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Supplemental Information

Bioselection Reveals miR-99b and miR-485 as Enhancers of Adenoviral Oncolysis in Pancreatic Cancer

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Supplemental Data Items:

A

miRNA	FC (PDAC vs HC)	FDR
miR-99b-5p	1,85	0,14
miR-99b-3p	**-1,50**	**0,03**
miR-485-5p	-1,10	1,00
miR-485-3p	**-2,35**	**0,0016**

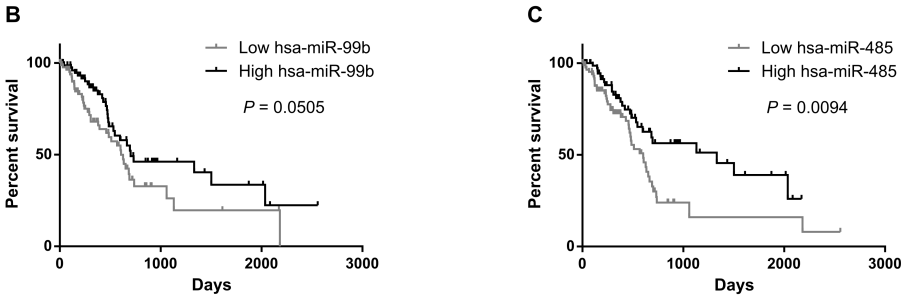


Figure S1. Overall survival and miR-99b and miR-485 expression correlation in PDAC patients. (A) Genome-wide miRNA profiling data of miR-99b and miR-485 represented as fold-change expression in PDAC patients (n = 11) versus healthy controls (n = 3). Differential expression was calculated with R version 2.13.0 using DESeq package 1.4.1 available in Bioconductor version 2.8[1]. Fold change was calculated as the ratio between normalized count data for tumor and normal samples. Significance is considered for miRNAs with a FDR< 0.05. **(B, C)** Kaplan-Meyer survival curves for patients with low or high miR-99b and miR-485 expression. Data were obtained from the TCGA database (https://tcga-data.nci.nih.gov) and divided in low and high miR expression using the median value as a cut-off. Statistical differences were evaluated with a non-parametric log-rank test.

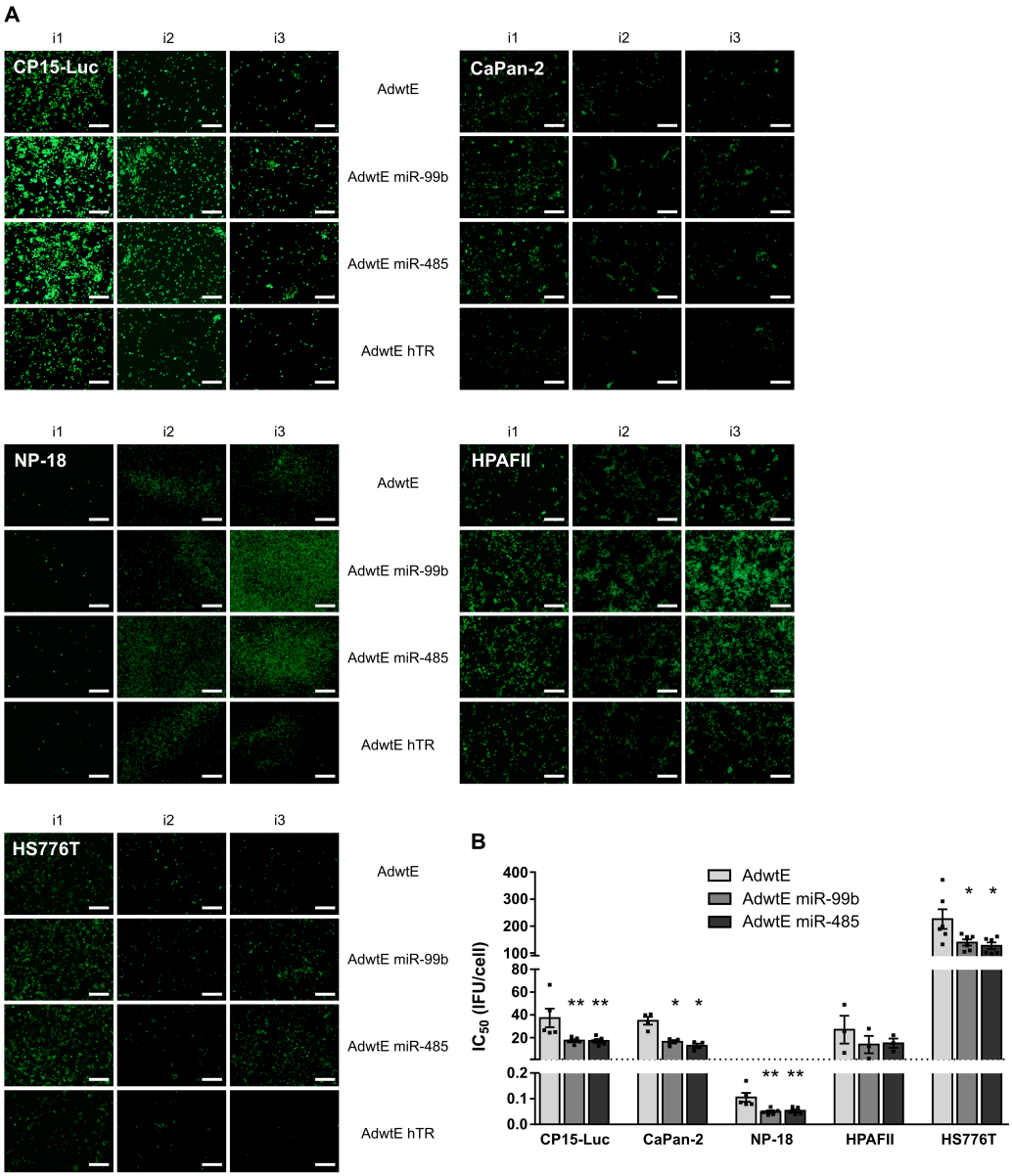


Figure S2. miR-99b and miR-485 encoding viruses have an increased activity in the patient-derived CP15-Luc and NP-18 cells and in CaPan-2, HPAFII and HS776T cell lines. **(A)** Cells were infected with AdwtE, AdwtE miR-99b, AdwtE miR-485, or AdwtE hTR (CP15-Luc, CaPan-2 and HPAFII – 10 IFU/cell; NP-18 – 0,05 IFU/cell; HS776T – 50 IFU/cell). At 48 h PI, virus-containing supernatants were collected and used to infect new cells (CP15-Luc – 50%; CaPan-2 and HS776T – 80%; HPAFII – 20%; NP-18 – 2%). EGFP expression was evaluated at the end of each infection. Representative fluorescent images are presented; original magnification, 4×; scale bar, 200 μm. **(B)** *In vitro* oncolytic activity in CP15-Luc, CaPan-2, NP-18, HPAFII and HS776T cells. Cells were seeded in triplicate and infected with a dose range of AdwtE, AdwtE miR-99b, or AdwtE miR-485. Cell viability was measured 7 days PI by MTT assay, and IC50 values were determined. Data are shown as mean ± SEM for at least three independent biological replicates. Significance was assessed by comparison to AdwtE infected cells using a two-tailed Mann-Whitney test. *P < 0.05, **P < 0.01.

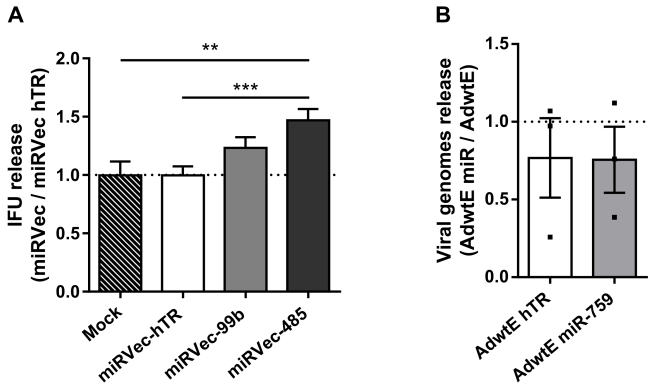
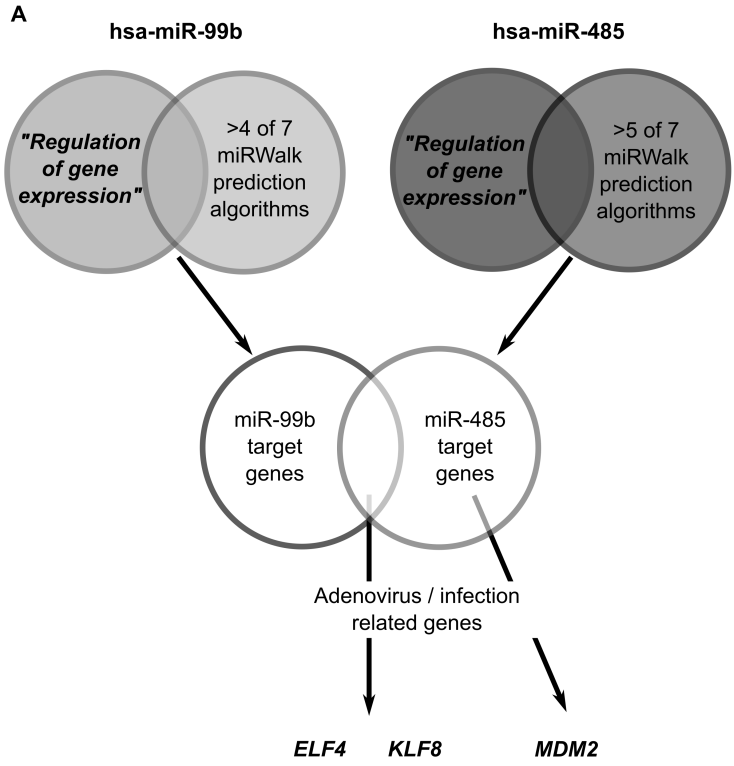


Figure S3. The increased viral release is specific for miR-99b and miR-485 expression. (A) Relative infective viral particles release in PANC-1 cells transfected with miRNA expression plasmids by using oligopeptide-modified poly(β-amino ester)s. PANC-1 cells were mock transfected or transfected with hTR or miRNA expression plasmids. At 24 h post-transfection, cells were infected with 5 IFU/cell of AdwtE. At 48 h PI, supernatants were collected and titrated by viral infectious units. Dashed line represents miRVec hTR transfected cells values. Data are shown as mean ± SEM for at least five independent biological replicates. Significance was assessed using a two-tailed Mann-Whitney test. $^{**}P < 0.01$, $^{***}P < 0.001$. **(B)** Viral genomes released in PANC-1 cells. Cells were infected with 1 IFU/cell of AdwtE, AdwtE hTR, or AdwtE miR-759. Supernatants were collected at 48 h PI, and viral genomes were quantified by qPCR. Dashed line represents AdwtE infected cells values. Data are shown as mean ± SEM for three independent biological replicates.



B

Gene	miR-99b-3p	miR-99b-5p	miR-485-3p	miR-485-5p
ELF4	1	-	-	1
KLF8	1	1	1	2
MDM2	-	-	1	7

Figure S4. Identification strategy of potential miR-99b and miR-485 target genes. (A) Bioinformatics analysis to identify potential miR-99b and miR-485 target genes (see Methods for detailed description). **(B)** Predicted target sites for miR-99b and miR-485 at the 3′ UTR of the *ELF4*, *KLF8*, and *MDM2* genes.

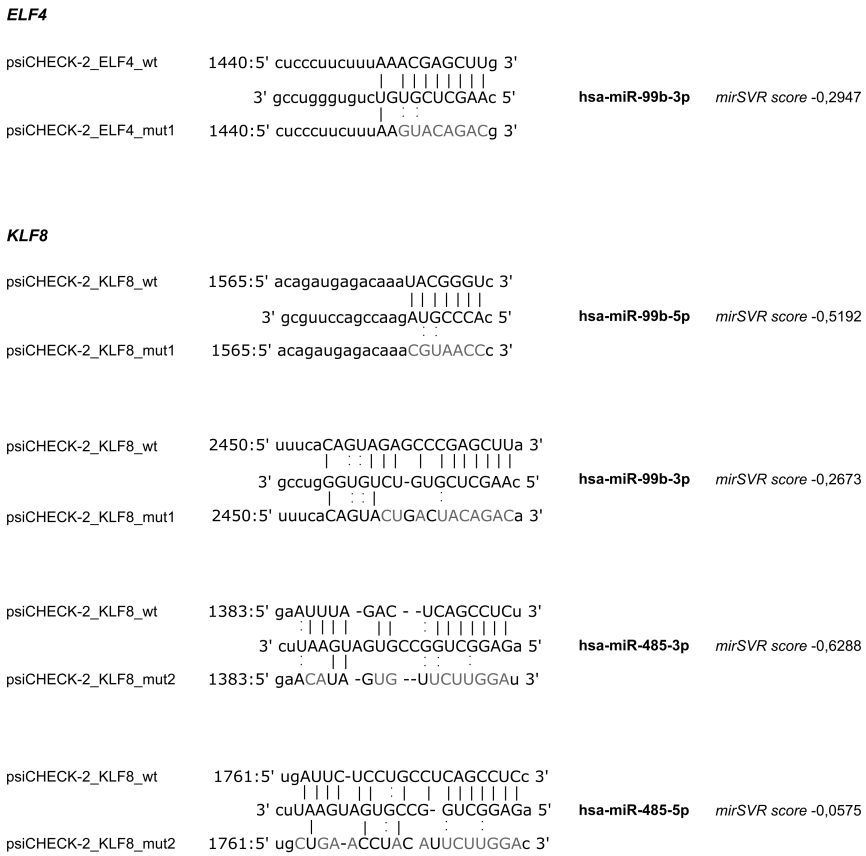


Figure S5. miR-99b and miR-485 target sites at the 3'UTR of ELF4, MDM2 and KLF8 genes. Wild type and mutagenized miR-99b and miR-485 target sites present at the3'UTR of ELF4 and KLF8 genes cloned in the psiCHECK-2 vector for further validation.

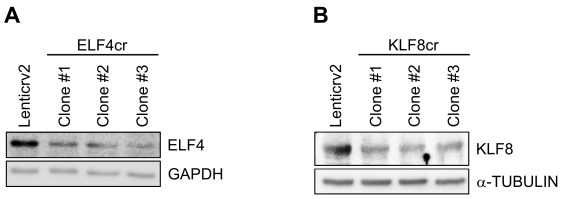
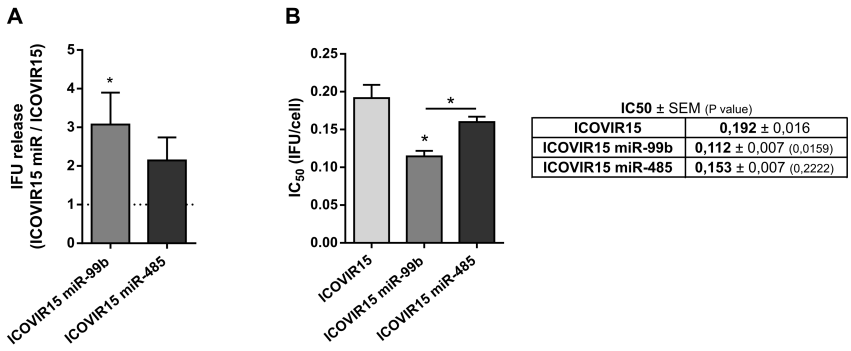


Figure S6. ELF4 and KLF8 downregulation in CRIPSR/Cas9 modified PANC-1 clones. (A, B) Representative ELF4 and KLF8 western blots of control PANC-1 cells (Lenticrv2) and three CRISPR/Cas9 modified PANC-1 clones.



IC50 ± SEM (P value)	
ICOVIR15	**0,192** ± 0,016
ICOVIR15 miR-99b	**0,112** ± 0,007 (0,0159)
ICOVIR15 miR-485	**0,153** ± 0,007 (0,2222)

Figure S7. miR encoding ICOVIR15 viral activity in MIA PaCa-2 cells. (A) Relative infective viral particles release. MIA PaCa-2 cells were seeded in triplicate and infected with ICOVIR15, ICOVIR15 miR-99b, or ICOVIR15 miR-485 (1 IFU/cell). Supernatants were collected at 48 h PI and titrated by viral infectious units. Dashed line represents ICOVIR15 values. **(B)** *In vitro* oncolytic activity in MIA PaCa-2 cells. Cells were seeded in triplicate and infected with a dose range of ICOVIR15, ICOVIR15 miR-99b, or ICOVIR15 miR-485. Cell viability was measured 7 days PI by MTT assay, and IC_{50} values were determined. Data are shown as mean ± SEM for at least five independent biological replicates. Significance was assessed using a two-tailed Mann-Whitney test. $*P < 0.05$.

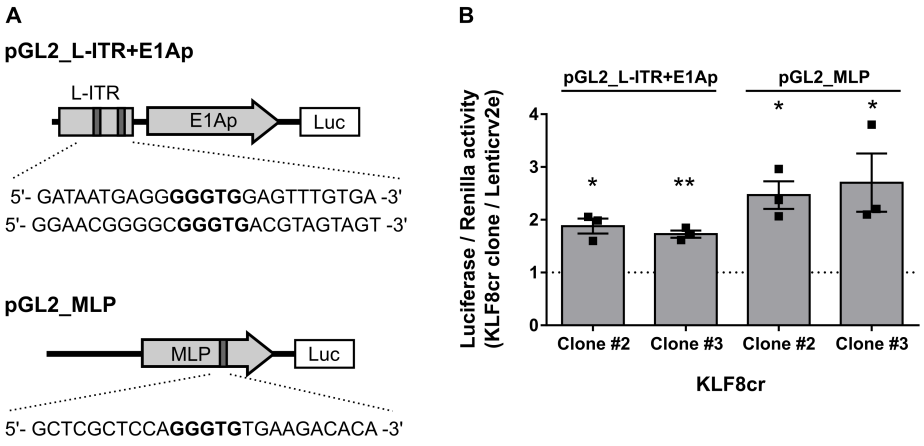


Figure S8. L-ITR+E1A and MLP transcriptional activation in low KLF8 expressing cells (A) Schematic diagram of pGL2 reporter vectors containing the L-ITR and E1A promoter regions (pGL2_L-ITR+E1Ap) or the Major Late Promoter (MLP) region (pGL2_MLP) controlling the expression of the *Luciferase* gene. The sequences surrounding the GT-boxes (KLF8 binding sites) are shown. **(B)** Control PANC-1 cells (Lenticrv2) and two CRISPR/Cas9 modified PANC-1 clones for KLF8 were co-transfected with pGL2_L-ITR+E1Ap or pGL2_MLP luciferase reporter vectors and a pGL4.75 control vector (Renilla gene). Luciferase and Renilla activities were evaluated 48 h post-transfection.

Table S1. miRNA encoding adenovirus isolated at the end of the bioselection process

PANC-1

miRNA	% clones
miR-99b	**7,1**
miR-485	**28,6**
miR-200c	21,4
miR-183	14,3
miR-324	14,3
miR-511	7,1
miR-345	7,1

MIA PaCa-2

miRNA	% clones
miR-99b	**87,9**
miR-485	**9,1**
miR-142	3,0

Table S3. Primers used for adenovirus and reporter plasmids generation

	Name	Sequence
1	psi-CHECK-2 Fw	GCTAAGAAGTTCCCTAACACCGAG
	psi-CHECK-2 Rv	CGCGTCAGACAAACCCTAACCAC
2	RpsLNeo Fiber Fw	caattggtactaagcggtgatgtttctgatcagccaccGGCCTGGTGATGATG
	RpsLNeo Fiber Rv	gacttgaaattttctgcaattgaaaaataaagtttattaTCAGAAGAACTCGTCA
3	Fiber-GFP Fw	caattggtactaagcggtgatgtttctgatcagccaccATGGTGAGCAAGGGCGAGG
	Fiber-GFP Rv	gacttgaaattttctgcaattgaaaaataaagtttattaCTTGTACAGCTCGTCCATGC
4	E4-RpsLNeo Fw	gaaaactacaattcccaacacatacaagttactccgccctaag*cagtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatt*TCA GAAGAACTCGTCA
	E4-RpsLNeo Rv	cgtggcgcggggcgtgggaacggggcgggtgacgtaggttGGCCTGGTGATGATG
5	E4-miRNA cua 2 Fw	gaaaactacaattcccaacacatacaagttactccgccctaaCTTTTATTTTATCGAATCTGC
	E4-miRNA cua Rv	cgtggcgcggggcgtgggaacggggcgggtgacgtaggttACATTGATTATTGACTAG
6	GFP_seq Fw	CTGGTCTGGCCACAACTACA
	GFP_seq Rv	GGAGGTGGCAGGTTGAATAC
7	Step3/4 Fw	CCAGAAACGAAAGCCAAAAA
	Step3/4 Rv	TAATGAGGGGGTGGAGTTTG

Lowercase - homology tails
Lowercase italics - polyA sequence

Table S4. Primers used for qPCR reactions

Set	Name	Sequence
1	E1A Fw	ATCGAAGAGGTACTGGCTGA
	E1A Rv	TTCCTCCGGTGATAATGACAAGACCTGCAAC
2	qPCR-Hexon Fw	GTCTACTTCGTCTTCGTTGTC
	qPCR-Hexon Rv	TGGCTTCCACGTACTTTG
3	qPCR-Fiber Fw	CTCCAACTGTGCCTTTTC
	qPCR-Fiber Rv	GGCTCACAGTGGTTACATT
4	Hexo01	GCCGCAGTGGTCTTACATGCACATC
	Hexo02	CAGCACGCCGCGGATGTCAAAG
5	Albumin Fw	GCTGTCATCTCTTGTGGGCTGT
	Albumin Rv	GGCTATCCAAACTCATGGGAG
6	5' E4 Fw3	GCCAAGTGCAGAGCGAGTAT
	Adwt R-ITR Rv2	CGGGGCGGGTGACGTAGGTTTTA
	AdmiR-99b Rv	CTATACGGCCTCCTAGCTTTCCGAATTC
	AdmiR-485 Rv	CTGGGGCACTACCAACTTTAGGCAATTC
7	Elf4 Fw	TCCTGGATGAGAAGCAGATCTTCA
	Elf4 Rv	ATGGTGCTGCCTTTGCCATC
8	Klf8 Fw	TGCTGGATCAGTGAAAGTTGACC
	Klf8 Rv	TTTATAAGGCTTCTCTCCTGTATGGATTCTG
9	Mdm2 Fw	ACAAGAGACCCTGGTTAGACCAAAG
	Mdm2 Rv	CCTGAGTCCGATGATTCCTGCTG
10	Gapdh Fw	TGTCAAGCTCATTTCCTGGTATGA
	Gapdh Rv	TTACTCCTTGGAGGCCATGTGGG

Table S5. Antibodies and conditions for western blot analysis

Antibody	Provider	Reference	Molecular size (KDa)	Dilution	Hybridization conditions
Adenovirus-2/5 E1A [M73]	Santa Cruz Biotechnology	sc-25	48-54	1/200	1h - RT
Adenovirus type 5 (hexon)	Abcam	ab6982	107	1/200	1h - RT
Adenovirus type 5 (penton)	Abcam	ab6982	64	1/200	1h - RT
Adenovirus fiber trimer [2A6]	GeneTex	GTX23232	62	1/200	1h - RT
ELF4 [AB1]	Sigma Aldrich	AV38028	71	1/500	O/N - 4ºC
KLF8	Aviva Systems Biology	ARP32859_P050	39	1/1000	O/N - 4ºC
MDM2 [SM14]	Santa Cruz Biotechnology	sc-965	90/60	1/200	O/N - 4ºC
GAPDH	Merck Millipore	ABS16	38-40	1/10000	1h - RT / O/N -4ºC
Tubulin	Sigma Aldrich	T9026	50	1/500	O/N - 4ºC

Secondary antibody	Provider	Reference	Molecular size (KDa)	Dilution	Hybridization conditions
Polyclonal Rabbit Anti-Mouse Immunoglobulins HRP	Agilent Technologies (Dako)	P0161	-	1/2000	45 min - RT
Polyclonal Goat Anti-Rabbit Immunoglobulins HRP	Agilent Technologies (Dako)	P0160	-	1/2000	45 min - RT

Supplemental Materials and Methods:

EGFP fluorescence quantification. EGFP fluorescence intensity in adenovirus infected cells was quantified from images obtained using the microscope Olympus IX51 (at a wave length of 480 nm) and ImageJ v10.2 software.

Reporter gene assays. HEK293T cells were seeded in triplicate (4×10^4 cells/96-well plate); 24 h later, transient transfections were performed using CalPhos Mammalian Transfection Kit (Clontech). About 50 ng of psiCHECK-2_ELF4_wt, psiCHECK-2_ELF4_mut1, psiCHECK-2_KLF8_wt, psiCHECK-2_KLF8_mut1, or psiCHECK-2_KLF8_mut2 were co-transfected with 100 ng of miRVec control, miRVec-99b, or miRVec-485. Renilla and firefly luciferase activities were measured at 48 h post-transfection using the DualGlo® Luciferase Assay (Promega).

Western blot analysis. Total protein extracts were obtained using lysis buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, and 10% glycerol) containing 1% Complete Mini Protease Inhibitor (Roche). Lysates were boiled for 10 min at 98ºC and centrifuged 5 min at 16,000 × *g*. Protein concentration was determined by BCA Protein Assay kit (ThermoFisher Scientific). Forty mg of total protein was resolved in 7.5%–10% SDS-PAGE and transferred to nitrocellulose or PVDF membranes by standard methods. Membranes were blocked with TBS-Tween 10% milk (1 h at room temperature), immunoblotted with the corresponding antibody diluted in TBS-Tween 5% milk (Table S5), rinsed with TBS-Tween, and incubated with HPR-conjugated goat anti-rabbit or rabbit anti-mouse secondary antibodies (1/2000 in TBS-Tween 5% milk, 45 min at room temperature). Antibody labelling was detected by ECL Amersham Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences).

cDNA synthesis and RT-qPCR of viral and cellular RNA. Total RNA from tumors and cell cultures was obtained using miRNeasy Mini RNA Extraction Kit (Qiagen). About 500 ng of total RNA was reverse-transcribed to generate cDNA using Moloney murine leukemia virus reverse transcriptase and Oligo dT from PrimeScript RT Reagent Kit (Takara), according to manufacturer's instructions. qPCR reactions were performed on a ViiA7 System using SYBR Green I Master Mix (Roche), using 1 ml of cDNA and primers specific for each reaction (Table S4).

In vitro cytotoxicity assays. PANC-1 and MIA PaCa-2 cells were seeded in triplicate (5×10^3 cells/well in 96-well plates) and infected at different viral doses. At 4 h PI, virus-containing medium was replaced with fresh medium. Cell viability was measured at 7 days PI with the MTT colorimetric assay (Affymetrix, USB® Products).

CRISPR/Cas9-modified PANC-1 cells generation. Single guide RNA sequences targeting *ELF4* and *KLF8* were designed using the CRISPR/Cas9 MIT webtool (http://crispr.mit.edu/) in combination with Breaking Cas software (http://bioinfogp.cnb.csic.es/tools/breakingcas/). The oligonucleotides containing sgRNAs were synthesized by IDT (Integrated DNA Technologies) and cloned in lenticrv2 plasmid (Addgene #52961) following protocol described by Zheng Lab at MIT (available online at https://media.addgene.org/data/plasmids/52/52961/52961-attachment_B3xTwla0bkYD.pdf); sgELF4 (exon 3) 5′-CGCGGTTGACATGGTGTGCG-3′ and sgKLF8 (exon 3) 5′-TATGACTTCTCCAACACTCC-3′. Plasmids containing sgRNAs were transfected to HEK293T cells together with packaging pCMVAR 8.91 and envelope pVSV plasmids to obtain lentiviral particles. Transduction of PANC-1 cells with these lentiviruses generated pools of cells genetically modified via CRISPR/Cas9 technology. Cells were selected with puromycin (8 μg/ml) for one week, and single cell clones were isolated. Alterations of the genomic sequence were analyzed via Sanger sequencing using the corresponding primers for *ELF4* EX3 (Fw: 5′-GGACTTTGAGGACATCGTGC-3′, Rw: 5′-CGGGCTGGCCCTGATTATAG-3′) and *KLF8* EX3 (Fw: 5′-GCTTGCATGTCTCTTCAGGG-3′, Rw: 5′-CCATCTCCTCCAATGAGTGGG-3′). In the selected clones, absence of protein expression was validated by Western blotting.

Promoter reporter gene assay. pGL2_LITR+E1Ap and pGL2_MLP were generated by cloning the corresponding promoter region (Ad5 L-ITR+E1Ap – 1bp to 559bp, MLP – 5787bp to 6053bp) into a pGL2 vector. Promoter regions were amplified by PCR with primers containing a HindIII restriction site at their 5' end. PCR products were digested with HindIII (Thermo Fisher Scientific) and introduced into the pGL2 vector, digested with the same restriction enzyme. Plasmid constructions were tested by colony PCR and validated by Sanger sequencing. Control PANC-1 cells (Lenticrv2) and two CRISPR/Cas9 modified PANC-1 clones for KLF8 were seeded in triplicate (4×10^4 cells/96-well plate); 24 h later,

transient transfections were performed with Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific). About 100 ng of pGL2_LITR+E1Ap and pGL2_MLP were co-transfected with 50 ng of pGL4.75 vector. Firefly and Renilla activities were measured at 48 h post-transfection.

Immunofluorescence detection of adenovirus E1A protein in tumors. OCT-embedded sections of PANC-1 tumors, obtained at the end of the antitumoral efficacy study, were fixed in a 4% paraformaldehyde solution for 5 min, rinsed three times with PBS 1× + 0.1% Triton™, and incubated for 1 h at room temperature with PBS 1× and 20% goat serum. Sections were incubated overnight at 4ºC with anti-adenovirus-2/5 E1A [M73] antibody (Santa Cruz Biotechnology) diluted 1:50 in PBS 1× and 5% goat serum, rinsed with PBS 1× and 0.1% Triton™, and incubated for 1 h at room temperature with goat anti-rabbit igG (H+L) Secondary Antibody Alexa 633 (Invitrogen) diluted 1:200 in PBS 1× and 5% goat serum. Finally, sections were rinsed three times with PBS 1× and 0.1% Triton™, incubated with 100 ng/ml DAPI, rinsed with PBS 1×, and mounted using Vectashield® (Vector Laboratories). Images were visualized under a fluorescent microscope (Nikon Eclipse 50i), captured using a digital camera (Cool Cube1, MetaSystems), and processed with ImageJ v10.2 software.

miRNA target genes identification analysis. A list of potential target genes predicted by different algorithms (miRWalk, miRanda, miRDB, Pictar2, PITA, RNA22, and Targetscan) in the miRWalk database (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) was obtained for miR-99b and miR-485. Each list was loaded into the GO enrichment analysis tool (http://www.geneontology.org/page/go-enrichment-analysis) and classified in terms of biological processes. The first commonly enriched GO term for both miRNAs was selected. From the genes belonging to that term, those predicted by at least 4 (miR-99b) or 5 (miR-485) prediction algorithms in the miRWalk database were selected for further study. miRNA target site scores were obtained from the microRNA.org database (miRDB algorithm) (http://www.microrna.org/microrna/getMirnaForm.do).

Supplemental References:

1. Anders S, Huber W. (2010). Differential expression analysis for sequence count data. *Genome Biol*. **11**:R106.