. GAPDH serves as a loading control. (C) Polysome profiles of cell extracts prepared from HeLa cells treated with CHX or DMSO for 16h duration as described in panel (A). (D) CHX treatment increases miRNA association with polysomes. Real time analysis of miRNA content (represented by C_t values) in polysomes isolated from HeLa cells expressing miR-122 mimic or endogenously expressed let-7a in control or CHX treated cells Low and High stands as 25 and 100 ng of plasmids used for transfection of cells present per well of a six well plate. Results depict mean values from at least three experiments.

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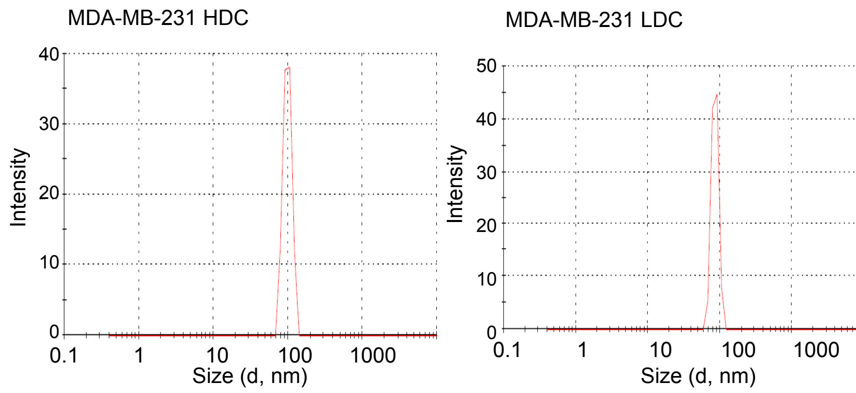

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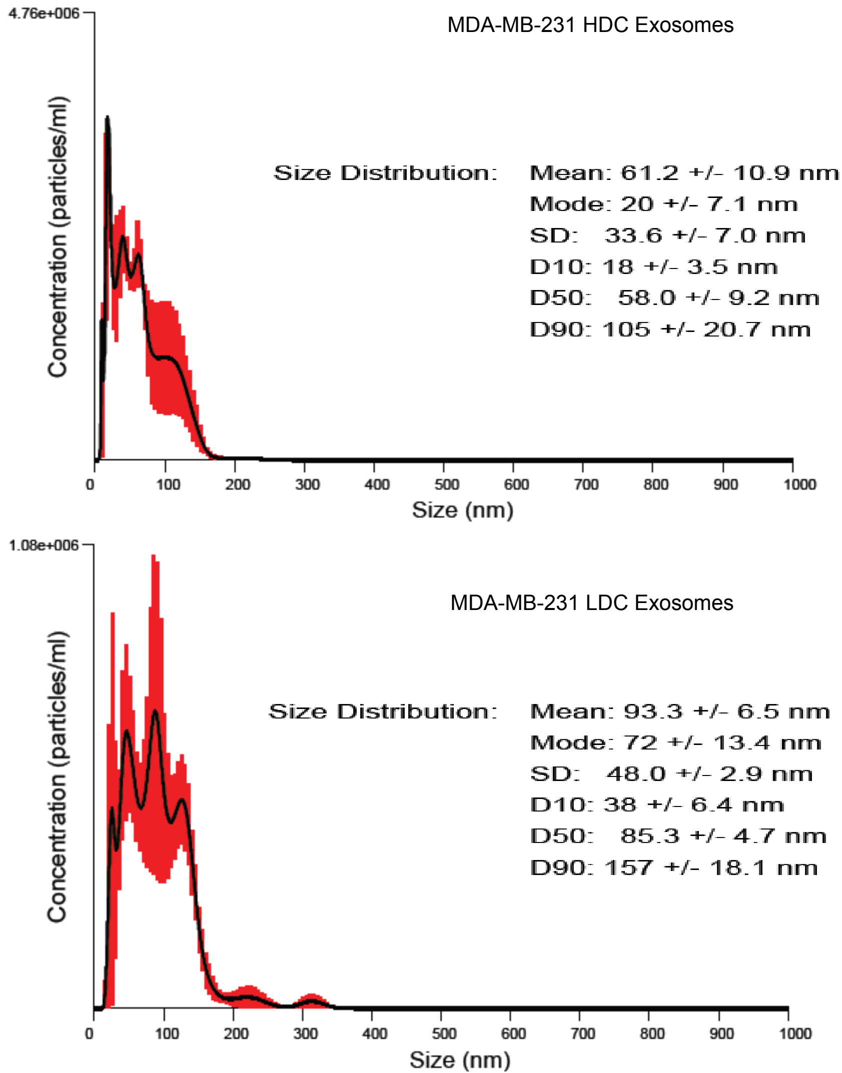

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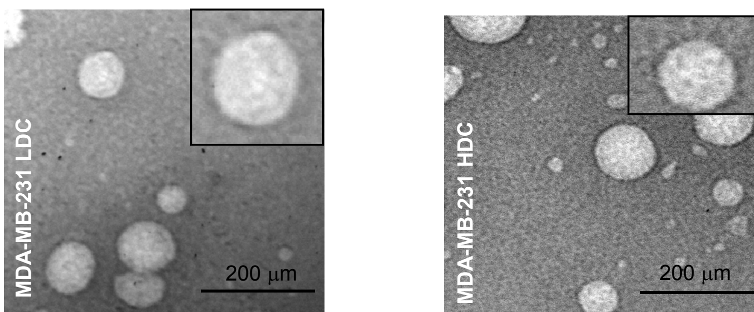

Figure S6.
Characterization of exosomes from HDC and LDC cells. (a) Dynamic light scattering analysis of purified exosomes from MDA-MB-231 cell supernatants grown to LDC and HDC states.Mean histogram profiles reveal the avarege diameter of the exosomal population from each growth type. (b) Nanoparticle Tracking Analysis (NTA) for automatic tracking and size estimation of LDC and HDC cell derived exosomes. Results are displayed as a frequency size distribution graph from three independent analyses. (c)Transmission Electron microscope imaging for visualization of exosomes released b y HDC and LDC MDA-MB-231 cells.

Supplemental Table 1 List of Plasmids and siRNAs

Name of Plasmid	Reference/Source	Plasmid Description
pRL-3xbulge-let-7a	From Witold Filipowicz (Pillai *et al.,* 2005)	Three let-7a binding sites downstream of Renilla Luciferase (RL) coding region.
pRL-Con	From Witold Filipowicz (Pillai *et al.,* 2005)	Humanized Renilla Luciferase coding region.
pRL-3xBulge-miR-122	From Witold Filipowicz in (Bhattacharyya *et al.,* 2006)	Three miR-122 binding sites downstream of Renilla Luciferase (RL) coding region.
pGL3FF	From Promega	Firefly Luciferase (FL) under SV40 promoter
pRL-per-let-7a	From Witold Filipowicz (Pillai *et al.,* 2005)	One let-7a perfect binding sites downstream of Renilla Luciferase (RL) coding region.
RL-HMGA2 3'UTR	From Anindya Dutta (Lee and Dutta 2007)	3 Kb wild type 3' UTR of the HMGA2 gene with intact miRNA binding sites cloned downstream of the RL in pRL-con plasmid.
RL-HMGA2 3'UTR mut	From Anindya Dutta (Lee and Dutta 2007)	Mutated let-7 binding sites in the HMGA2 3'UTR cloned downstream of the pRL-con plasmid.
FLAG-HA-AGO2 (FH-AGO2)	From Tom Tuschl(Meister *et al.,* 2004)	Expressing FLAG & HA tagged human AGO2
GFP-3xbulge-let-7a		GFP replacing RL in pRL-3xbulge-let-7a
NHA-GW182B, NHA-AGO2, NHA-AGO3, NHA-AGO4	From Witold Filipowicz(Zipprich *et al.,* 2009)	NHA tagged version of GW182B and other proteins
GFP-AGO2	From Witold Filipowicz (Cougot *et al.,*, 2008)	AGO2 cloned in frame with GFP in peGFP-C2 (Clontech)
pCIneo-let-7a	As described by Belasco, J.G.(Wu *et al.,* 2006)	Plasmid expressing pre-let7a, let-7a coding region was amplified and cloned in pCIneo vector
pmiR-122	As described by Chang J. (Chang *et al.,* 2004)	Plasmid encoding pre-miR-122 under a constitutive U6 promoter
miR 122 mimic and let-7a mimic	PM11012 and PM10050	Ambion Pre miR miRNA Precursors
siRL	Synthesized from Eurogentech	siRLsense strand GCGAGAUCCCUCUCGUUAATT siRLantisense strand UUAACGAGAGGGAUCUCGCGG
si All Star Negative	Qiagen	

Supplemental Table 2 List of primers used

Target	Forward Primer	Reverse Primer
18S rRNA	5' TGACTCTAGATAACCTCGGG 3'	5' GACTCATTCCAATTACAGGG 3'
Pre-Let7a	5’ AATGGCCCAAATAGGTGAC 3’	5’CCATCAATCTAGTGACCCC 3’
RL Reporters	5' CCAAGCAAGATCATGC 3'	5' GCTCTTGATGTACTTACCC 3'
GAPDH	5’ AAAAGCGGGGAGAAAGTAGG 3’	5’ AAGAAGATGCGGCTGACTGT 3’
Pri-Let-7a	5'-CAAGCAGGCGATTGGTGGA-3'	5'-GACGCAGCTTCGAAGAGTTCTGTC-3'
Lamin B	5’ TCAGGGTTGCTGGTTTTTCT 3’	5’ CTTGAGTCCGAGGTTCCAAA 3’

Supplemental Table 3 List of Antibodies used

Name of Antigens	Raised in	Source	Dilutions used for Western Blot
Hsp90	Rabbit Monoclonal	Cell Signalling Technology	1,1000
β-Actin	Mouse Monoclonal (HRP conjugated)	Sigma Aldrich	1,10000
HA	Rat Monoclonal (clone 3F10)	Roche	1,1000

GFP	Mouse Monoclonal (clone 7.1 and 13.1)	Roche	1,1000
AGO2 (eIF2C2)	Mouse Monoclonal	Novus Biologicals	1,500
XRN1	Rabbit	Bethyl Laboratories	1,10000
RCK/p54	Rabbit	Bethyl Laboratories	1,10000
Dcp1a	Mouse Monoclonal	Novus Biologicals	1,1000
β Tubulin	Mouse	Sigma	1,1000
GW182	Rabbit	Bethyl Laboratories	1,1000
eEF2	Rabbit	Bethyl Laboratories	1,5000
Dcp2	Rabbit	Bethyl Laboratories	1,5000
Phospho 4E-BP1	Rabbit	Cell Signal Technology	
Dicer	Rabbit	Bethyl Laboratories	1,5000
Xrn2	Rabbit	Bethyl Laboratories	1,5000
Drosha	Rabbit	Bethyl Laboratories	1,5000
GAPDH	Mouse Monoclonal	Sigma	1,30000
HRS	Rabbit	Bethyl Laboratories	1,2000
Alix	Mouse	Santa Cruz	1,200
CD 63	Mouse	BD Pharmingen	1,1000
Ribosomal S3	Rabbit	Cell Signalling	1,1000

Supplemental Materials and Methods

Immunoprecipitation (IP) and analyses of mRNA and miRNA by qRT-PCR

Immunoprecipitation (IP) of AGO2 and subsequent analysis of the IPed materials for RNA and proteins were done as per published procedures described earlier (Bhattacharyya *et al.,* 2006; Kundu *et al.,* 2012). For IP reactions, FLAG-HA-AGO2 expressing cells were lysed in Lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT), 1% Triton X-100 and 1X EDTA-free protease inhibitor cocktail (Roche) for 30 min at 4º C. The lysates, clarified by centrifugation, were treated with Calbiosorb (Calbiochem) to remove detergent and were incubated with pre-blocked anti-FLAG M2 affinity gel (Sigma; A2220), overnight at 4º C. Subsequently, the beads were washed thrice with IP buffer (20 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT), and the bound proteins were analysed by western blot. Parallely from half of the materials, separated during washing steps, RNA was extracted with Trizol LS (Invitrogen) followed a by DNase I treatment (Invitrogen) to remove residual DNA contamination. Real time analyses by two-step RT-PCR was performed for quantification of miRNA and mRNA levels on a 7500 REAL TIME PCR SYSTEM (Applied Biosystems) or Bio-Rad CFX96™ real time system using Applied Biosystems Taqman chemistry based miRNA assay system. mRNA real time quantification was generally performed in a two step format using Eurogentec Reverse Transcriptase Core Kit and MESA GREEN qPCR Master Mix Plus for SYBR Assay with Low Rox kit from Eurogentec following the suppliers' protocols. The comparative C_t method which typically included normalization by the 18S rRNA levels for each sample was used for relative quantitation.

miRNA assays by real time PCR was performed with 25-200 ng of total RNA unless specified otherwise, using specific primers for human let-7a (assay ID 000377), human miR-122 (assay ID 000445) human miR-16 (assay ID 000391). U6 snRNA (assay ID 001973) was used as an endogenous control. One third of the reverse transcription mix was subjected to PCR amplification with TaqMan® Universal PCR Master Mix No AmpErase

(Applied Biosystems) and the respective TaqMan® reagents for target miRNA. Samples were analyzed in triplicates. The miRNA levels were defined from the cycle threshold values (C_t) for representation of exosomal miRNA or immunoprecipitated miRNA levels. The comparative C_t method which included normalization by the U6 snRNA or a non-relevant miRNA, for each sample was used for all other instances.

in vitro DICER activity assay

The Dicer activity assay was carried out with a synthetic human premiR122 (5'-UGGAGUGUGACAAUGGUGUUUGUGUCUAAACUAUCAAACGCCAUUAUCACACUAAAU A 3') 5' end labelled using [γ- 32P] ATP and T4 polynucleotide kinase. HeLa cells transiently expressing FLAG-DICER were grown to HDC and LDC states and FLAG-DICER1 immunoprecipitated with anti-FLAG M2 Agarose beads (SIGMA). The washed FLAG-DICER1 beads were incubated with 10 nM radiolabelled pre-miR122 at 37°C for 1h in DICER1 reaction buffer, and the products were analyzed on a 12% denaturing (8 M Urea) polyacrylamide gel and visualized in a phosphoimager.

Cell cycle analysis

Cells were analyzed for their DNA content with the BD Cycletest Plus reagent kit. Briefly, the procedure involved permeabilizing the cell membrane with a nonionic detergent, eliminating the cell cytoskeleton and nuclear proteins with trypsin, digesting the cellular RNA with Ribonuclease A, and stabilizing the nuclear chromatin with spermine (BD Biosciences). Nuclei are stained with Propidium Iodide solution provided and analyzed in a BD FACS CALIBUR instrument.

DNA isolation

Total DNA content of cells was recovered with Wizard® Genomic DNA Purification Kit as per manufacturers protocol (Promega)

Transmission Electron microscopic imaging of MDA-MB-231 exosomes

Exosomes of MDA-MB-231 cells obtained after ultracentrifugation of cell culture supernatants from $2x10^6$ cells were resuspended in 500 µl PBS and 5µl of the same was spotted onto Formvarcoated grids (200 mesh). The exosomes were then directly visualized. Grids were examined by a FEI Technai G2 Spirit BioTWIN electron microscope at 100 kV.

Nanoparticle tracking analysis

MDA-MB-231 exosomes (from approx. ~2 x 106 cells) isolated by ultracentrifugation at 100,000xg were resuspended in 1 ml of PBS and were used for NTA. 1,10 diluted sample was then resuspended in 700 µl of PBS and was loaded into the sample chamber of an NS500 unit (Nanosight, Amesbury, UK) and three videos of 30 seconds were recorded for each sample. Data analysis was performed with NTA 2.3.5 software (Nanosight). Data are presented as the average and standard deviation of the three video recordings of each sample.

Dynamic light scattering of Huh7 exosomes

Exosomes of MDA-MB-231 cells obtained after ultracentrifugation of cell culture supernatants were resuspended in 1000µl PBS. Dynamic Light Scattering was then performed in a Malvern Zetasizer Nano (model Zen 3600). The operating procedure was programmed (using the DTS software supplied with the instrument) such that there are average 20 runs, each being averaged for 10 s, and a particular hydrodynamic radius (R_h) is computed in each case and ultimately the result is presented as the distribution of R_h

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SUPPLEMENTAL REFERENCES

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