iScience, Volume *23*

Supplemental Information

BxPC-3-Derived Small Extracellular Vesicles Induce FOXP3+ Treg through ATM-AMPK-Sirtuins-Mediated FOXOs Nuclear Translocations

Tao Shen, Shengnan Jia, Guoping Ding, Dongnan Ping, Liangjing Zhou, Senhao Zhou, and Liping Cao

TRANSPARENT METHODS

Ethics Statement

The research protocol was reviewed and approved by the Research Ethics Committee of Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University (Reference Number: ZJU20170222-23). All experiments were conducted in accordance with approved guidelines of the Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University. Written informed consent for scientific research statement was obtained from all participants.

Reagents

The antibodies used in the study were: p-AMPK (T183/T172) (Abcam, Cat#ab133448), SIRT1 (Abcam, Cat#ab32441), SIRT2 (Abcam, Cat#ab134171), SIRT6 (Abcam, Cat#ab176345), p-ATM (S1981) (Cell Signaling Technologies, Cat#5883), ATM (Abcam, Cat#ab32420), β-Actin (Abcam, Cat#ab179467), γ-H2AX (Abcam, Cat#ab26350), RAD51 (Abcam, Cat#ab133534), RPA2 (Abcam, Cat#ab2175), 53BP1 (Abcam, Cat#ab175188), FOXO1A (Abcam, Cat#ab52857), FOXO3A (Abcam, Cat#ab23683), FOXP3 (Abcam, Cat#ab20034), Histone H3 (Abcam, Cat#ab201456), anti-mouse AlexaFluor 488 secondary antibody (ThermoFisher, Cat#A-21202), anti-mouse AlexaFlour 594 secondary antibody (ThermoFisher, Cat#A-21203), and anti-rabbit AlexaFlour 594 secondary antibody (ThermoFisher, Cat#A-21207).

The chemicals, peptides, and recombinant proteins used in the study were: KU-60019 (MedChemExpress, Cat#HY-12061), Compound C (MedChemExpress, Cat#HY-13418A), EX-527 (MedChemExpress, Cat#HY-15452), AGK2 (MedChemExpress, Cat#HY-100578), OSS_128167 (MedChemExpress, Cat#HY-107454), anti-CD3ε antibody (clone OKT3) (Miltenyi Biotec, Cat#130-093-387), and recombinant human interleukin 2 (PeproTech, Cat#200-02).

The critical commercial assays used in the study were: Transcription Factor Buffer Set (BD Bioscience, Cat#562574), Enhanced BCA Protein Assay Kit (Beyotime, Cat#P0010S), Rabbit Polymer Detection System (ZSbio, Cat#PV-6001), Mouse Enhanced Polymer Detection System (ZSbio, Cat#PV-9002), DAB kit (ZSbio, Cat#ZLI-9017), Human TGF-beta 1 ELISA Kit (RayBiotech, Cat#ELH-TGFb1), and TB-green Premix Ex Taq II (TAKARA, Cat#RR820Q).

Cell Culture

Human PDAC cell lines BxPC-3 and PANC-1 were obtained from American Type Culture Collection. Human pancreatic stellate cell line HPaSteC (HPSC) were obtained from BeNa Culture Collection. BxPC-3 cells were maintained in RPMI 1640 medium (GIBCO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, GIBCO), 100U/mL penicillin (Solarbio), and 100mg/mL streptomycin (Solarbio) at 37°C in a humidified atmosphere with 5% CO_2. HPSC and PANC-1 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCO) supplemented with 10% (v/v) heat-inactivated FBS (GIBCO) at 37°C in a humidified atmosphere with 5% CO_2.

Patients and tissue samples

Forty formalin-fixed and paraffin-embedded lymph node specimens including 20 tumor-infiltrated and paired 20 normal lymph nodes, and forty pancreatic specimens including 20 tumor and paired 20 normal tissue, were obtained from 20 PDAC patients (5 female and 15 male patients with a median age of 63.4 years; age range, 49-79 years) who underwent R0 surgical resection at the Department of General Surgery, Sir Run Run Shaw Hospital of Zhejiang University School of Medicine between 2011 and 2016. All cases were confirmed by pathological diagnosis. None of them had received radiotherapy, chemotherapy, hormone therapy or other related anti-tumor therapies before surgery.

Generation of tdTomato/EGFP-BxPC-3 cells

sEVs-CD63-toTomato virus and sEVs-TGF-β1-EGFP virus were obtained from S&E Shanghai Medical Biotechnology Co., Ltd. For labeling CD63 (or TGF-β1) with fluorescence, BxPC-3 cells were transfected with a CMV promoter-driving tdTomato-tagged CD63 gene (or EGFP-tagged TGF-β1 gene) using lentivirus vector system. Culture medium added with 1μg/mL puromycin (Beyotime) was using to purify stably transfected BxPC-3 cells.

Purification and Verification of sEVs

sEVs were purified from serum-free supernatants of human HPSC, BxPC-3, PANC-1, tdTomato-BxPC-3, Ctrl/CD63-BxPC-3, or TGF-β1/CD63-BxPC-3 cells. Briefly, the supernatants were filtered through a 0.22μm sterile filter (EMD Millipore) and centrifuged at 200 × *g* for 10 min, 2,000 × *g* for 20 min, 10,000 × *g* for 30 min, and 100,000 × *g* for 1 hour at 4°C (Optima XPN-100). After the last centrifugation step, the pellets were suspended in PBS and then centrifuged at 100,000 × *g* for 2 hours at 4°C. The purified sEVs were re-suspended in PBS and stored at -80°C. To evaluate the morphology of isolated sEVs, the sEVs were settled on carbon-coated 400-mesh copper grids, stained with 2% uranyl acetate, air-dried,

and imaged by transmission electron microscopy (Tecnai T10). Nanoparticle tracking analysis was performed using the Dynamic Light Scattering System of the NanoSight LM10 (NanoSight Ltd).

For separation of subtypes of PC derived extracellular vesicles, we centrifuged the supernatants of BxCP-3, PANC-1 and HPSC cells at 200 × g for 10 min and 2,000 × g for 20 min at 4℃ to remove the cell debris. Debris-free supernatants were then centrifuged at 10,000×g for 30 min at 4℃ to extract lEVs. The pellets were re-suspended in PBS and then centrifuged at 10,000×g for 1 hours at 4℃ to purify lEVs. Meanwhile, the lEVs-free supernatants were filtered by 0.22μm sterile filter and centrifuged at 100,000×g for 1 hour at 4℃ to extract small size (<200nm) extracellular vesicles (sEVs). After the last centrifugation step, the pellets were re-suspended in PBS and then centrifuged at 100,000×g for 2 hours at 4℃ to purify sEVs. Finally, sEVs-free supernatants were prepared as lyophilized powders at -80℃ using FreeZone Freeze dryer (Labconco). The lEVs, sEVs and supernatant lyophilized powders were re-suspended in PBS and their protein concentrations were detected using BCA method.

Preparation of human peripheral T lymphocytes

This experiment was conducted in accordance with approved guidelines of the Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University. Written informed consent for scientific research statement was obtained from all healthy volunteers. At Day 0, human peripheral blood mononuclear cells (PBMCs) from 32 age-matched healthy volunteers were isolated using Ficoll-Paque PLUS Medium (GE Healthcare) density gradient centrifugation. PBMCs were cultured with RPMI 1640 medium (GIBCO) at 37°C in a humidified atmosphere with 5% CO_2. After 2 hours culture, the adherent PBMC were discarded as a monocytes fraction, and the nonadherent PBMC were collected as a T lymphocytes fraction, as reported previously (Wieckowski et al., 2009). Subsequently, T lymphocytes were stimulated by 100ng/mL anti-CD3ε antibody (clone OKT3, Miltenyi Biotec) overnight and maintained in RPMI 1640 medium (GIBCO) supplementing with 10% (v/v) heat-inactivated volunteer-derived autologous serum and 100U/mL recombinant human interleukin 2 (rhIL-2, PeproTech) at 37°C in 5% CO_2. Fresh rhIL-2 and medium were added every 2 days. Serum sEVs were depleted from autologous serum by ultracentrifugation.

***In vitro* T lymphocytes Treatment**

Peripheral T lymphocytes which is isolated from PBMC and pre-stimulated by anti-CD3 antibody were adjusted to the density of 1×10^6/mL, and the isolated sEVs were added to each T lymphocytes sample at the concentration of 100μg/mL or at titration dose at Day 1 and Day 3 (sEVs-T). Isovolumetric PBS was added as a control (Ctrl-T). At Day 4, the T lymphocytes were harvested for follow-up experiments,

such as cytotoxicity assay, flow cytometry, CyTOF and western blotting. For ATM, AMPK, SIRT1, SIRT2 or SIRT6 inhibition, at Day 2, peripheral T lymphocytes were adjusted to the density of 1 × 10^6/mL, and stimulated with 50 μM KU60019 (MedChem Express), 20 μM Compound C (MedChem Express), 10 μM EX-527 (MedChem Express), 40 μM AGK2 (MedChem Express) or 100 μM OSS_128167 (MedChem Express), respectively.

Fluorescent Tracing of sEVs Transfer

For tracing of sEVs transfer, tdTomato-sEVs were added to peripheral T lymphocytes at Day 1 and Day 3. Stimulated T lymphocytes were twice rinsed, re-suspended in 200μL PBS, and adhered onto a poly-L-lysine solution (Sigma-Aldrich) -charged coverslip. T lymphocytes were fixed by 4% paraformaldehyde for 20 minutes at room temperature, mounted in Fluoroshield Mounting Medium with DAPI (Abcam) and then visualized using ZEISS LSM 800 confocal microscope (Zeiss).

Cytotoxicity Assay

The *in vitro* cytotoxicity of the harvested T lymphocytes against the HPSC, BxPC-3 or PANC-1 cells was determined using the Real-Time Cell Analyzer (RTCA)-DP xCELLigence system (Roche Applied Science), which operates by tracking electrical impedance signals and enables the cell growth status to be monitored in real time on microelectrode-coated plates. HPSC, BxPC-3 or PANC-1 cells (n=5000) were seeded on each well of E-Plate 16 (Roche Applied Science) and incubated in normal cell culture medium for 24 h. In the next step, the T lymphocytes were collected and co-incubated with the HPSC, BxPC-3 or PANC-1 cells in the proportion of 20:1 for 48 h. During the time of co-incubation, the viability of HPSC, BxPC-3 or PANC-1 cells were monitored in real time, and expressed by means of cell index.

TGF-β1 ELISA Assay

For supnatant:

In day 4, supernatant of Ctrl-T, sEVs-T were collected and centrifuged at 200 × g for 10 min and 2,000 × g for 20 min at 4℃ to remove the cell debris. Debris-free supernatants were then centrifuged at 10,000×g for 30 min at 4℃ to remove lEVs. lEVs-free supernatants were centrifuged at 100,000×g for 1 hour at 4℃ to remove sEVs. sEVs-free supernatants were then used to perform ELISA according to the manufacturer's protocol.

For subtypes of BxPC-3-derived extracellular vesicles:

The lEVs, sEVs and supernatant lyophilized powders were re-suspended in PBS, underwent

ultrasonic decomposition, and their protein concentrations were detected using BCA method. lEVs, sEVs and supernatant were then used to perform ELISA according to the manufacturer's protocol. According to the protein concentration, the finally measured TGF-β1 concentration was converted to pg/mg.

CyTOF analysis

CyTOF analyses were performed by PLTTech Inc. as previously reported (Han et al., 2018). In brief, at Day 4, T lymphocytes were collected, fixed, permeabilized, stained with antibody mix and rinsed. The marker signals were detected by CyTOF system (Helios), and the types of T lymphocytes were identified via non-linear dimensionality reduction algorithm [t-distributed stochastic neighbour embedding (tSNE)] and k-means-clustering algorithm, as previously reported (Lim et al., 2019).

Gene set enrichment analysis

GSEA3.0 software was used for identification of enriched signatures obtained from the MSigDB 6.2 hallmark and curated gene datasets.

Western Blotting

Cells were lysed with RIPA buffer (Beyotime) supplemented with protease and phosphatase inhibitor cocktail (Beyotime) on ice for 15 min. Lysates were centrifuged at 12,000 × *g* for 5 min to remove cell debris. The protein concentrations were measured by BCA protein assay kit (Beyotime). And then, the supernatant was taken up in SDS/PAGE sample loading buffer (Beyotime) and boiled for 10 minutes at 95°C. Twenty μg protein was separated by SDS-PAGE and transferred to 0.2μm polyvinylidene fluoride membrane (Bio-Rad Laboratories). Membranes were blocked for 60 min in tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) supplemented with 5% (w/v) non-fat dry milk or bovine serum albumin for 1 hour at room temperature and incubated with primary antibodies overnight at 4°C. The next day, membranes were thrice washed in TBST, incubated with horseradish-peroxidase conjugated secondary antibodies (Thermo Fisher Scientific) for 1 hour at room temperature, and then thrice washed in TBST. Bands were imaged using ChemiDoc Touch Imaging System (Bio-Rad Laboratories) and the band intensities were quantified using the Image Lab 5.2.1 software (Bio-Rad Laboratories).

Flow cytometry

The expression markers on Treg were determined by flow cytometry analyses after surface staining with FITC-conjugated mouse anti-CD4 (555346, BD Biosciences) BV421-conjugated mouse anti-CD25 (562442, BD Biosciences) or intracellular staining with PE-conjugated mouse anti-FOXP (560046, BD

Biosciences). The transcription factor buffer set was utilized for fixation/permeabilization of cells before FOXP3 staining. All stained cells were analyzed using BD LSRFortessa (BD Bioscience) and the data were analyzed by FlowJo 10.0.7 software.

Immunofluorescence and confocal microscopy

At Day 4, stimulated T lymphocytes were twice rinsed, re-suspended in PBS at a density of 1×10^5 cells, adhered onto a Poly-L-Lysine-charged coverslip at 37℃ for 30 minutes. The cells were fixed with formalin for 20 minutes at room temperature, blocked using 5% (w/v) Bovine serum albumin (BSA) in PBS supplemented with 0.3% (v/v) Triton X-100 (Sigma-Aldrich) for 30 minutes at room temperature. After blocking, cells were incubated with mouse anti-γ-H2AX (S139) antibody (ab26350, Abcam) at dilution 1:200, mouse anti-RPA2 antibody (ab2175, Abcam) at dilution 1:300, rabbit anti-53BP1 antibody (ab175188, Abcam) at dilution 1:200 or mouse anti-RAD51 antibody (ab133534, Abcam) at dilution 1:200 for 1 hour at room temperature. Next, cells were washed gently using PBS and incubated with anti-mouse AlexaFluor 488 secondary antibody (1:2000, A-21202, ThermoFisher), anti-mouse AlexaFlour 594 secondary antibody (1:2000, A-21203, ThermoFisher) or anti-rabbit AlexaFlour 594 secondary antibody (1:2000, A-21207, ThermoFisher) for 45minutes at room temperature. After washing, the coverslips were mounted in Fluoroshield Mounting Medium with DAPI (Abcam) and images were taken using ZEISS LSM 800 confocal microscope (Zeiss).

Quantitative PCR

Quantitative PCR (qPCR) was performed with a TB-green Premix Ex Taq II Kit (TAKARA) on the LightCycle 480 II (Roche) as follows: 95°C for 3 minutes; followed by 40 cycles of 95°C for 10 seconds; and 60°C for 20 seconds. GAPDH was used as the control genes. The target genes and primers were:

SIRT1 (F5'-AAGGGATGGTATTTATGCTC-3' and R5'-ACAAGGCTATGAATTTGTGA-3');

SIRT2 (F5'-GTTCAAGCCAACCATCTGTCA-3' and R5'-CTTAGCGGGTATTCGTGCC-3');

SIRT3 (F5'-TTGGCTTGGCATCCTC-3' and R5'-GTCCTCCTCAGCAGTCTGTA-3');

SIRT4 (F5'-TCCCAACCTGCGTTCA-3' and R5'-CCAGGCAGTGAGGATAA-3');

SIRT5 (F5'-TGCCAGCATCCCAGTTGAG-3' and R5'-CACAGAGGAAGTGCCCACC-3');

SIRT6 (F5'-CCCACGCAGACCCACAT-3' and R5'-TTGGCACATTCTTCCACAAA-3');

SIRT7 (F5'-CAGCACGGCAGCGTCTATC-3' and R5'-CTCATGTGGGTGAGGGTTGG-3');

GAPDH (F5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and R5′-AGCCTTCTCCATGGTGGTGAAGAC-3′).

Structured illumination microscopy

At Day 4, stimulated T lymphocytes were twice rinsed, re-suspended in PBS at a density of 1×10^5 cells, adhered onto a Poly-L-Lysine-charged coverslip at 37℃ for 30 minutes. The cells were fixed with formalin for 20 minutes at room temperature, blocked using 5% (w/v) Bovine serum albumin (BSA) in PBS supplemented with 0.3% (v/v) Triton X-100 (Sigma-Aldrich) for 30 minutes at room temperature. After blocking, cells were incubated with rabbit anti-FOXO1A antibody (ab52857, Abcam) at dilution 1:200, rabbit anti-FOXO3A antibody (ab23683, Abcam) at dilution 1:150, or mouse anti-FOXP3 antibody (ab150117, Abcam) at dilution 1:100 for 1 hour at room temperature. Next, cells were washed gently using PBS and incubated with anti-rabbit AlexaFlour 594 secondary antibody (1:2000, A-21207, ThermoFisher) or anti-mouse AlexaFluor 488 secondary antibody (1:2000, A-21202, ThermoFisher) for 45minutes at room temperature. After washing, the coverslips were mounted in Fluoroshield Mounting Medium with DAPI (Abcam) and images were taken using a 3D structural illumination microscope equipped with a 100× objective lens. For z-stack analysis, optical sections were obtained from the interface along the Z-axis at 0.2 μm intervals. Image analysis and mean fluorescence intensity (MFI) calculation were performed by Imaris v9.5 software. Briefly, the overall spatial structure of FOXO1A, FOXO3A or FOXP3 was reconstructed according to the channel information of AlexaFlour 488 or 594, the spatial structure of nucleus was reconstructed according to the channel information of DAPI, and the co-localized area of AlexaFlour and DAPI was segmented as the nuclear expression of FOXO1A or FOXO3A. And then, FOXO1A or FOXO3A nuclear expression ratio was calculated using the following equation: Ratio of nuclear/total (%)=co-localized area MFI / overall spatial structure MFI.

Human pancreatic tissues and lymph nodes immunohistochemical analysis

Paraffin-embedded lymph node tissue sections were dewaxed by dimethylbenzene and rehydrated through a gradient ethanol series and washed by PBS. Then, the sections were bathed on citric acid buffer (PH6.0) at 95–98 °C for 15 min for antigen retrieval. Naturally cooled to room temperature, the sections were incubated in 5% (w/v) BSA in PBS supplemented with 0.3% (v/v) Triton X-100 (Sigma-Aldrich). Following the serum block, sections were incubated with primary antibody for FOXO1A (1:200, Abcam), FOXO3A (1:150, Abcam) or FOXP3 (1:100, Abcam) at 4°C overnight. Isotype-matched antibodies were used as negative controls. Next day, sections were washed with PBS, incubated secondary antibodies using Rabbit Polymer Detection System (ZSbio) or Mouse Enhanced Polymer Detection System (ZSbio), and stained by DAB kit (ZSbio). Counterstaining of sections was performed with hematoxylin and dehydrated. The slides were mounted using neutral resin (Biosharp) and imaged using Leica DM4000 microscope (Leica). In each case, we checked that the secondary antibodies did not cross-react with the

isotype. The positive expression score of FOXO1A, FOXO3A or FOXP3 in nuclear was quantified using ImageJ software with the plugin of IHC Profiler according to manuscript. The positive expression rate of FOXO1A, FOXO3A or FOXP3 was calculated as the follow equation: positive expression rate (%)= positive expression score / the number of T lymphocytes in 40x objective field. Two authors, blinded for clinical data, independently calculated the positive expression rate of FOXO1A, FOXO3A or FOXP3.

Statistical analysis

Statistical analyses were performed using SPSS software version 23.0 (IBM Corp.). Two-tailed Student's *t*-tests were used for comparisons between two groups from immunofluorescence data, flow cytometry data, CyTOF data or Western blotting data. Pearson's correlation coefficient was used to evaluate the correlation matrices. Paired *t*-test was used to compare the FOXO1A, FOXO3A or FOXP3 expression between matched negative and positive lymph nodes. The p-value<0.05 was considered statistically significant. Graphical representations were performed GraphPad Prism 6 software (GraphPad Software, Inc.).

Supplementary Figure Titles and Legends

Figure S1 (related to Figure 1): The effect of HPSC- and PANC-1-derived sEVs on T lymphocytes.

(A) DLS system measured the average size of HOSC- or PANC-1-derived sEVs.

(B) TEM image showing the morphology of sEVs (black arrowheads).

(C) Western blotting assays were used to analyze the expression of sEVs biomarkers.

(D) Line chart showing the HPSC (above) or PANC-1 (below) survival curve. Cell index (Y-axis) indicating the number of live HPSC or PANC-1 cells. The survival curve reflected the cytotoxic activity of Ctrl-T, lEVs-T and sEVs-T. Each point in the graph constituting the curve represented the average of 3 biological replicates. Quantifications of 24h, 48h and 72h were expressed as mean ± SD of 4 biological replicates.

(E) TGF-β1 secreted in supernatant were detected by ELISA (n=3).

Data shown are mean ± standard deviation. **$p < 0.01$; ns, no significant difference (two-tailed, unpaired Student's t test).

Figure S2 (related to Figure 2): CyTOF for immune markers in Ctrl-T and sEVs-T.

(A) Heat map showing the expressions of 42 immune markers in the 37 cell clusters in Ctrl-T (above) or sEVs-T (below). The label on the left showing the T cell types of clusters according to typically expressed markers.

(B) Flow cytometry showing the T lymphocytes activation biomarker. The graphs are from a single experiment which is representative of 5 independent experiments. Data shown are mean ± standard deviation. **$p < 0.01$; ns, no significant difference (two-tailed, unpaired Student's t test).

Figure S3 (related to Figure 3 and 4) BxPC-3- and PANC-1-derived exosomes induce Treg.

(A) Scatter plots showing correlation between immunosuppressive biomarkers and Treg abundance. Data derived from the tumor-immune system interactions database (TISIDB) (http://cis.hku.hk/TISIDB/). R value meaning correlation coefficient, $p < 0.05$ meaning statistically significant (Spearman's correlation).

(B) Flow cytometry showing the FOXP3 expression at different time after 100μg/mL PANC-1-derived sEVs stimulation (n=3).

(C) Flow cytometry showing the FOXP3 expression at different time after 100μg/mL HPSC-derived sEVs stimulation (n=3).

(D) Flow cytometry showing the FOXP3 expression after 3 days of 100μg/mL HPSC-, PANC-1- or BxPC-3-derived sEVs stimulation (n=3).

(E) Western blot showing the upregulated FOXP3 expression in T lymphocytes treated by HPSC- PANC-

1- or BxPC-3-derived exosomes for 3 days (n=3).

(B-E) The graphs are from a single experiment which is representative of 3 independent experiments. Data shown are mean ± standard deviation. *p < 0.05; **p < 0.01; ns, no significant difference (two-tailed, unpaired Student's t test).

Figure S4 (related to Figure 5): TGF-β1-SMAD pathway is involved in exosomes-induced Treg.

(A) GSEA showing the enriched gene sets of TGF-Beta-signaling in sEVs-T. P value < 0.05 and q value < 0.25 meaning statistically significant.

(B) ELISA showing the content of TGF-β1 in BxPC-3-derived supernatant, lEVs and sEVs (n=3).

(C) Western blot showing the expression of TGF-β1 in BxPC-3 and BxPC-3-derived sEVs. HSP70 was considered as a control.

(D) Confocal microscope showing the uptaken of Ctrl/CD63-sEVs or TGF-β1/CD63-sEVs by T lymphocytes. DAPI (blue) pointing the nuclei of T lymphocytes, tdTomato (orange) pointing the CD63, and EGFP (green) pointing the TGF-β1. Three biological replicates were made in each group.

(E) Flow cytometry showed the ratio of TGF-β1/CD63-sEVs-positive T lymphocytes. Data shown are mean ± standard deviation. Three biological replicates were made in each group.

(F) Flow cytometry showing the FOXP3 expression during treatment with PBS, 50 μg/mL Disitertide, 100μg/mL BxPC-3-derived sEVs, and sEVs pre-treated with Disitertide (n=3).

(C-F) The graphs are from a single experiment which is representative of 3 independent experiments. Data shown are mean ± standard deviation. **p < 0.01; ***p < 0.001; ns, no significant difference (two-tailed, unpaired Student's t test).

Figure S5 (related to Figure 5): SIRTs mRNA expressions, effect concentration of selective inhibitor, and ATM phosphorylation.

(A) Quantitative PCR showing the mRNA expression of SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7 in Ctrl-T and sEVs-T

(B) Western blot showing the effect concentration for inhibition of AMPK phosphorylation by 20 μM Compound C (red point).

(C) Western blot showing the effect concentration for inhibition of SIRT1 expression by 10 μM EX-527 (red point).

(D) Western blot showing the effect concentration for inhibition of SIRT2 expression by 40 μM AGK2 (red point).

(E) Western blot showing the effect concentration for inhibition of SIRT6 expression by 100 μM

OSS_128167 (red point).

(F) Western blot showing the effect concentration for inhibition of ATM phosphorylation by 50 μM KU-60019 (red point).

(G) Western blot showing the ATM phosphorylation during different dose of sEVs-treatment.

Western blot graphs are from a single experiment which is representative of 3 independent experiments. Fold changes are relative to control. Data shown is mean ± standard deviation. n=3. *p<0.05; **p < 0.01; ns, no significant difference (two-tailed, unpaired Student's t test).

Figure S6 (related to Figure 8): Translocations of FOXO1A and FOXO3A in subcellular fractions under the sight of SIM.

(A) Reconstructed 3D-images showing the FOXO1A translocation (red meaning total expression, yellow meaning nuclei expression) and FOXP3 (green) expression in treated T lymphocytes. Scale bar was indicated in image.

(B) Ratio of Nuclear/Total expression of FOXO1A and mean fluorescence intensity (MFI) of FOXP3 from Imaris analysis as in (A) (n=3).

(C) Reconstructed 3D-images showing the FOXO3A translocation (red meaning total expression, yellow meaning nuclei expression) and FOXP3 (green) expression in treated T lymphocytes. Scale bar was indicated in image.

(D) Ratio of Nuclear/Total expression of FOXO3A and mean fluorescence intensity (MFI) of FOXP3 from Imaris analysis as in (C) (n=3).

(A and C) Images are from a single experiment which is representative of three independent experiments.

(B and D) Fold changes are relative to sEVs-T. Data shown are mean ± standard deviation. n=3. *p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001 (two-tailed, unpaired Student's t test).

Figure S7 (related to Figure 9): Translocations of FOXO1A and FOXO3A in subcellular fractions under the sight of SIM.

(A-C) Immunohistochemistry images showing the expression of FOXO1A (A), FOXO3A (B) and FOXP3 (C) in pancreatic normal and tumor tissues. Arrowhead indicating the positive cell. Scale bar was indicated in image.

Figure S1 (related to Figure 1)

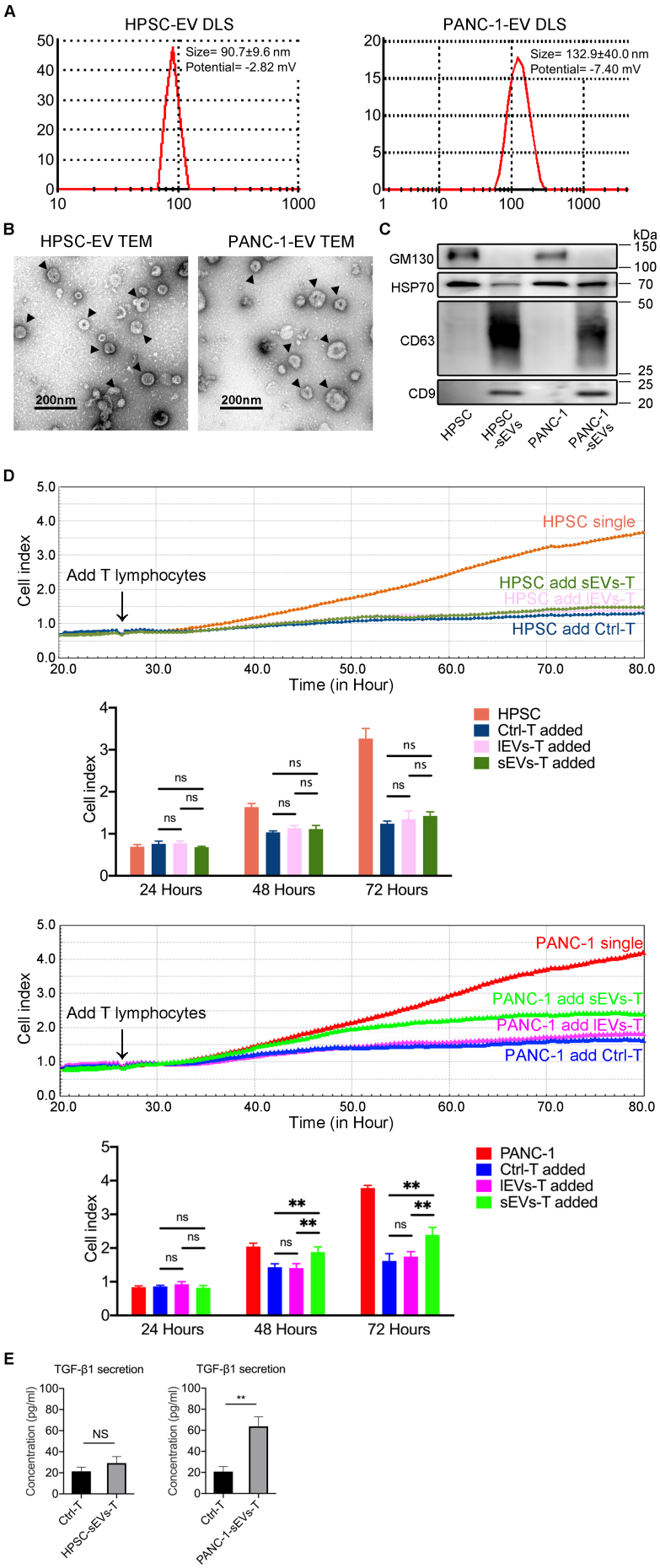

Figure S2 (related to Figure 2)

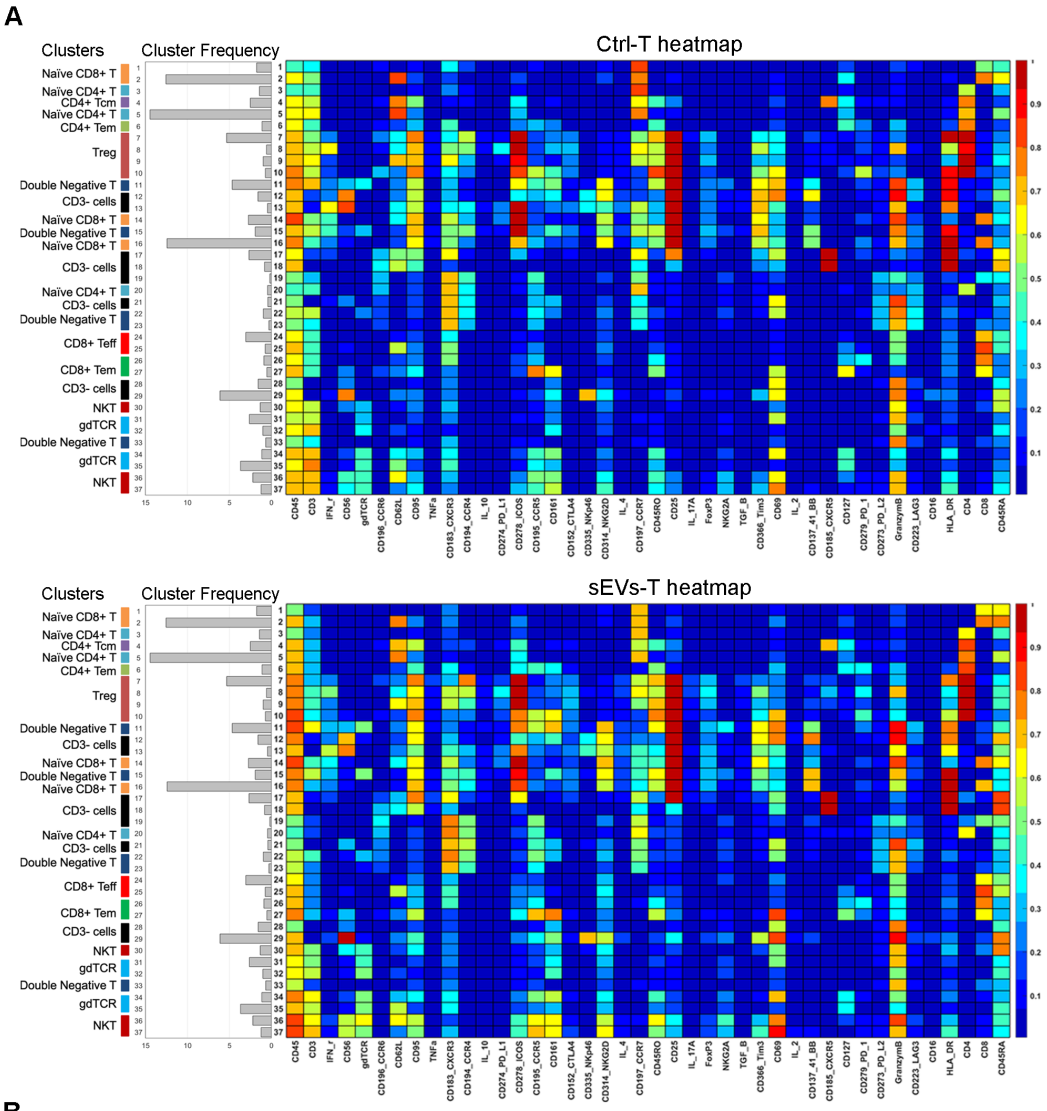

Figure S3 (related to Figure 3 and 4)

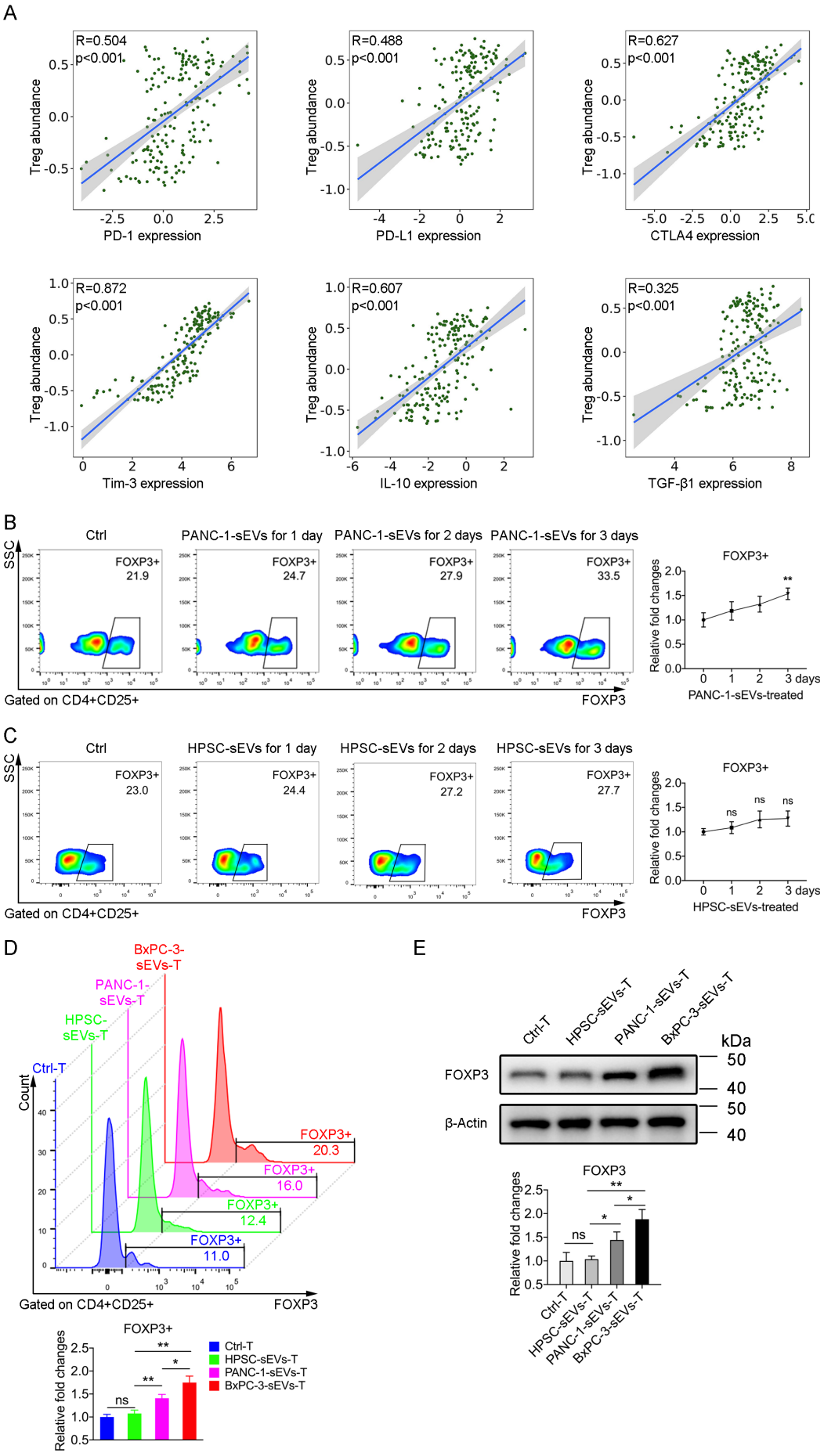

Figure S4 (related to Figure 5)

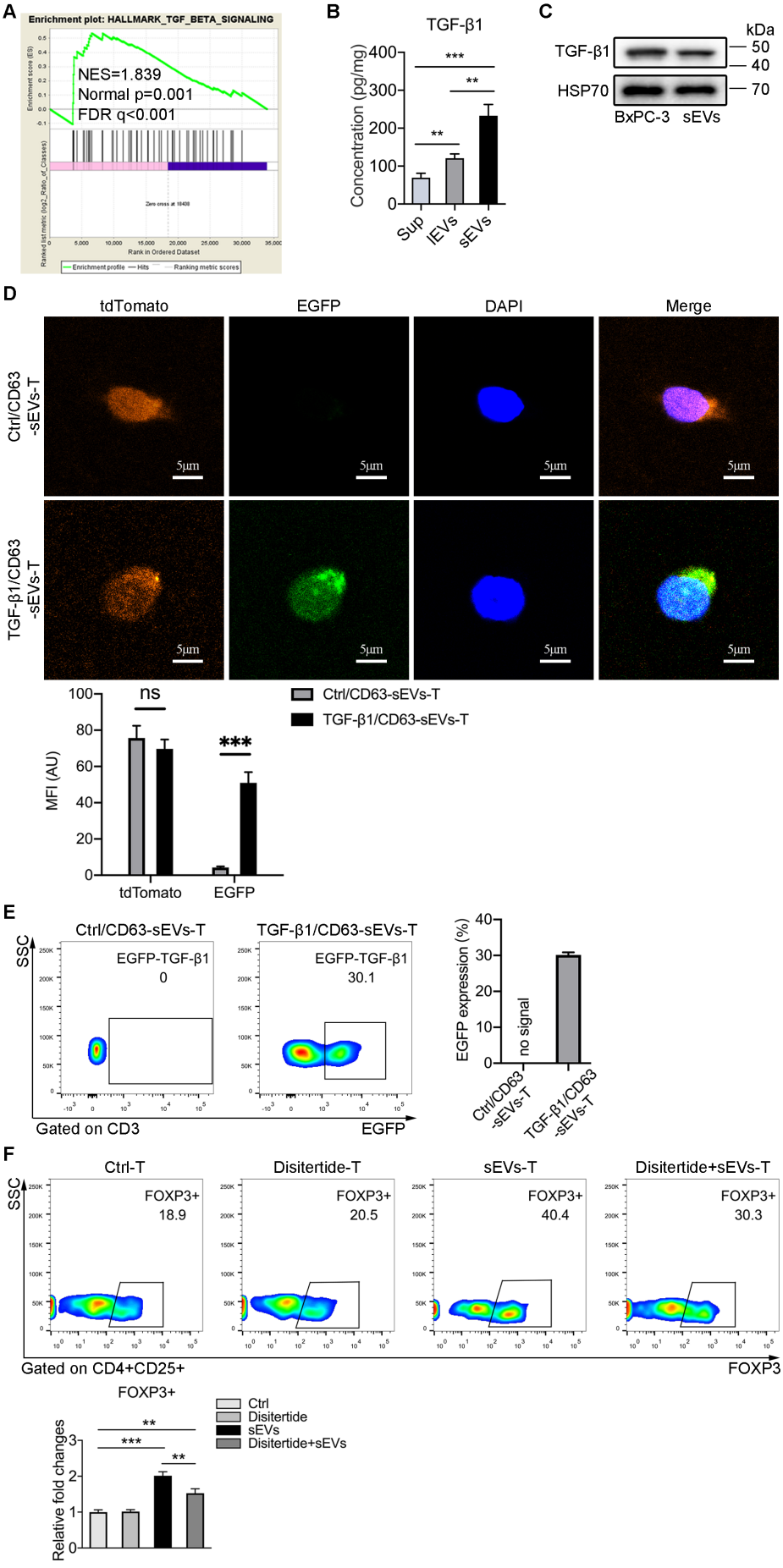

Figure S5 (related to Figure 5)

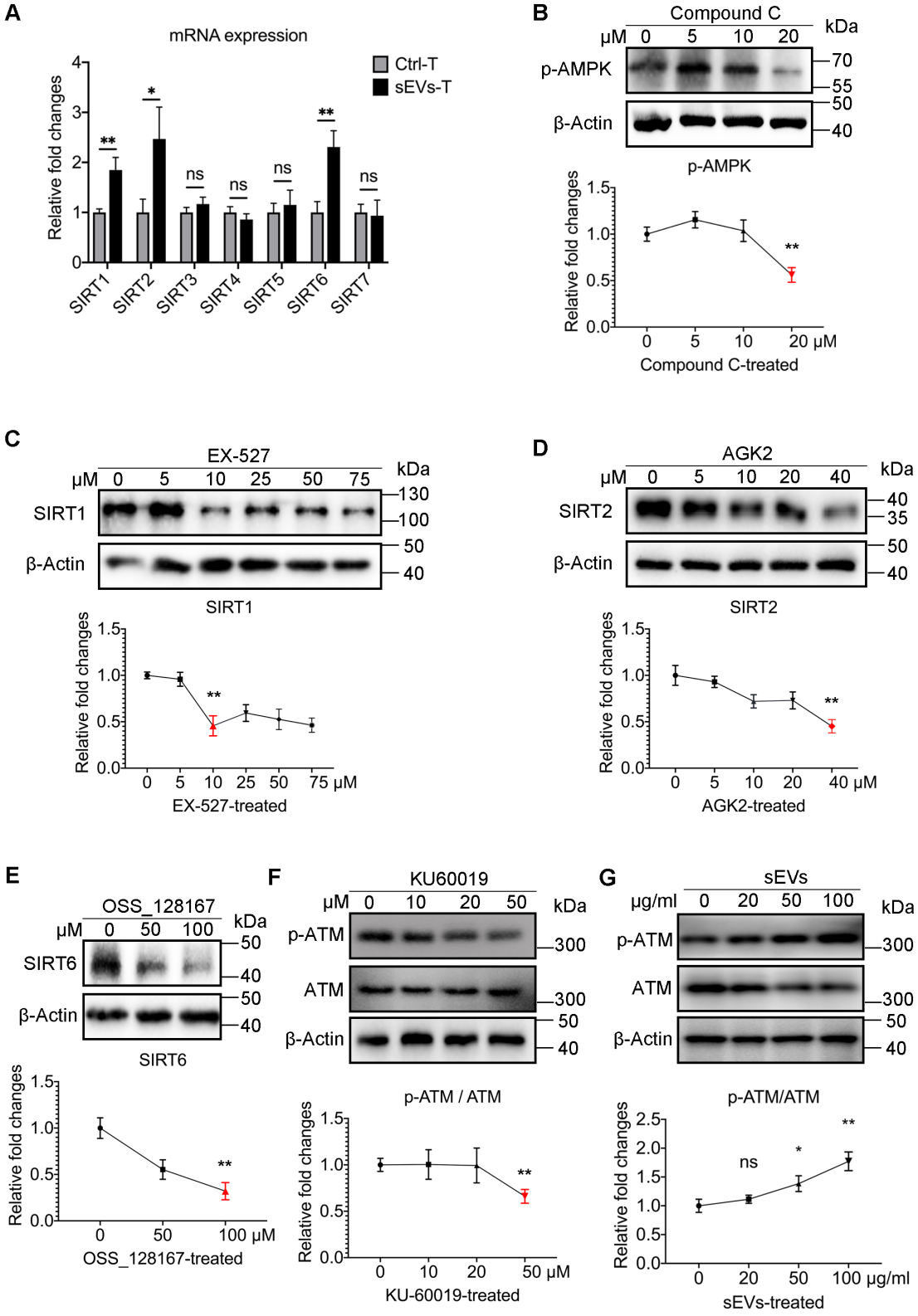

Figure S6 (related to Figure 8)

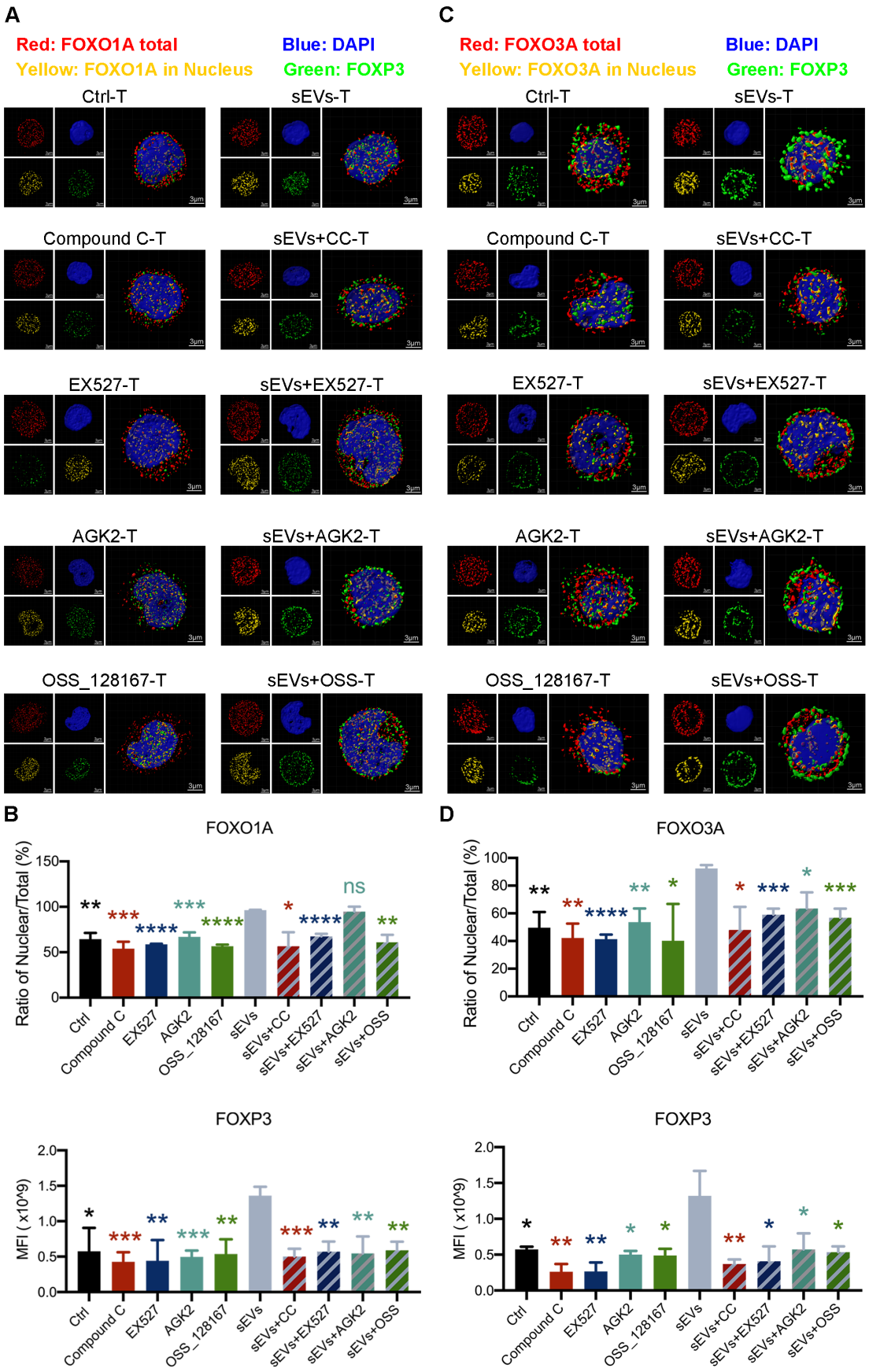

Figure S7 (related to Figure 9)