

Supplementary Figure Legends

Supplementary Figure 1. Knockdown of KDM3A results in decreased malignant properties of PDAC cells.

(A, B) Quantitative RT-PCR analyses for KDM family genes with or without knockdown of KDM3A (shRNA 2 and shRNA3) in MaPaca-2 (A) and S2-007 (B) cells. Arrows indicate KDM3A.

Supplementary Figure 2. Knockdown of KDM3A results in decreased migration potential of PDAC cells.

(A-B) Wound closure assays for 24 hours, showing that knockdown of KDM3A inhibited migration of both S2-013, MiaPaCa-2 and S2-007 cells ($p < 0.01$).

Supplementary Figure 3. Phenotypic rescue by manipulating KDM3A levels in S2-007.

(A) Reintroduction of KDM3A in KDM3A-knockdown S2-007 cells rescued KDM3A protein expression.

(B) Spheroid-formation assays. KDM3A reintroduction rescued pancosphere formation reduced by KDM3A knockdown in S2-007 cells.

Supplementary Figure 4. Overexpression of KDM3A in HPNE.

(A-B) Wound closure assays for 48 hours. Representative images and quantification graph showing 24 and 48 hours after the wound were generated. Both Trans18 and Trans21 HPNE clones accelerated migration or wound closure ($p < 0.01$).

(C) Invasion assays using HPNE cells with (Trans18 and Trans21) or without (EV) overexpression of KDM3A, showing HPNE clones Trans18 and Trans21 cells invaded faster than control HPNE EV cells.

Supplementary Figure 5. Knockdown of KDM3A in KDM3A-transformed cells affects spheroid formation.

(A) Western blot for KDM3A using KDM3A-overexpressing Trans18 HPNE cells transfected with two different siRNAs for KDM3A in comparison to EV.

(B) Spheroid-formation assays. Knockdown of KDM3A in Trans18 HPNE cells nullified increased sphere-forming potential of Trans18 HPNE cells by KDM3A overexpression.

Supplementary Figure 6. Western blot analyses of KDM3A overexpressing HPNE cells shows increased phosphorylation of signaling proteins.

Trans18 and Trans21 HPNE clones, as well as S2-007 and MiaPaCa-2 cells, showed increased levels of pEGFR, pAkt, 14-3-3 σ , and CA-19-9, compared with parental HPNE cells.

Supplementary Figure 7. DCLK1 is upregulated through KDM3A.

(A) Immunohistochemistry for DCLK1 showing that DCLK1 was overexpressed in PDAC tissues.

(B) Co-expression of KDM3A and DCLK1 observed by immunofluorescence using confocal microscopy was performed with human PDAC tissue sections shows that KDM3A (green) and DCLK1 (red) co-express (yellow) in the same cells.

(C) Immunofluorescence studies for KDM3A and DCLK1 using KDM3A-overexpressing Trans18 HPNE and S2-007 cells. Merged images for KDM3A and DCLK1 are shown.

(D) Chromatin Immunoprecipitation coupled Sequencing (ChIP-Seq) by comparing the results between HPNE-KDM3A cells and S2-007 PDAC cells. Red letters show common genes potentially regulated by KDM3A, one of which was DCLK1.

Supplementary Figure 8. Co-expression of KDM3A and DCLK1 in orthotopic tumors.

(A) Immunohistochemistry for KDM3A, DCLK1, PCNA, and Ki67, showing a positive correlation of KDM3A with DCLK1 expression as well as PCNA, proliferation markers, in xenografts derived from Trans18 HPNE and S2-007 cells.

(B) Western blot analysis for KDM3A using Trans18 HPNE and S2-007 orthotopic model and their metastases in the lungs and liver.

(C) Immunofluorescence for KDM3A and DCLK1 in S2-007 xenografts, showing co-expression of KDM3A and DCLK1 in S2-007 xenografts. Merged images co-expression of KDM3A and DCLK1 in those animal tumors.

(D) Immunohistochemistry for KDM3A and DCLK1 showing a positive correlation of KDM3A with DCLK1 expression in PanIN and tumor tissue derived from KPC mice.

Supplementary Figure 9. Novel KDM3A inhibitors inhibit tumor growth in orthotopic PDAC model in mice

(A) Structure of proposed KDM3A inhibitors: The compound PNSA was synthesized based on the structure of N-oxalyl glycine (NOG, a known KDM3A inhibitor).

(B) Molecular Docking studies using Autodock Vina showed the binding of PNSA in the protein cavity of KDM3A with lower binding energy and more hydrogen bonds formation as compared to NOG.

(C) Enzyme inhibition assay showed that PNSA is a potent inhibitor of the enzyme activity as compared to NOG.

(D) PNSA inhibited the proliferation of MiaPaCa-2 and S2-007 cell lines in a dose- and time-dependent manner.

(E) PNSA inhibited the colony formation of MiaPaCa-2 and S2-007 cell lines.

(F) The western blot showing the treatment of PNSA inhibited the expression of DCLK1 and KDM3A in the MiaPaCa-2 and S2-007 cell line.

(G) PNSA (100 mg/kg) inhibited the tumor growth in S2-007 orthotopic tumors in athymic nude mice

(H) PNSA (100 mg/kg) reduced the tumor weight in S2-007 orthotopic tumors in athymic nude mice ($p < 0.05$).

Supplementary Methods

Immunohistochemistry

Paraffin-embedded human pancreatic tissue sections were placed in a 58-60°C oven for two hours for the tissue to adhere. The sections were deparaffinized in xylene, rehydrated through graded ethanol and washed with PBS before being treated with 1X Reveal in a Decloaking Chamber (Biocare Medical, CA) for antigen retrieval following the manufacturer's protocol. After rinsed in PBS for 15 min, the tissue sections were soaked in 3% H₂O₂ in PBS for 30 min to quench endogenous peroxidase activity. Sections were incubated for 60 min in 3% normal rabbit serum (Vector Laboratories, CA) in PBS at room temperature to block non-specific binding sites and then probed with primary antibodies (JMJD1A Abcam). Sections were incubated with diluted primary antibodies (1 µg/ml) prepared in PBS containing 1.5% normal rabbit serum for one hour in a hydrated chamber at room temperature. Tissue sections incubated with 1 µg/ml normal goat IgG (Santa Cruz Biotechnologies, CA), were run in parallel with each stain, as negative controls. Following extensive washing, antigen-antibody complexes were detected using the Vectastain Elite ABC kit (Vector Laboratories, CA) according to the manufacturer's protocol. Staining was performed with ImmPact™ DAB peroxidase substrate kit (Vector Laboratories, CA). Sections were then counterstained in Mayer's hematoxylin and dehydrated in ascending grades of ethanol before clearing in xylene and mounting under a coverslip using Cytoseal XYL. The levels of JMJD1A expression in each specimen were scored according to the extent (percent of stained cells) and intensity of staining.

KDM3A Knock-down

JMJD1a lentiviral plasmids derived from Mission shRNA (Sigma-Aldrich, MO). Lentiviral vector packaged into CMV8.4 and VSVG envelop vectors. The vectors transfected into 293T cells, the viral particles were harvested and infected into MiaPaCa-2, S2-007 and S2-013 cells. The knock-down cells were selected by Puromycin (1 µg/ml).

Quantitative PCR (qPCR)

Total RNA was isolated from the shRNA knock-down KDM3A MiaPaCa-2, S2-007 cells, including scrambled by using the RNeasy Mini kit (Qiagen, Cat No./ID: 74104) and treated with RNase-free DNase (Qiagen, Cat No./ID: 79254). The integrity of the RNA was determined by the denaturing formaldehyde agarose gel electrophoresis and reverse-transcribed with Superscript II reverse transcriptase in the presence of random hexanucleotide primers (Invitrogen, CA). cDNAs were then used for real-time PCR using Jumpstart Taq DNA polymerase (Sigma-Aldrich, MO) and SYBR Green Nucleic Acid Stain (Molecular Probes). Crossing threshold values for individual genes were normalized to β -actin. Changes in mRNA expression were expressed as fold change relative to control. Primers used in this study were as follows: β -actin: 5'-CTGATCCACATCTGCTGG-3' and 5'-ATCATTGCTCCTCCTCAGCG-3'; cyclin D1: 5'-AATGACCCCGCACGATTTTC-3' and 5'-TCAGGTTTCAGGCCTTGAC-3'.

The isolated total RNA was used to determine the expression profiles of the genes associated with stem cell markers, hypoxia and angiogenesis markers, according to the procedure recommended by the supplier (Qiagen PCR Array Biosciences).

Chromatin immunoprecipitation (ChIP). ChIP study was done using the kit ChIP-IT Express from Active Motif, CA (Cat no: 53008). First, intact cells were fixed using formaldehyde, which cross-linked and therefore preserved protein/DNA interactions. DNA was then sheared into small uniform fragments and the DNA/protein complexes were immunoprecipitated using KDM3A antibody (Cat no: ab91252, Abcam). Following immunoprecipitation, the DNA was washed, cross-linking was reversed, and the proteins were removed by Proteinase K treatment. Eluted DNA was purified using Active Motif's Chromatin IP DNA Purification Kit (Cat no: 58002) then downstream analysis via qPCR or Next-generation sequencing was done.

ChIP-Seq. For ChIP sequencing, samples were sent to BGI Americas Corporations, MA. They used the BGISEQ-500 sequencing platform to perform the study. Standard bioinformatics analysis and production statistics were performed which includes read alignment, genome-wide distribution of ChIP-Sequencing reads, peak scanning, distribution peak related gene scanning, GO function analysis and difference analysis of multi-samples. Further bioinformatics analysis was performed.

RNA-Seq. RNA was isolated from the cells using the kit RNeasy Mini kit from Qiagen (Cat no: 74104). Then from the purified RNA Rapid Read RNA-Seq was prepared and bioinformatic analysis was performed.

Proliferation assay

5X10³ cells per well of MiaPaCa-2 and S2-007 cells were seeded in 96-well culture plates. (Corning, NY). After 24 h, cells were treated with various concentrations NOG, and PNSA. Cell proliferation was determined up to 72 hours by an enzymatic hexosaminidase assay¹.

Pancosphere formation assay

Pancosphere formation assay was performed according to the method described². Briefly, cells (1 × 10³/well) were plated in ultra-low attachment plates 6 well plates in cells were cultured in DMEM-F12 and supplemented with B-27 and bFGF. Numbers of pancosphers formation, after 10-14 days culture, were counted under light microscopy and quantified by Celigo software (Nexcelom Bioscience, MA).

Colony formation assay

Briefly, 6 well dishes were seeded with 5x10² viable cells and allowed to grow for 24 h. The control and shRNA knock-down cells were then for kept in culture for 10 days in complete medium. Each treatment was done in triplicate. The colonies obtained were washed with PBS and fixed in 10% formalin for 10 min at room temperature and then washed with PBS followed by staining with Crystal violet. The colonies were counted and compared with control cells.

Wound-healing assay

The wound-healing assay was done to assess cell migration. Cells were grown in complete medium to a confluent monolayer and were scrapped by 200- μ l pipette tips to

create an artificial wound. Each experiment was performed in triplicate. The wound wait for close for 24 h and measured cells were migrated from initial-wound.

Western blot analysis

The cell lysates were prepared in RIPA buffer with Pierce protease inhibitor cocktail (Thermofisher Scientific, MA). The total protein was quantified by Pierce BCA protein assay (Thermofisher Scientific, MA). The proteins were resolved in denatured SDS-PAGE system. The proteins were transferred onto PVDF membrane. The proteins were probed against anti-human antibodies such as DCLK1, KDM3A, H3K9me3, H3K9me2, H3K9me1 and H3.

Immunofluorescence

Mice and human pancreatic tissue (normal and cancer) sections, as well as PDAC cell lines (HPNE, TRANS18 and S2-007), were stained for KDM3A and DCLK1.

Sample preparation

Tissue sections: Pancreatic tissues (human /mice) were harvested and fixed with normal buffered formalin. After fixing, pancreatic tissues were embedded in paraffin wax. Later 8 μ m thick tissues were sectioned and were mounted on microscopic slides. Sections were dried for approximately 8-10 hours and stored at room temperature until processing. At the time of processing, xylene, ethanol and phosphate buffered saline (PBS) were used to perform serial hydration. Antigen retrieval was performed in a 0.01 M citrate buffer, pH 6.2, with 0.002 M EDTA. Slides were allowed to cool down and then washed with PBS. Permeabilization was performed in 1.0% Triton X-100 in 0.1 M PBS,

pH 7.4 for 30 min. Tissue sections were then used for immunofluorescence staining and imaging.

Cell lines: Cells were laid down on the microscopic slides and were fixed with normal buffered formalin. Fixed cells were stored at +4°C until processed. At the time of processing, slides were washed with PBS three times for 5 minutes each and then permeabilized in 1.0% Triton X-100 in 0.1 M PBS, pH 7.4 for 30 min. Slides were then used for immunofluorescence staining and imaging.

Staining

Tissue sections/ cells lines were blocked using 1%BSA, 10% goat serum, 0.03% Triton X-100 and PBS for 30 minutes. Tissue sections/ cell lines were incubated overnight with primary antibodies against KDM3A (1:200, Abcam, UK, Cat no # ab 91252) and DCLK1 (1:200, Abcam, UK Cat no # ab 37994) in a humidified chamber at +4°C. After incubating with primary antibody, tissue sections/ cell lines were washed with PBS three times for five minutes each and then incubated in a mix of fluorophore-conjugated secondary antibodies consisting of Alexa Fluor 488 (1:400, Molecular Probes,OR, Cat no: A11029), Alexa 555 (1:400, Molecular Probes,OR, Cat no: A21429) and in a dark wet chamber for approximately 40 minutes. Tissue sections/ cell lines were then washed with PBS three times for five minutes each and mounted with anti-fading agent Gel/Mount (Biomedex, CA). Primary and secondary antibodies were diluted in the solution containing: 1% BSA, 0.03% Triton X-100 and PBS. Images were captured on a Nikon C1Si confocal microscope.

The cancer genome atlas (TCGA)

The gene expression data of TCGA was analyzed by using GEPIA (Gene Expression Profiling Interactive Analysis) online³. The survival curve of PDAC patients relative to KDM3A expression was extracted from the human protein atlas⁴.

Molecular docking

The X-ray crystal structure of the Human KDM3A was developed using Swiss modeller based on PDB ID: 4C8D. The predicted structure was evaluated by Ramachandran's plot. All the molecular docking calculations were performed using AutoDock Vina software⁵ to study the interaction of NOG and PNSA with the generated structure of KDM3A. Autodock Vina is a free molecular docking program that offers high performance and improved accuracy. The software is developed in Molecular Graphics Lab at The Scripps Research Institute (<http://vina.scripps.edu/>). The 3D grid box is generated around the NOG binding site with a grid spacing 1.0 Å and 60X60X60 point size. The default parameters of the Autodock tools were used for all docking calculations. The protein and the ligand were prepared by adding total Kollman and Gasteiger charges and polar hydrogens. Lamarckian GA was utilized to search the potential conformations. The most stable conformer of the compound-protein complex was selected based on the lowest binding energy and visualized using Pymol (<https://pymol.org/2/>)⁶.

Statistical analysis

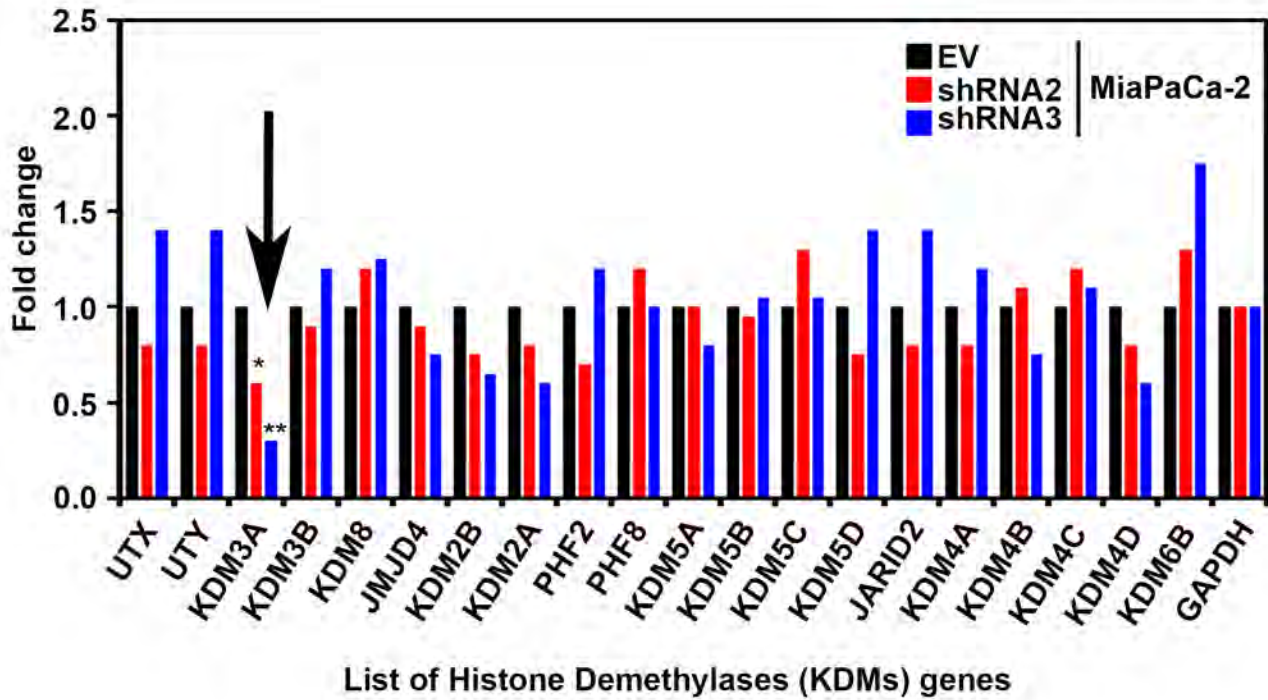
All values are expressed as the mean \pm SEM. Data were analyzed using an unpaired 2-tailed *t*-test. A *P* value of less than 0.05 was considered statistically significant.

References for Supplementary Methods

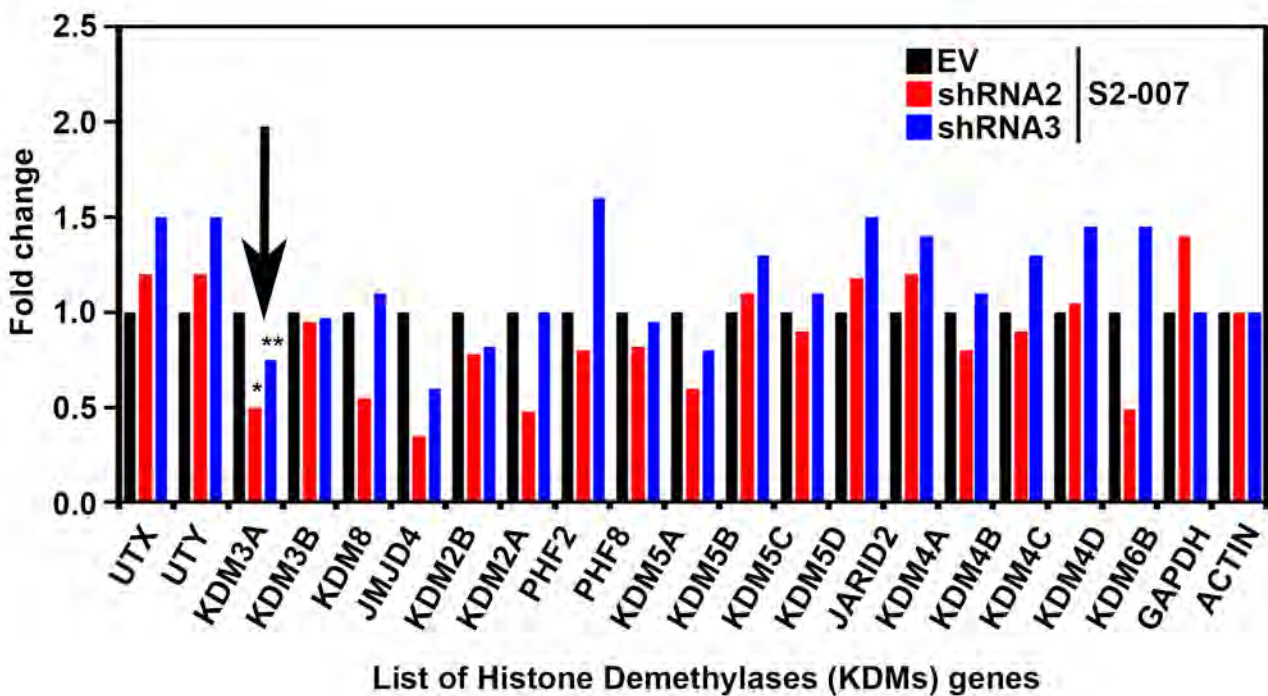
1. Subramaniam D, Nicholes ND, Dhar A, et al. 3,5-bis(2,4-difluorobenzylidene)-4-piperidone, a novel compound that affects pancreatic cancer growth and angiogenesis. *Mol Cancer Ther* 2011;10:2146-56.
2. Ponnurangam S, Dandawate PR, Dhar A, et al. Quinomycin A targets Notch signaling pathway in pancreatic cancer stem cells. *Oncotarget* 2016;7:3217-32.
3. Tang Z, Li C, Kang B, et al. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res* 2017;45:W98-W102.
4. Uhlen M, Fagerberg L, Hallstrom BM, et al. Proteomics. Tissue-based map of the human proteome. *Science* 2015;347:1260419.
5. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 2010;31:455-61.
6. Alexander N, Woetzel N, Meiler J. bcl::Cluster : A method for clustering biological molecules coupled with visualization in the Pymol Molecular Graphics System. *IEEE Int Conf Comput Adv Bio Med Sci* 2011;2011:13-18.

Supplementary Figure 1

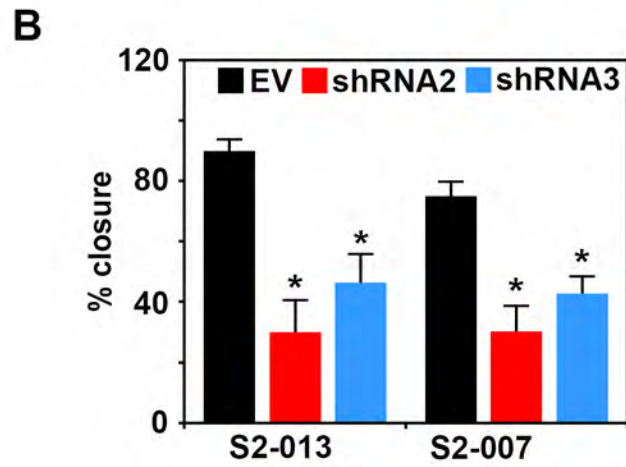
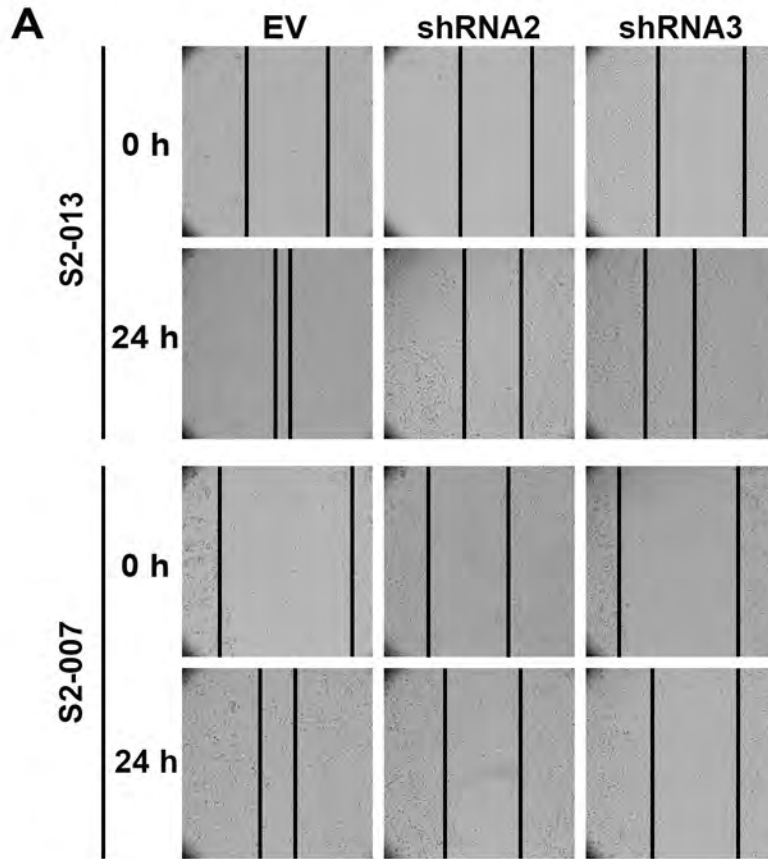
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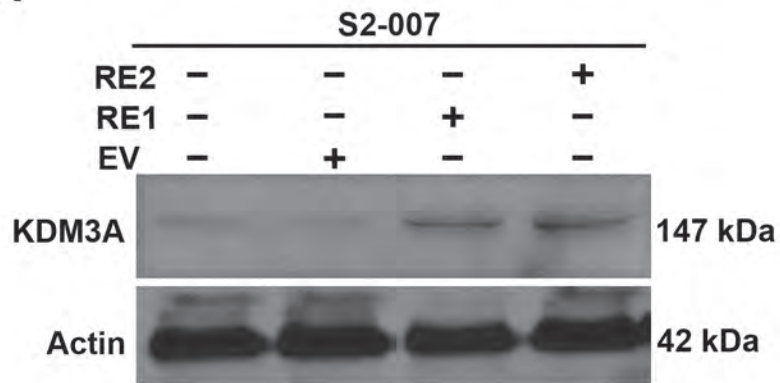


Supplementary Figure 2

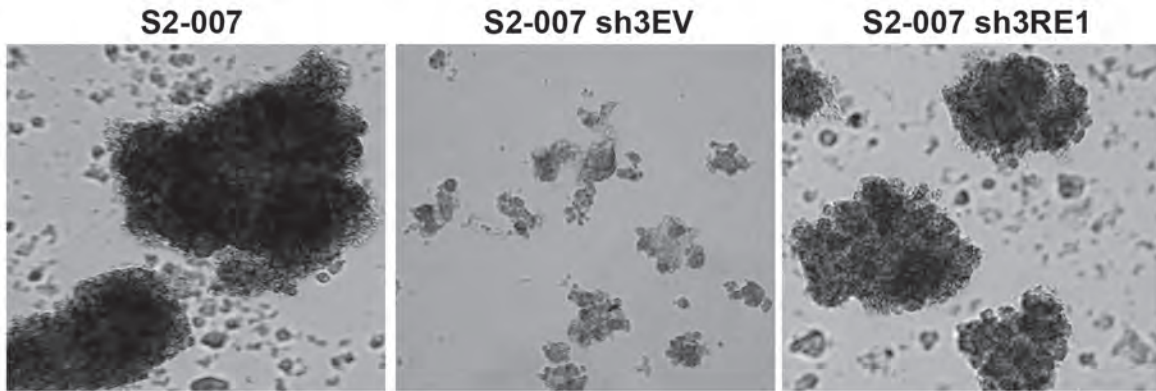


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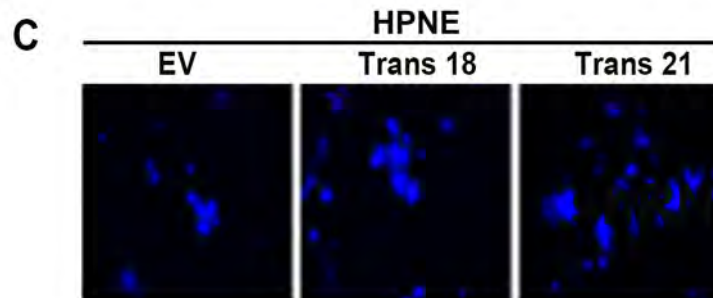
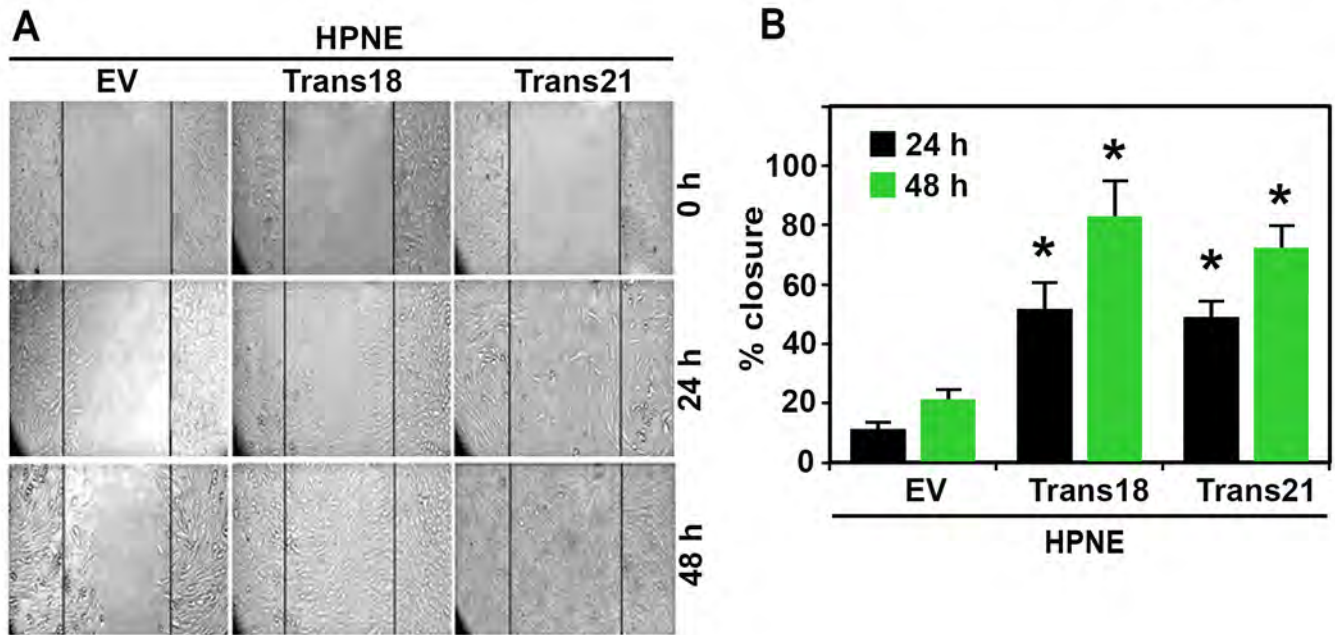
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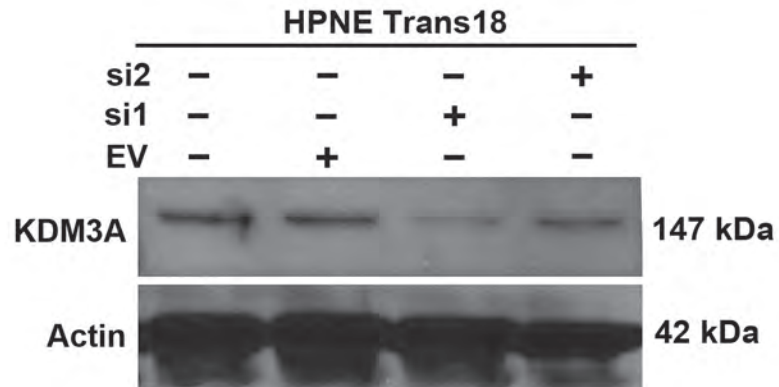


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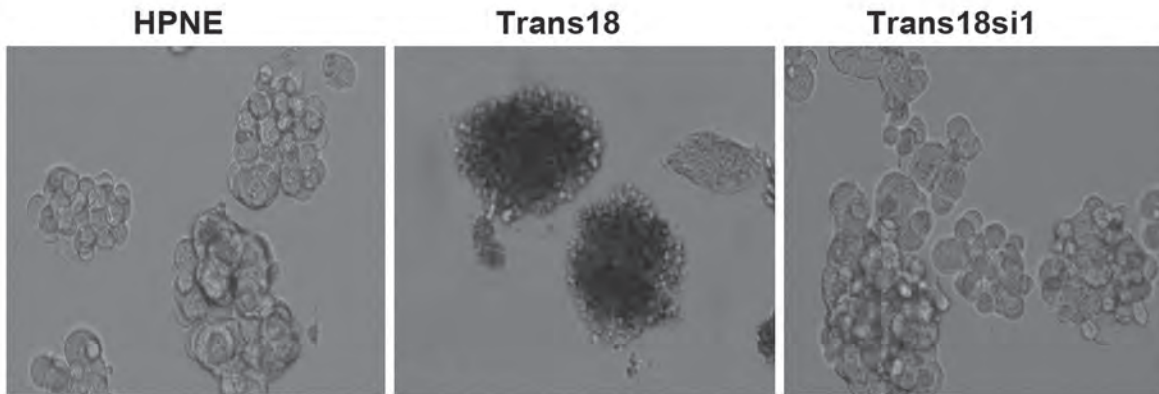


Supplementary Figure 5

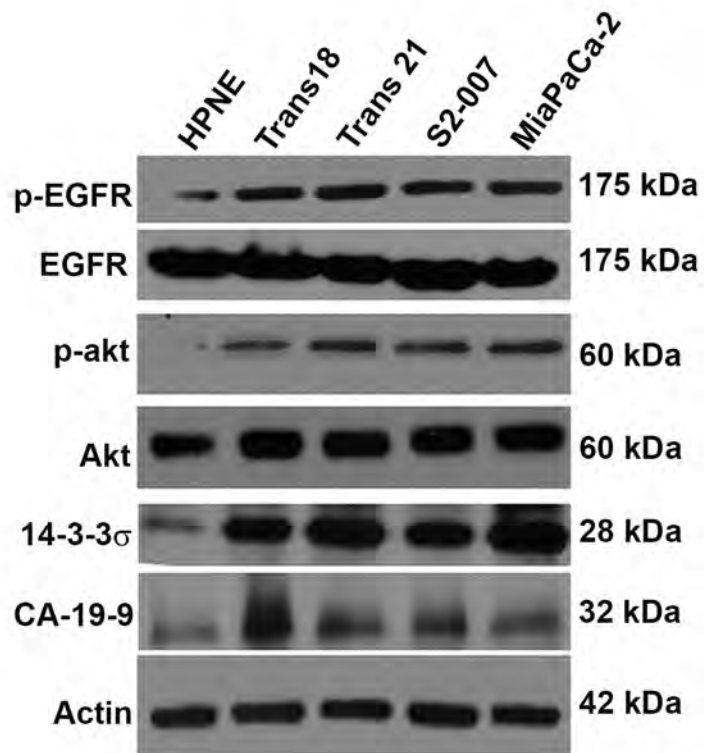
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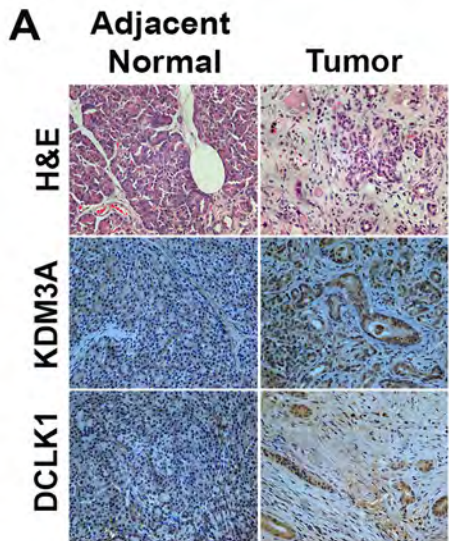


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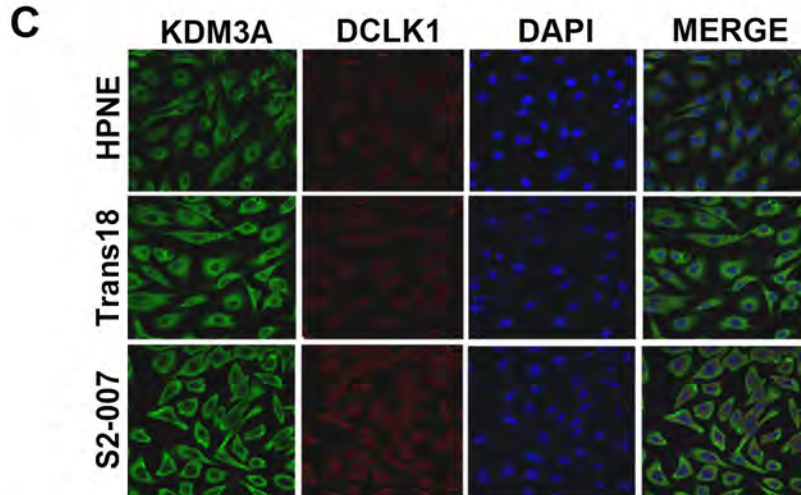
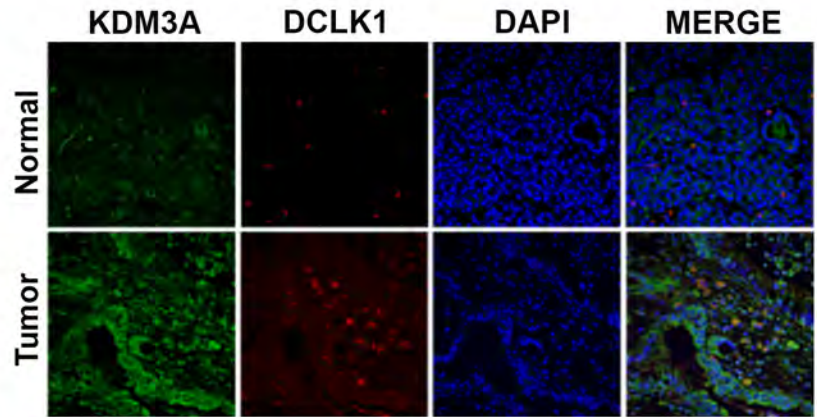


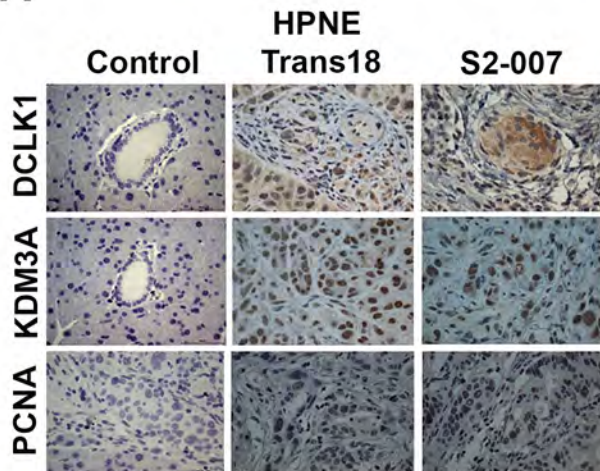
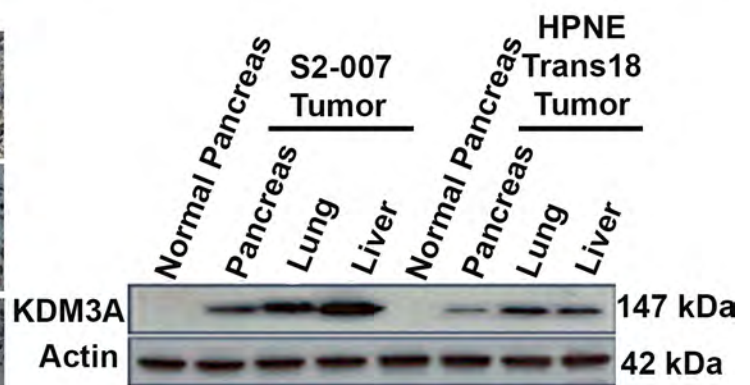
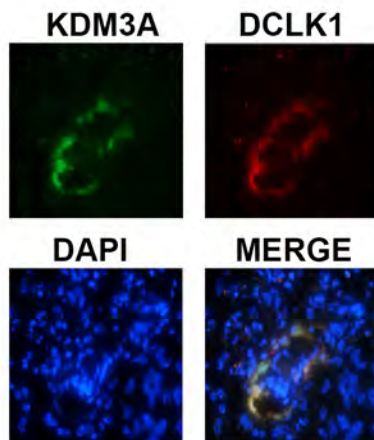
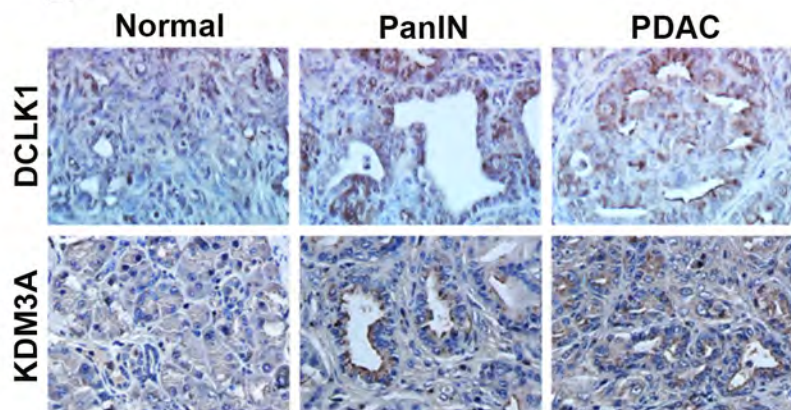
Supplementary Figure 6





B **Supplementary Figure 7**



A**B****Supplementary Figure 8****C****D**

Supplementary Figure 9

