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Supplemental Information

A miRNA-Mediated Approach to Dissect the Complexity of Tumor-Initiating Cell Function and Identify miRNA-Targeting Drugs

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Supplementary Methods

Lentiviral transduction

For lentiviral transfection, Lenti Starter Kit (System Biosciences, CA) was used. Briefly, $3x10^6$ 293T cells were plated in 10cm plate with antibiotic free DMEM media supplemented with 10% FBS. At 50-70% confluence, 2μg of lentiviral plasmid and 10μg of pPACKH1-plasmid mix were co-transfected with Lipofectamine 2000 (Life Technologies, CA) following manufacturer's protocol. 48hr later, virus particles were harvested and precipitated. Target cells were transduced by plating 100,000 cells/well in a 6 well plate with virus particles and 4μg/mL polybrene (Santa Cruz Biotechnologies, CA) and were analyzed 72 hrs later.

RNA extraction and real-time PCR

Total RNA was extracted using the Total RNA Purification Plus Kit (Norgen Biotek, ON, Canada) according to manufacturer's instructions. For mRNA analysis, cDNA synthesis from 1μg of total RNA was done using the Transcriptor Universal cDNA Master kit (Roche, IN, USA). SYBR green-based Real-time PCR was subsequently performed in triplicate using SYBR green master mix (Roche) on the Light Cycler 480 II real time PCR machine (Roche). For miRNA expression assays, cDNA synthesis was done using Taqman gene expression assays (Life Technologies, Carlsbad, CA) and subsequent Real-time PCR was performed using Taqman universal PCR mastermix, no AmpErase UNG buffer (Life Technologies, Carlsbad, CA) with corresponding probes and primer mix (Taqman gene expression assays).

Transient transfection assays

For *miR-181a* promoter assays, *miR-181a* promoter (1µg) and renilla (150ng) were co-transfected using lipofectamine RNAiMAX (Life Technologies). Luciferase activities were analyzed using the Dual-Luciferase Reporter Assay System (Promega) with data normalization to the corresponding renilla values. For *miR-181a* antagomiR experiments, the control antagomiR and *miR-181a* antagomiRs were both purchased from Dharmacon. OVCAR3 cells were transfected with 50nM of the antagomiRs using lipofectamine RNAiMAX. 24hrs after transfection the cells were plated in 384 well imaging plates at different cell densities and mCherry fluorescence was determined 24hrs later.

3D on top matrigel sphere formation assay

250µl matrigel was plated in 24 well plates (Corning) and after 30 min incubation at 37^0C, 5000 cells were plated in 250µl mammocult on top of the first matrigel layer and incubated at 37^0C for 1hr after which 500µl of 10% matrigel in mammocult was added on top. After 3 weeks, 10x10 stitch imaging was done at 10x (100 random images acquired) using a Retiga Aqua Blue camera (Q Imaging, Vancouver, BC) connected to a Leica DMI6000 inverted microscope. Individual images were taken and then a composite image was generated using the scan slide function in Metamorph Imaging Software (Molecular Devices, Downington, PA). Subsequent integrated analysis also used Metamorph software.

ALDEFLOUR Assay

ALDH activity was determined by using ALDEFLOUR assay kit (Stem cell technologies). Briefly, $1x10^6$ OV81.2-Control and OV81.2-*miR181a* cells were resuspended in ALDEFLOUR reagent at 37^0c for 45 minutes (after trypsinization and

PBS wash) in the presence or absence of the ALDH inhibitor DEAB. Flow data was acquired using LSRII (BD Biosciences).

microarray and GSEA analysis

Total RNA was extracted from cell lysates of OCI-P5X *miR-181a* ^{high} and OCI-P5X *miR-181a* ^{low} cells in triplicate and submitted for Microarray using Affymetrix Human Clariom S array and the WT Plus chemistry. In brief, for the WT Plus assay, 150ng of total RNA was labeled using a reverse transcription priming method to prime the entire length of each RNA transcript, including both polyA and non-polyA mRNA to provide complete and unbiased transcriptome coverage. This protocol efficiently generated amplified and biotinylated sense-stranded DNA targets, avoiding loss of specificity due to antisense strand interference. Data was check for quality before being assessed on the Affymetrix Clariom S Human MicroArray. Changes in mRNA expression were then identified using the Clariom S Human MicroArray. On this array expression for each gene was assessed by approximately 11 probes, which were tiled throughout the transcript. The array provides basic gene level coverage of known genes. Labeled samples were hybridized to the arrays overnight in a rotating Hybridization Oven. Arrays were stained and washed in Affymetrix FS45U Fluidics Stations according to Affymetrix automated procedures. Data is collected using the GC3000 scanner with autoloader. The Clariom S Assays for the microarray data were downloaded and all data has been uploaded to GEO (Accession GSE52077). The data was pre-processed with RMA (Robust multichip average algorithm) using the R/Bioconductor package *Oligo* (42) where background subtraction, quantile normalization, and summarization (via median-polish) was accomplished. Gene set Enrichment Analysis was performed between the 3

mCherry High samples and 3 mCherry low Controls, using the *web application of GSEA at Broad website*(43) with the Hallmark Gene sets that represents established biological states and Processes and shows coherent expression(44).

miR-181a promoter acetylation analysis

H3K27ac ChIP with rabbit anti-H3K27ac (Abcam no. 4729) was performed using 10 million cross-linked cells and sequencing libraries were prepared as previously described (Schmidt et al, Methods, 2009). ChIP–seq libraries were sequenced on a HiSeq 2500 platform at the Case Western Reserve University Genomics Core Facility. Data analysis was performed as previously described (Morrow et al, Nature Medicine 2018).

Screening with chemical library

Chemical Compounds

The Collection of Biologically Active Molecules (Collection3114) was compiled from LOPAC library (Millipore Sigma, USA) and Bioactive Compound Library (Selleckchem). A total of 3114 mechanistically annotated partially redundant compounds were used for screening. All compounds were dissolved in DMSO at 10mM. A final DMSO concentration of 0.1% was not exceeded in the screening assay and in hit validation experiments. Upon hit identification, all compounds were retested as 10mM stock solutions purchased from original vendor.

Measurement of drug activity

Both mCherry^{low} and mCherry^{high} cells were seeded at 600 cells/well in Corning 3712 384-well plates using growth media. Cells were seeded in 50μL of media using

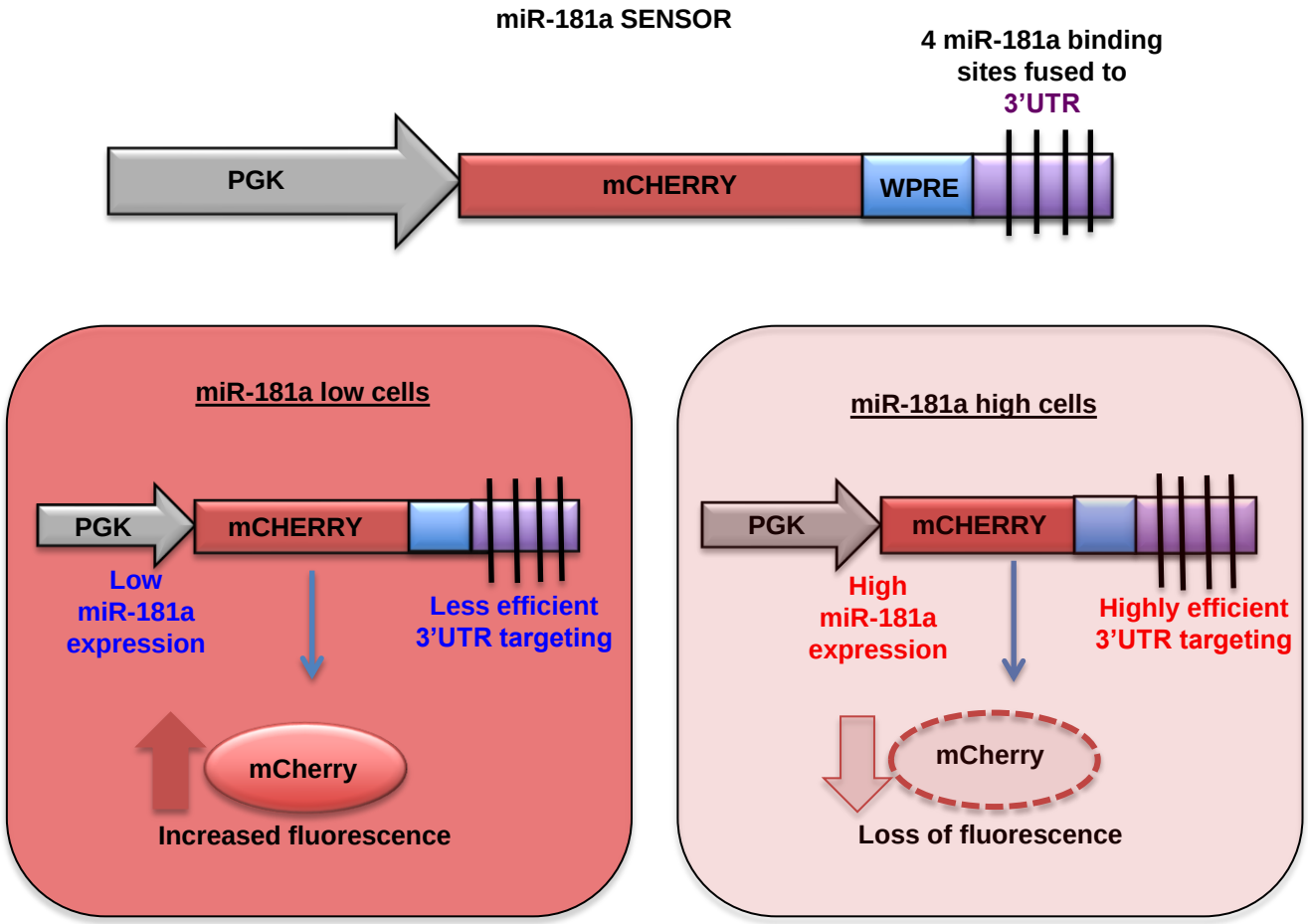
automated dispenser (MultiFlo FX, BioTek). During screening campaign, a single column (16 wells) on each plate were seeded with mCherry^{high} cells and served as positive control and another single column was seeded with Cherry^{low} cells to serve as a negative control. The negative and positive controls contained the same percentage of vehicle. During re-screening in each plate, two columns (32 wells) on each plate were seeded with mCherry^{high} cells and served as positive control and another two columns were seeded with Cherry^{low} cells to serve as a negative control. For the screening, 384-well assay plates were prepared with final test concentrations of 10μM using a Janus liquid handling platform (Perkin Elmer) equipped with 50nL pin transfer tool (V&P Scientific). For hit validation in dose–response studies at eight concentrations in two-fold dilutions, final test plates were prepared from stock solutions at 10 mM using Janus liquid handling platform (Perkin Elmer) equipped with a standard 96 tip head. In both screening and hit validation experiments cells were incubated with compounds for 48 hr. After incubation cells were fixed with 2% paraformaldehyde and stained with nucleic acid binding dye Hoechst 33342 (H342, Millipore Sigma, 5μg/mL). To test possible auto-fluorescence of hit candidate compounds dose–response studies were also performed in untransfected human osteosarcoma cells U2OS.

Cell imaging and image analysis

Fixed and stained cells were subjected to imaging and image analysis. The Operetta high-content imaging system with a 10x objective (PerkinElmer) was used for cell imaging. mCherry [Ex(560-580)/Em(590-640)] and Hoechst [Ex(360-400)/Em(410-480)] fluorescent images were obtained from a single field for each well. Image acquisition and storage were performed using Harmony 4.1 and Columbus software suites (PerkinElmer).

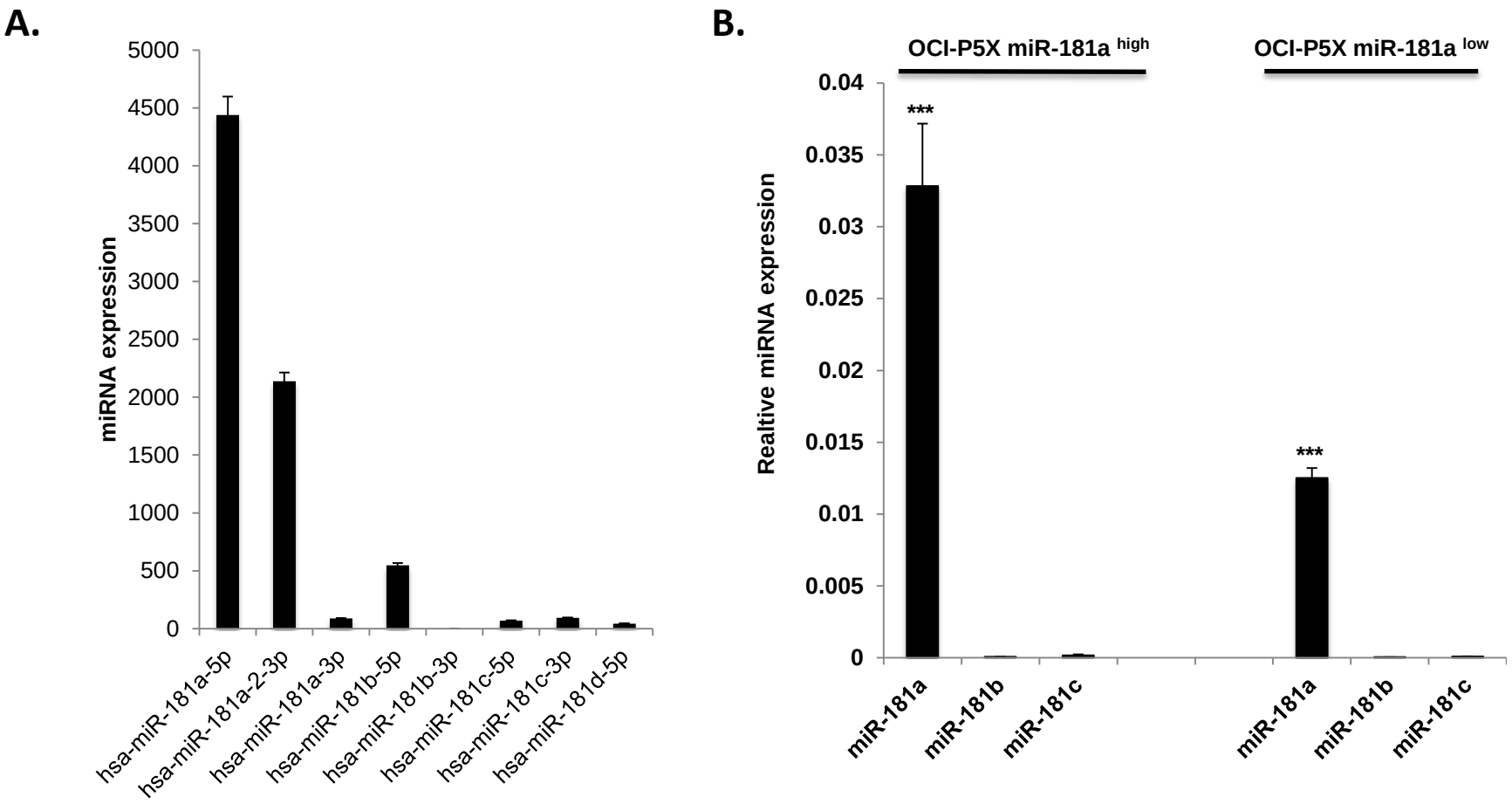
Image analysis and calculations were performed using Acapella software suite (PerkinElmer). Cells nuclei were identified based on fluorescence intensity (FI) of DNA-bound H342 as well as shape and area. Cell number was then determined for each well. Relative viability was calculated on per-plate basis as follows: Viability = $CN_{compound}/MEAN\_CN_{vehicle}$, where $CN_{compound}$ is a cell number for a given compound treated well and $MEAN\_CN_{vehicle}$ is an average (N=16) of DMSO-only treated wells on that particular plate. Cell cytoplasm was detected around each nucleus using low-intensity RNA-bound H342 stain. After that, for each cell mCherry fluorescence intensity was determined within cytoplasm mask. Relative mCherry FI was calculated for each well as follows: $mCherry\_FI_{compound}/MEAN\_mCherry\_FI_{vehicle}$, where $mCherry\_FI_{compound}$ is an average mCherry FI for a given compound treated well and $MEAN\_mCherry\_FI_{vehicle}$ is an average (N=16) of DMSO-only treated wells on that particular plate.

Figure S1 Illustration of miR-181a sensor platform



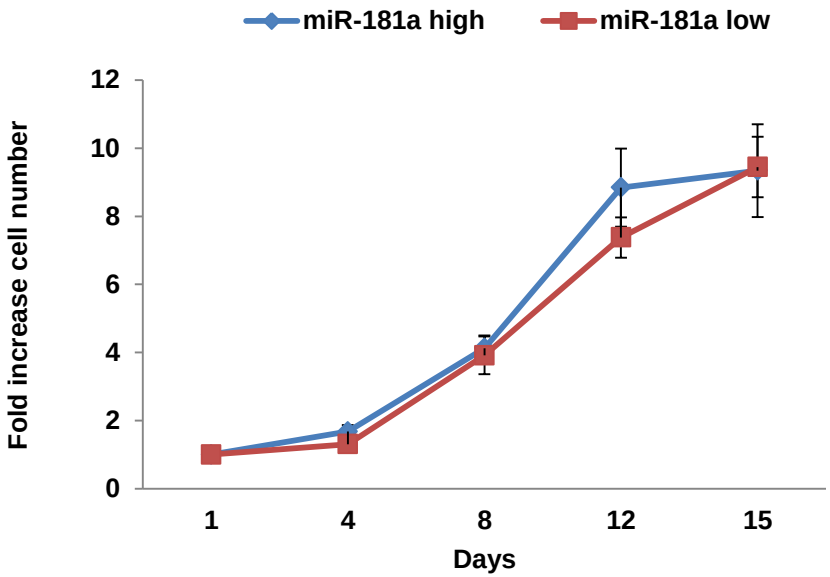
The *miR-181a* sensor contains 4 miR-181 recognition elements in the 3'UTR of mCherry. In this sensor the 3'UTR activity inversely correlates with mCherry fluorescence. Hence, low mCherry expression would represent the *miR-181a*^{high} population of cells. Conversely, high mCherry expression would represent the *miR-181a*^{low} population of cells that can be sorted out by transducing tumor cells with the *miR-181a* sensor.

Figure S2 Relative expression of miR-181 family members in TCGA cohort and OCI-P5X primary HGSOC cells



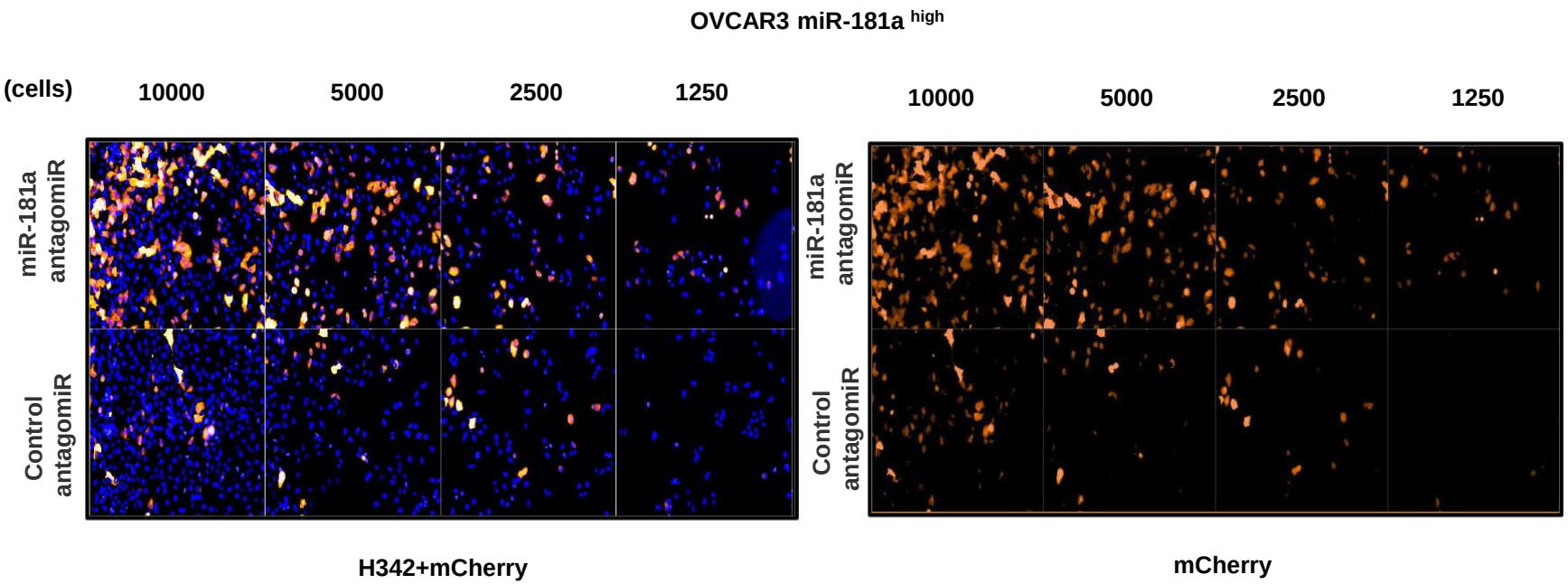
A. TCGA analysis of 476 HGSOC tumors revealed that miR-181a-5p and miR-181a-2-3p were the most highly expressed miR-181 family members. **B.** Taqman miRNA expression assay (three-independent experiments) showing significantly higher expression of *miR-181a* as compared to *miR-181b* and *miR-181c* in *miR-181a* sensor sorted primary HGSOC cells (*** $p<0.0005$) (Student's *t*-test two-tailed).

Figure S3 miR-181a ^{high} and miR-181a ^{low} cells do not differ in *in-vitro* proliferation rate



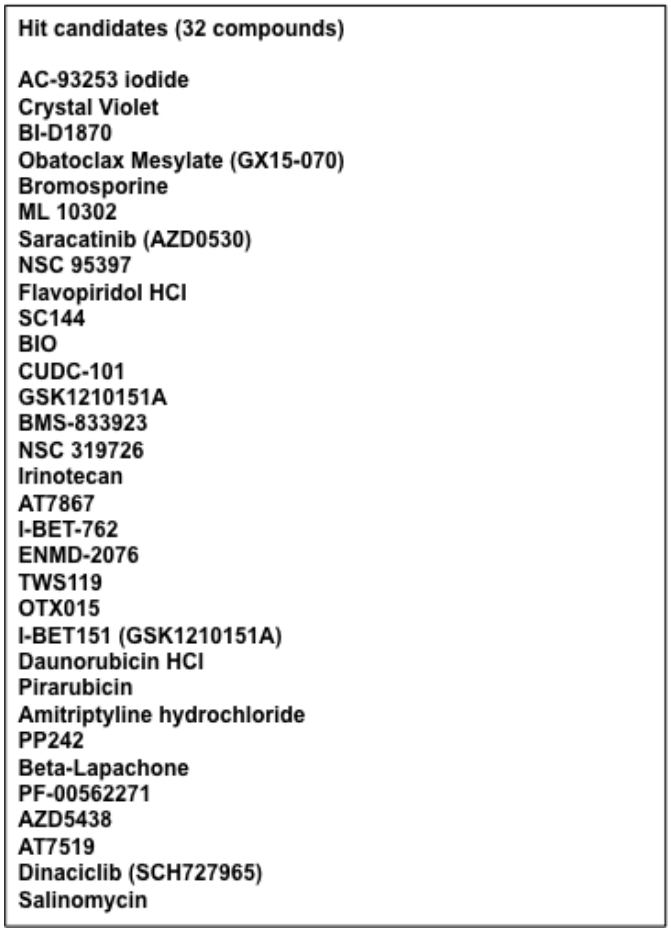
Growth curves for HEY miR-181a ^{low} and miR-181a ^{high} cells in microfluidic culture over 15 days. For these studies, a photograph was taken of each well of the plate on the indicated days. Total cell number is counted manually in the first 40 wells of each plate. Wells which had either no cell, 2 cells, or a contaminating cell (miR-181a low cell that contaminated the facs ~1% of cells) captured on the first day were excluded from analysis. Three independent isolates for each genotype were measured. The plots show mean ± SEM

Figure S4 Establishing *miR-181a* sensor based screening platform



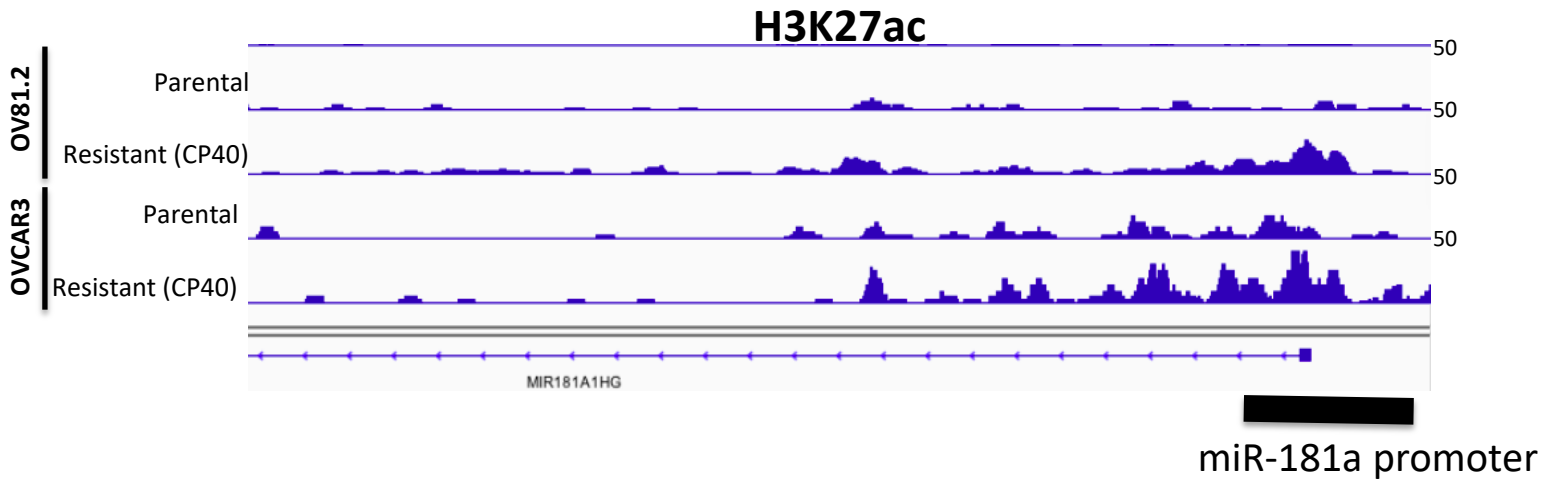
Fluorescence imaging showing increased mCherry fluorescence upon transfection with *miR-181a* antagomiR (50nM) (, *miR-181a* antagomiR was purchased from Dharmacon, Lipofectamine 2000 transfection protocol was used) as compared to control antagomiR in OVCAR3 cells. H342 dye was used to visualize cell nuclei.

Figure S5 32 Preliminary hits obtained from *miR-181a* screen



List of 32 candidate drugs from preliminary screening done in OVCAR3 *miR-181a* sensor sorted cells, which were further evaluated in secondary validation assays

Figure S6 *miR-181a* promoter acetylation



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Supplementary Table 1 Identification of *miR-181a* predicted targets in top 100 downregulated genes in miR-181a ^{high} HGSOC cells : *miR-181a* predicted target list was downloaded the miRWalk database. Common genes between this list and the top 100 downregulated genes in *miR-181a* ^{high} HGSOC cells were analyzed to identify 27 potential miR-181a targets to be downregulated in miR-181a ^{high} HGSOC cells

Supplementary Table 2 *miR-181a* miRNA sensor-based high-throughput screen and validation of active hit candidates identified in *miR-181a* miRNA sensor-based high-throughput therapeutic screen : A total of 3114 mechanistically annotated partially redundant compounds were used for screening. Relative viability and mCherry FI were calculated for each well on per-plate basis. Top 32 compounds (approximately 1% of the library) were identified as potential hits using relative mCherry FI parameter. Upon hit identification, all compounds were validated as a stock solutions purchased from original vendor. Compound titration experiments demonstrated that eight compounds induce *miR-181a* miRNA sensor-driven mCherry FI in a specific and concentration-dependent manner.