Nucleolin-aptamer therapy in retinoblastoma: Molecular changes and mass spectrometry based imaging

1. Supporting information

1.1. SI MATERIALS AND METHODS

RB tumor cells, cell lines –RNA extraction and qPCR

Real time PCR was performed to check the expression levels of NCL in RB tumors vs. normal retina and between the cell lines used in the study. RNA extraction from the tumor cells, normal retina, Y79, WERI-Rb1 and MIO-M1 cell lines was performed using Trizol based method followed by cDNA synthesis using Verso cDNA synthesis kit (ABgene, Epsom, Surrey, United Kingdom) with oligo dT primers and 1 μg of the total RNA, qPCRs were performed using SYBR green based method using ~ 50 ng of cDNA for NCL and β2M, β2-micoglobulin. The thermal profiles are initial denaturation at 95 °C for 10 min, cycle denaturation for 95 °C for 10 s, annealing and extension at 60 °C for 1 min. Data collections were set at extension step as well in the melt curve step. The quantitative PCR was performed in Applied Biosystem 7500 by using Sybr-green Dynamo HS master mix (Thermo Scientific, Mumbai, India). The primer sequences for NCL and β2M were as follows; FP: 5'-GACTTCAACAGTGAGGAGGATGCCA-3'and RP: 5'-CAAAGCCGCCTCTGCCTCCAC-3' for NCL and FP: 5'-TATCCAGCGTACTCCAAAGA-3' and RP: 5'-GACAAGTCTGAATGCTCCAC-3' for β2M. Other primers used in the study were listed in Table S2. Comparative quantification (normal retina vs. primary RB tumors or cell lines) was determined using the formula $2^{-\Delta\Delta Ct}$ and relative expression values normalized to the β2M endogenous control were used for plotting. Experiments were performed in triplicate for each sample.

Expression of nucleolin on tumors and cell lines

The expression level of NCL was studied by immunohistochemistry (IHC) of the RB tissue sections and normal retina, flow cytometry of the respective cells and by Western blotting of the nuclear and cytoplasmic fractions extracted from the RB and normal retina (NR) cells. IHC was performed on de-paraffinized tissue section, post-antigen retrieval by pressure cooker method, using anti-nucleolin antibody (sc-56640, Santacruz biotech) at 1:200 dilution with Novolink minpolymer detection system (Leica biosystems). Briefly, post-primary block, followed by polymer incubation for 90 min followed by diaminobenzidine (DAB) chromogen based detection. Slides were mounted and observed under light microscope. To check the expression levels of NCL in nuclear and cytoplasmic compartments of RB and NR cells, nuclear and cytoplasmic proteins were extracted following the manufacturer's instruction using Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, USA). Western blotting was performed on nuclear and cytoplasmic lysate for NCL (anti-nucleolin, ab13541, Abcam) and β-actin (raised in mouse, Sigma Aldrich). To study the expression of surface NCL, flow cytometry was performed on unfixed RB cell lines as well on RB tumor cells obtained from enucleated eyes. Briefly, $2X10^5$ cells were washed with 1X PBS followed by the addition of rabbit anti-NCL (abcam, 0.8 μg/ml) for 2 h at 4 °C, FITC conjugated anti-rabbit (sc-2012, 1:300 dilution) for an hour followed by washing and flow cytometry analysis.

Cellular uptake of nucleolin aptamer on RB tumor cells and cell lines

The NCL-APT with and without FITC at the 5' end of 5'-GGTGGTGGTGGTGGTTGTGGTGGTGGTGG-3' and LNA-NCL-APT with and without Cy5.5 carrying LNA modifications at 3 positions (2 modifications in the termini and one internally) in the NCL-APT sequence was purchased from Sigma Aldrich, India and Exiqon, Denmark respectively. The scrambled aptamer carrying C's instead of G's in NCL-APT was used as control aptamer. Unlabeled aptamer was used for studying the functional effect of aptamer. The aptamer binding and uptake was performed on live, fresh, unfixed or non-permeabilized cells. Titration using various concentration of aptamer was performed by

flow cytometry (0.5 μM, 1 μM, 5 μM) on Y79 cells. 500 nM and 1 μM were found to be the ideal concentration as it exhibited saturation. 500 nM of aptamer was added to RB tumor cells or cell lines and incubated for 45 min and the cells were thoroughly washed with 1X PBS twice and analysed by flow cytometry.

Treatment of cells with NCL-APT in RB cells

For studying the NCL mediated regulation and cellular events in RB, NCL-APT treatment or knock down of NCL was performed. The effect of NCL-APT on Y79, WERI-Rb1 and MIO-M1 cell lines was studied by treating cells with various concentration of NCL-APT (0.5 μM to 20 μM). The effective dose or IC_{50} concentration or a concentration with lesser cytotoxicity was chosen for further studies. The RB primary cells were also treated with 10 μM or 15 μM of NCL-APT in complete media for 48 h. In RB primary cells, pEGFP plasmid was transfected using lipofectamine 2000 to evaluate the metabolic activity. The uptake of FITC-NCL-APT on primary cells was also visualized using fluorescent microscopy. 48 h post treatment were collected and subjected to cell cycle, cellular cytotoxicity and cell proliferation assay.

Cell cycle and cell proliferation assay

Cellular events, metabolic activity and the mode of cell death was analysed to understand the mechanism of aptamer action in RB cells. To study the effect of NCL-APT and LNA-NCL-APT aptamers' activity on the cell cycle phases, cell cycle assay was performed using BD cycle-test kit. As per manufacturer's protocol, 48 h treated cells were PI stained. Briefly, cells were washed twice with PBS followed by addition of solution A and incubated for 10 min at RT, solution B 200 μl was added and mixed, incubated for 10 min at RT. Finally 150 μl of PI solution was added and incubated for 10 min at RT and flow cytometry was performed. Cell proliferation was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Assay was performed on NCL-APT treated primary cells and cell lines. Cells were incubated with 100 μl of media containing 10 μl of MTT (5 mg/ml) for 4 h at 37 °C. Then media containing MTT was removed, 100 μl of DMSO was added to each well and the absorbance was measured at 570 nm. All experiments were performed in triplicate.

Western blotting

The changes in gene expression upon aptamer treatment were confirmed by measuring the protein concentration by Western blotting. The xenograft tissues and cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1%Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 1 mM PMSF and Protease inhibitor cocktail, Sigma Aldrich) for 30 min on ice followed by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatant containing protein was collected and used. Protein concentrations were measured and an equal concentration was loaded onto 10% or 12% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and electroblotted onto a nitrocellulose membrane. The membranes were blocked with 5% skimmed milk for an hour at RT and then incubated with primary antibody, rabbit anti-NCL (dilution: 1:250; Abcam), rabbit anti-FOXM1 (dilution: 1:250; Santacruz biotech) or mouse anti-Bcl2 (dilution: 1:200, Imgenex) or mouse anti-survivin (dilution: 1:200, santacruz) for respective blots overnight at 4 °C. For normalizing the protein loading, mouse anti-β-tubulin (dilution: 1:250; Santacruz biotech) or mouse anti-β-actin (dilution: 1:4,000; Sigma Aldrich) was used. The membranes were washed with 1X PBS-T and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit (Litchfield et al.) antibody (diluted to 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at RT. The blot was subjected to supersignal west femto maximum sensitivity substrate (Thermo Scientific, Rockford, USA) and images were developed by autoradiography or using chemiluminescent detection system (Fluorchem, Protein simple). The blots are representative of triplicate experiments.

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High throughput miRNA microarray analysis for WERI-Rb1 NCL-APT treated cells

miRNA microarrays were performed in duplicates for WERI-Rb1 untreated and NCL-APT treated cells. The RNA was extracted from biological duplicates of treated and untreated cells, followed by quality check using bioanalyzer. Hybridization was performed for the biological duplicates. The miRNA labelling was performed using miRNA complete labelling and hybridization kit (Agilent Technologies, Part Number: 5190-0456). The total RNA sample was diluted to 50 ng/μl in nuclease free water. About 100 ng of total RNA was dephosphorylated along with appropriate diluted spike in control; (Agilent Technologies, MicroRNA Spike-In Kit, Part Number 5190-1934) using calf intestinal alkaline phosphatase (CIP) master mix (Agilent Technologies, Part Number: 5190-0456) by incubating at 37 °C for 30 min. The dephosphorylated miRNA sample was denatured by adding dimethyl sulfoxide and heating at 100 °C for 10 min and transferred to ice-water bath. The ligation master mix (Agilent Technologies, Part Number: 5190-0456) containing cyanine 3-pCp was added to the denatured miRNA sample and incubated at 16 °C for 2 h. The cyanine 3-pCp labelled miRNA sample was dried completely in the vacuum concentrator (Eppendorf, Concentrator Plus, Catalog Number: 5305000) at 45 °C to 55 °C for 2 h. The dried sample was re suspended in nuclease free water and mixed with hybridization mix containing blocking solution (Agilent Technologies, Part Number: 5190-0456) and Hi-RPM hybridization buffer (Agilent Technologies, Part Number: 5190-0456) and incubated at 100 °C for 5 min followed by snap chill on ice for 5 min. The samples were hybridized on the human miRNA version 3 array (Slide no: 252182715127). The hybridization was carried out at 55 °C for 20 h. After hybridization, the slides were washed using gene expression wash buffer 1 (Agilent Technologies, Part Number 5188-5325) at room temperature for 5 min and gene expression wash buffer 2 (Agilent Technologies, Part Number 5188-5326) at 37 °C for 5 min. The slides were then washed with acetonitrile for 30 s. The microarray slide was scanned using Agilent scanner (Agilent Technologies, Part Number G2565CA). The data was further analyzed using GeneSpring software.

Real-time quantitative reverse transcription PCR of miRNAs

The effect of NCL-APT on miRNA expression of the cells was detected by quantifying the mature miRNAs using reverse transcription real-time PCR. All reagents, including the TaqMan microRNA individual assays hsa-miR-17 (assay ID 002308), hsa-miR-18a (assay ID 002422), hsa-miR-19b-1 (assay ID 002425), hsa-miR-20a (assay ID 000580), hsa-miR-92 (assay ID 002137), hsa-miR-330 (assay ID 000544), hsa-miR-373 (assay ID 000561), hsa-miR-196b (assay ID 002215), hsa-miR-1 (assay ID 000385), hsa-miR-152 (assay ID 000474), hsa-miR-206 (assay ID 000510), the TaqMan microRNA Reverse Transcription Kit and the TaqMan Universal PCR Master Mix without AmpErase UNG were purchased from Applied Biosystems (Joyvel, Chennai, India) and Solaris qPCR Gene Expression Master Mix (Cat. No. AB4352B, ABgene, Thermo Scientific). Quantification was performed following the manufacturer's protocol starting with 100-250 ng of the total RNA per cDNA synthesis. The RNU6B probe was employed to normalizing miRNA levels in untreated to treated samples. The ABI PRISM 7500 Real-time machine (Applied Biosystems) was used. The innate expression of mature miRNAs of the miR-17-92 cluster was studied on MIO-M1, Y79 and WERI-Rb1 and normal retina. Similarly the levels of expression of miR-330, miR-196b, mir-152 and miR-1 in RB tumors were studied and compared with the normal retina and the MIO-M1 cell line.

In vivo experiment: Y79 xenograft study

Animal study was performed by utilizing the facility of Syngene (Bangalore, India) and experimentations were approved by the Institutional Animal Ethics Committee (IAEC Protocol Approval No: SYNGENE/IAEC/430/10-2013). Animals were maintained in a controlled environment with ambient temperature, humidity and light, fed with irradiated rodent diet, RO filtered potable water and housed group wise. Following aseptic procedure, $1X10^7$ human retinoblastoma cells (Y79) resuspended in 200 μl of serum free media containing 50% of Matrigel was injected subcutaneously in the right flank or back of the 5 week old female nude mice (Hsd: Athymic *Nude-Foxn1nu/Foxn1nu*). Once the tumor was palpable, the animals were randomized into 4 groups with 6 animals per group, based on tumor volume (TV ≈ 80 mm³) and dosed with sterile water for injection for vehicle control or NCL-APT (12.5 nmol/animal) or LNA-NCL-APT (12.5 nmol/animal) subcutaneously near the tumor surrounding area in 2 cycles of treatment and additionally a group with *intraperitoneal (i.p.)* injection of LNA-NCL-APT (12.5 nmol/animal). For the *s.c.* group, cycle 1 consisted of alternate day dosing from day 0 to 14 and cycle 2 for daily dosing between day 17 to 24. The *i.p.* group were given alternate day dosing till 4 days followed by daily dosing from day 5 to 14. The body weights and tumor volume were measured once every three days and % change in body weight was calculated. Tumor volume was determined by two-dimensional measurement with a vernier caliper; the length (L) and width (W) of the tumor was measured. Tumor volume (TV) was calculated using the following formula: tumor volume (mm³) = LXW²/2, where, L = length (mm), W = width (mm). Mean and standard error of mean (SEM) were calculated for individual groups and plotted as graph. Tumor growth inhibition was calculated using the formula: TGI = (1-T/C)X100; where T = (mean TV of the test group on Day_X - mean TV of the test group on Day_0) and C = (mean TV of the control group on Day_X - mean TV of the control group on Day_0).

Serum miRNA isolation and real time qPCR

Serum miRNA acts as biomarker for the RB disease progression, especially the mir-17-92 cluster. For studying the changes in the serum miRNA levels, the isolation of serum miRNA was performed using Plasma/Serum circulating and exosomal RNA purification kit (Slurry Format) by following manufacturer's protocol from n=2, of each group studied and additionally normal serum from nude mice was also included. Briefly, by mixing the serum (100 μl) with PS Solution A, which contains the separation matrix followed by PS Solution B and ethanol are then added, and the mixture is centrifuged. PS Solution C is then added to the pellet, and the slurry is loaded onto a provided mini filter spin columns. This is followed by washing of the bound RNA to remove the remaining proteins and other impurities. Finally, the purified circulating RNA is eluted into the 75 μl of elution buffer. As mentioned in the real time PCR section, qPCR of the mature miRNA of miR-17-92 cluster was performed and Hs-18s rRNA was used for normalizing the fold expression.

Real time qPCR and Western blotting of the xenograft tumor tissues

For studying the changes in the cancer stem cell markers, apoptotic and oncogenes' mRNA level, qPCR was performed from the cDNA transcribed from the total RNA of tumor tissues (n=2 per group) extracted using RNeasy Qiagen kit method. qPCR was performed for the mature miRs of miR-17-92 cluster and for the miRNAs deregulated upon NCL-APT treatment (miR-330, miR-196b, miR-152 and miR-1). Western blotting was performed on the tumor tissues (n=2 per group) lysed with RIPA buffer and analysed for the expression of FOXM1, Bcl2, Survivin and β-tubulin (antibodies from Santa Cruz biotech) following the protocol given above.

Protein array for apoptotic markers and cytokines

To study the effect of LNA-NCL-APT aptamer treatment (*i.p.* mode of injection) on inflammatory response and the apoptotic pathway, protein arrays were performed–Mouse cytokine array panel A (catalog no. ARY006) and Human apoptosis array (catalog no. ARY009) (R&D Systems, Abingdon, UK) following the manufacturer's instruction. The xenograft mouse (vehicle control and LNA-NCL-APT treated for 15

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days) serum samples and the tumor tissues lysate were normalized in their protein concentration and used for the array. The arrays were performed at a same time; similar conditions were applied and developed together using chemidoc XRS$^+$ instrument (BioRad) at once exposure. The integrated pixel density of the spots and background were measured using the ImageJ software with the microarray profile plugin and signal normalization was performed. The differences between the duplicate spots were used for calculating the standard deviation and expressed as error bar in the histogram plot.

1.1. Supplementary figures
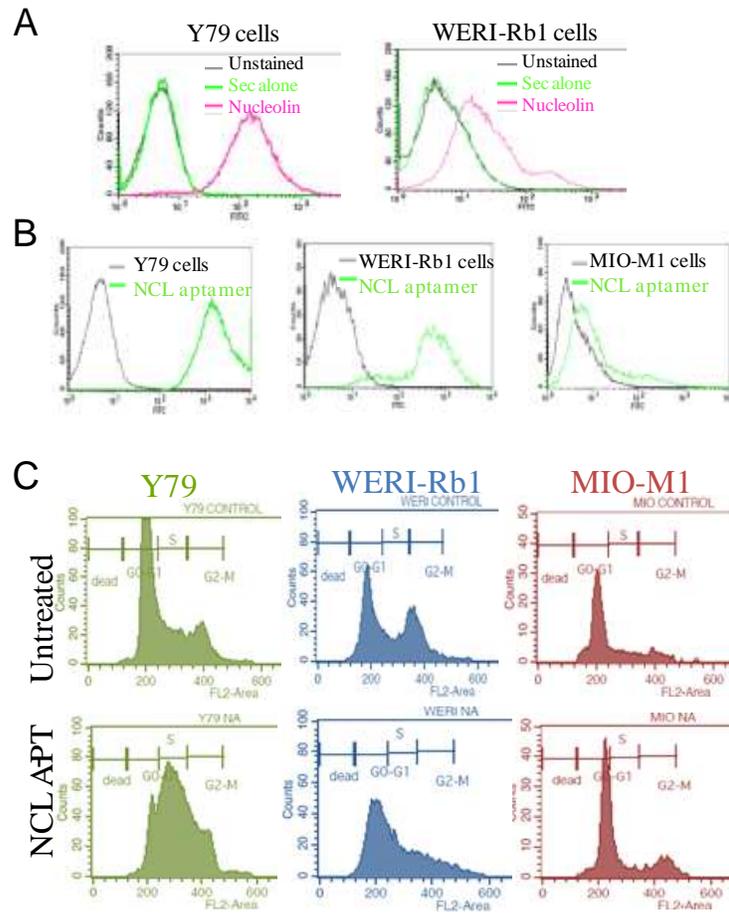
Figure S1. NCL expression, NCL-APT uptake and cellular effects on RB cell lines. A. Histogram overlay plots showing the expression of NCL in Y79 and WERI-Rb1 cell lines. B. Histogram overlay plots showing the binding of NCL-APT to MIO-M1, WERI-Rb1 and Y79 cell lines. The NCL-APT was used at concentration of 500 nM. C. Cell cycle changes in RB cell lines upon NCL-APT treatment. D. Graph representing the percentage viability of RB cell lines and MIO-M1 cells upon treatment with Scrambled aptamer.
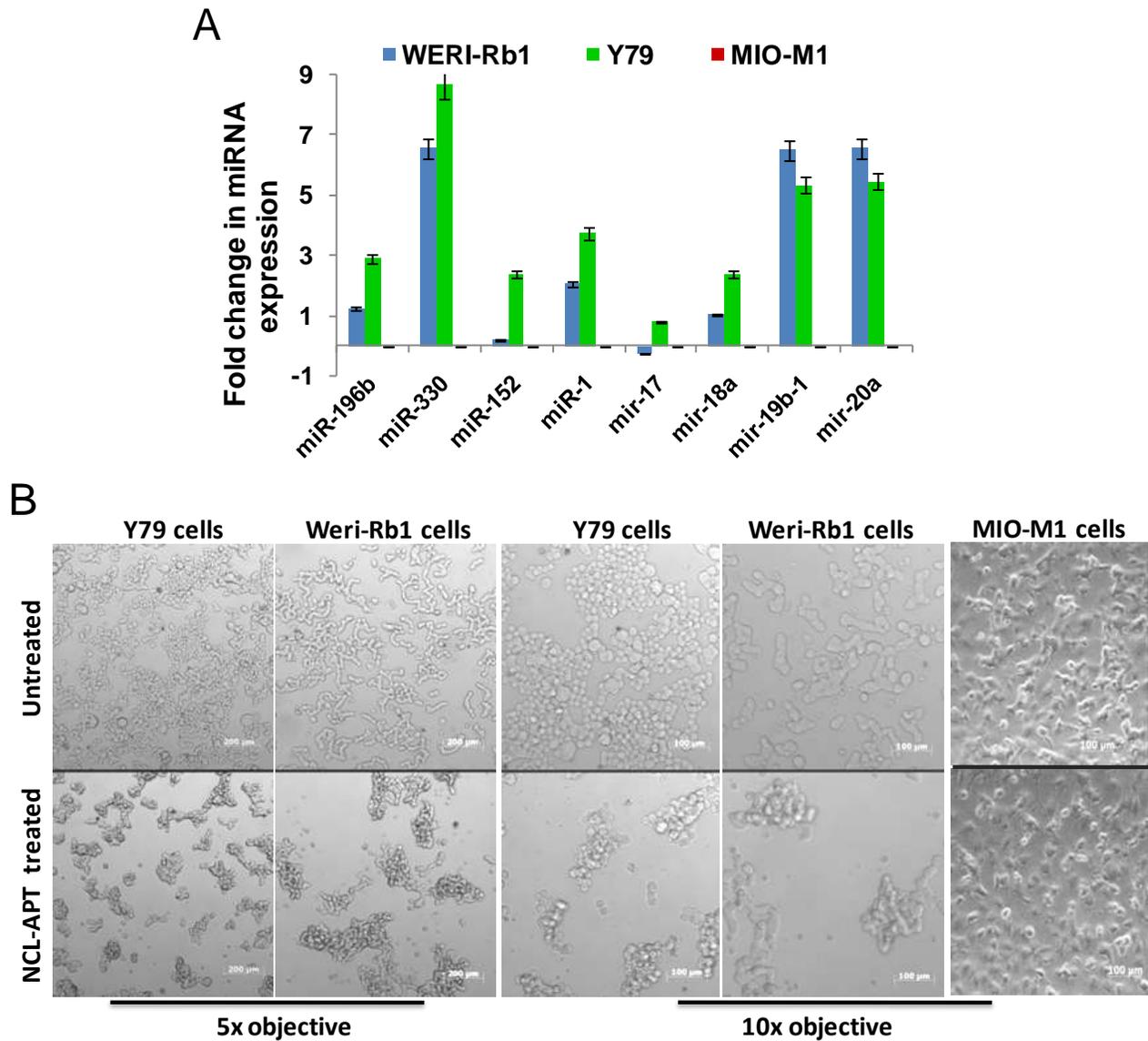
Figure S2. miRNA expression and effect of NCL-APT on the morphology of RB cell lines. A. Fold changes in the miRNA levels - mir-196b, mir-330, mir-152, mir-1, mir-17, mir-18a, mir-19-1 and mir-20a in RB cell lines normalized with MIO-M1. B. Phenotypic changes accompanying treatment of Y79, WERI-Rb1 and MIO-M1 cell lines with NCL-APT. The images shown are acquired at 5x and 10x objectives.
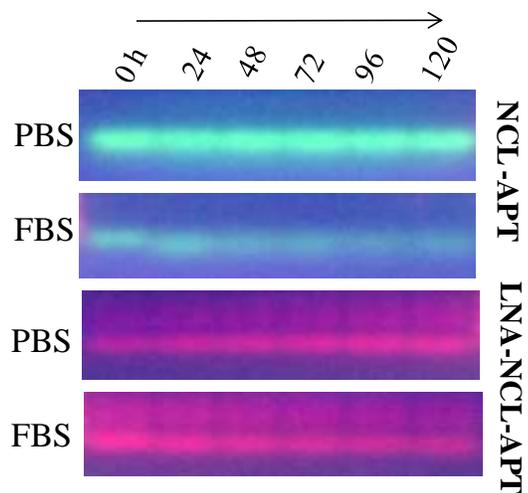
Figure S3. Stability and effect of NCL-APT and LNA-NCL-APT in cell lines. A. Stability of the NCL-APT and LNA-NCL-APT assessed by electrophoresing the aptamer reactions tested in the 1X PBS and 100% FBS at pH7.0±0.2 upto 120 h.
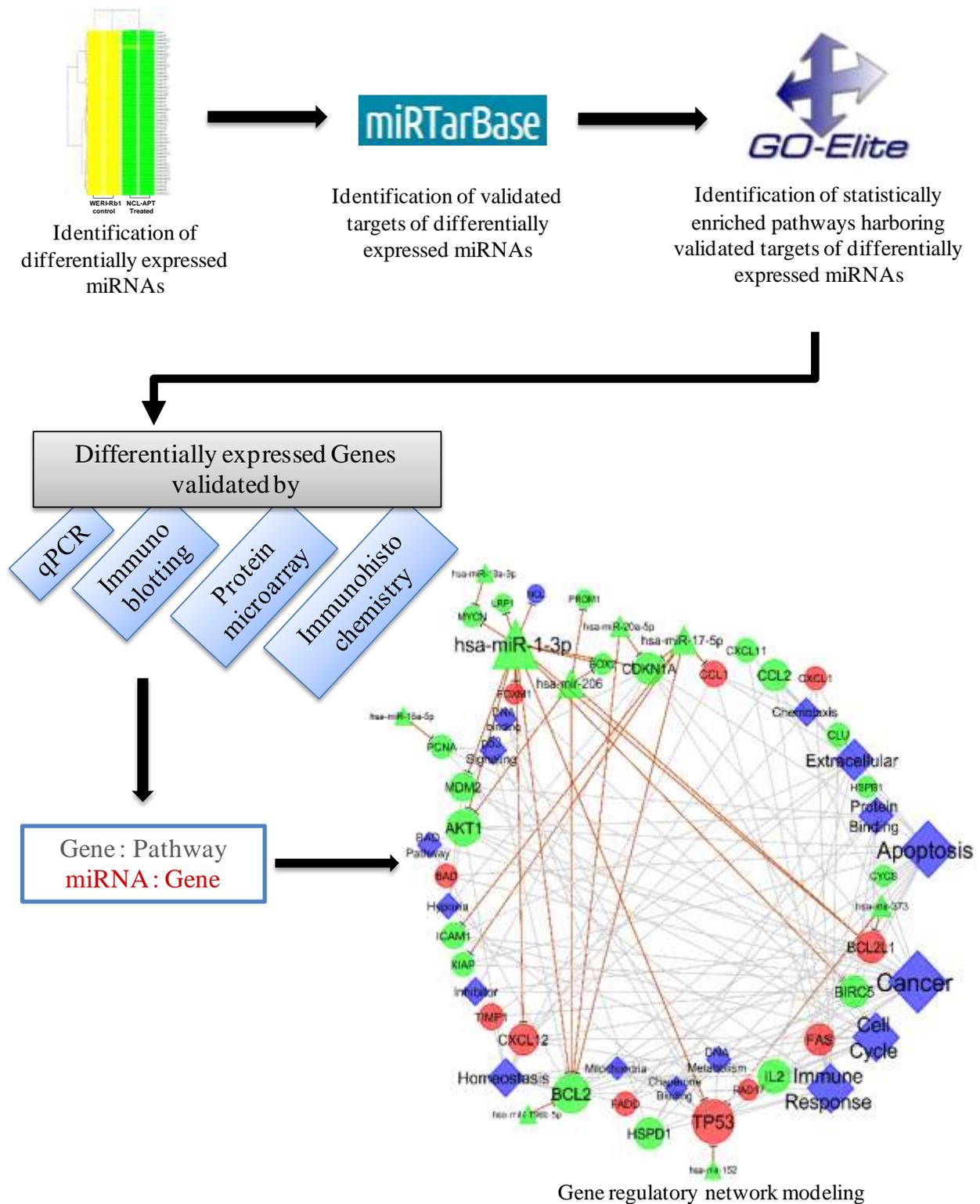
Figure S4. Schematic showing gene regulatory network upon NCL-APT treatment in RB. The flow of work shows right from miRNA microarray data to gene expression analyzed and validated to finally generate network model. Integrome regulatory network (shown at the end) depicts key pathways, genes and miRNAs (denoted by bigger the size to smaller the size) that underlays the transcriptional regulatory change established by NCL-APT treatment. Genes and miRNA is colored by their fold changes upon treatment with; upregulation indicated by red color and downregulation indicated by green color.
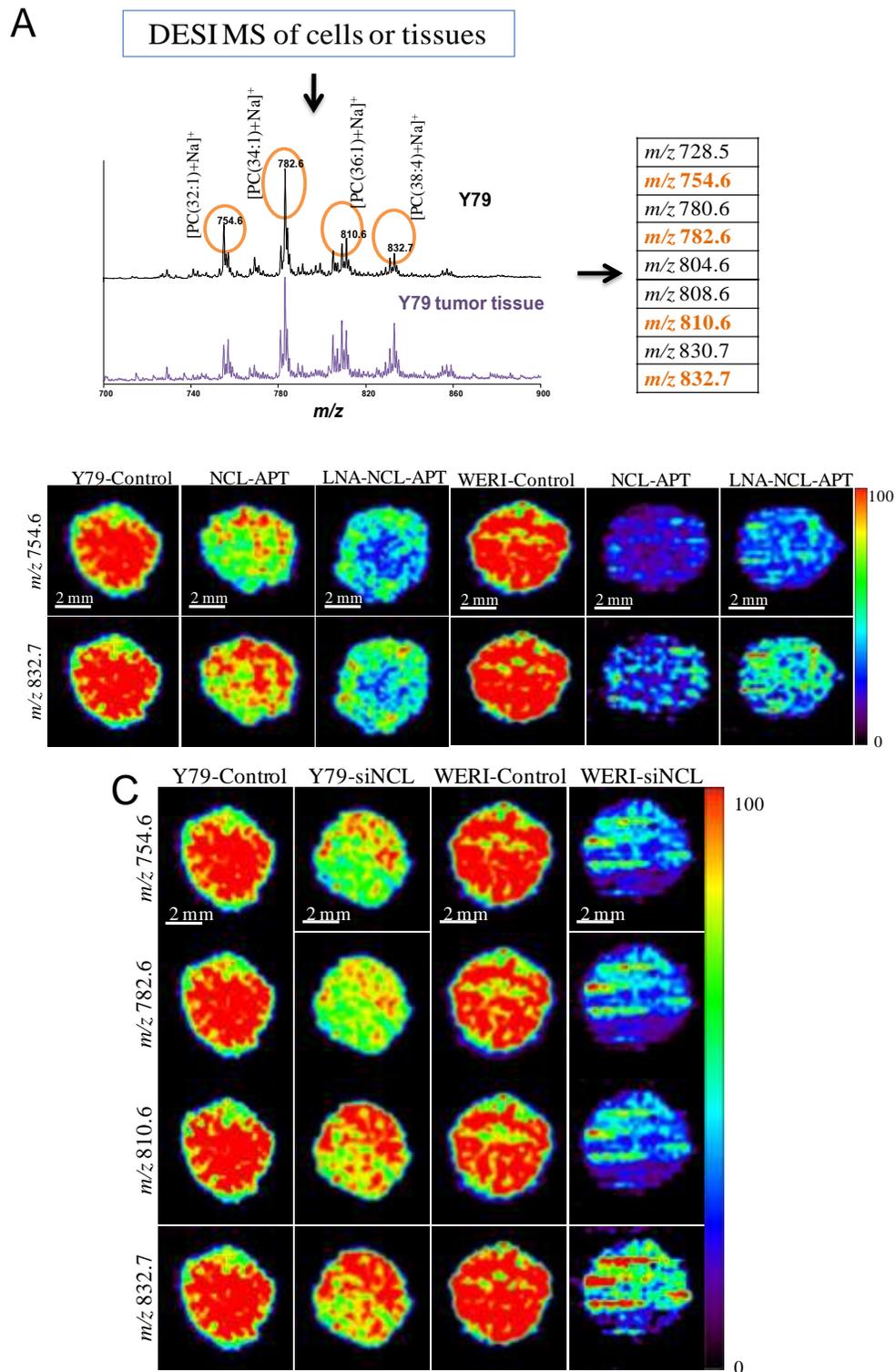
Figure S5. Effect of NCL-APT treatment and siRNA against NCL on lipids by DESI MS. A. DESI MS spectra obtained from cells spotted on Whatman 42 filter paper with major peaks highlighted and the list of peaks studied further are shown on right. B. DESI MS images of Y79 and WERI-Rb1 cells showing changes in m/z 754.6 and 832.7 upon NCL-APT and LNA-NCL-APT treatment. C. DESI MS images of Y79 and WERI-Rb1 cells transfected with siRNA against NCL showing changes in m/z 754.6, 782.6, 810.6 and 832.7.
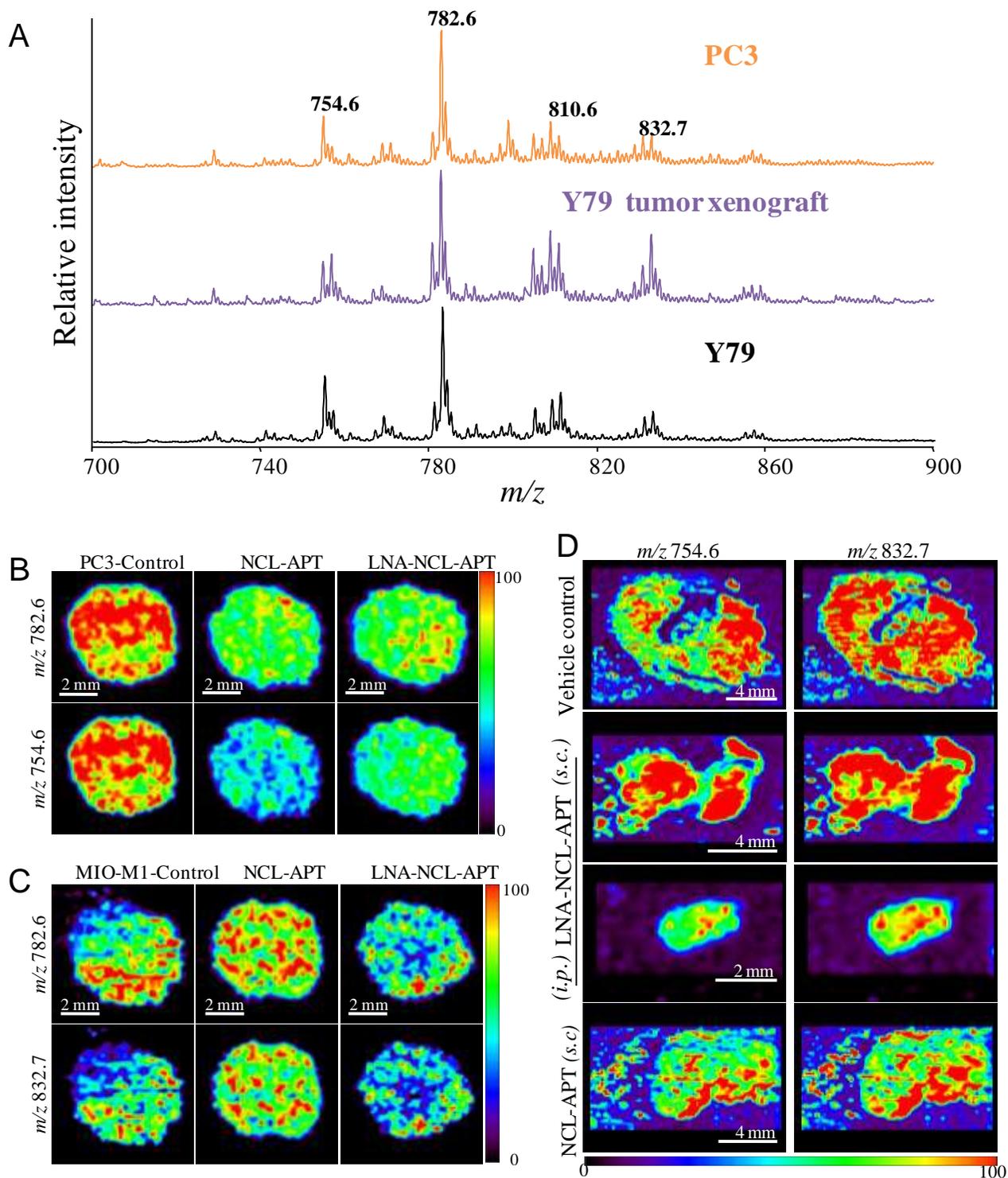
Figure S6. Effect of NCL-APT treatment on lipids by DESI MS. A. DESI MS spectra of PC3, Y79 tumor xenograft and Y79 cells. DESI MS images from PC3 (B), MIO-M1 (C): untreated or treated with NCL-APT (10 μM) or treated with LNA-NCL-APT (10 μM) for 48 h. D. DESI MS images of Y79 tumor tissues of the vehicle control, NCL-APT (s.c.), LNA-NCL-APT (s.c.) and LNA-NCL-APT (i.p.). Images represent the distribution of different lipids (PCs) across the cells and tissue sections.

Table S1. List of masses of the phosphatidylcholines predicted by MS/MS and database search

Mass: Phosphatidylcholines
m/z 728.5: [PC(30:0)+Na]$^+$
m/z 754.6: [PC(32:1)+Na]$^+$
m/z 780.6: [PC(34:2)+Na]$^+$
m/z 782.6: [PC(34:1)+Na]$^+$
m/z 804.6: [PC(36:4)+Na]$^+$
m/z 808.6: [PC(36:2)+Na]$^+$
m/z 810.6: [PC(36:1)+Na]$^+$
m/z 830.7: [PC(38:5)+Na]$^+$
m/z 832.7: [PC(38:4)+Na]$^+$