Supplementary Information

Quantitative secretomics identifies ligand-receptor pairs in pancreatic cancer that mediate bidirectional crosstalk between cancer stem cells and nociceptors

Material and Methods

Cell lines and cell culture

Five pancreatic adenocarcinoma (PDAC) cell lines, A13A and A13D cells were provided by Christine Iacobuzio-Donahue at The Memorial Sloan Kettering Cancer Center, L3.6pl and L3.6sl cells was provided by Isaiah J. Fidler at The University of Texas MD Anderson Cancer Center, and PANC1 cells was obtained from ATCC. A normal human pancreatic duct epithelial cell line, H6C7, was provided by Ming Tsao at the University of Toronto and human embryonic stem cell line, H9 ESC, was obtained from WiCell. For standard pancreatic adenocarcinoma cell cultures, cells were grown at 37°C humidified incubator containing 5% CO2 in Dulbeccos modified Eagles medium (DMEM), high glucose, GlutaMAX™ Supplement, pyruvate (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (Thermo Fisher Scientific, Inc.), MEM Non-Essential Amino Acids Solution (Life Technologies; Thermo Fisher Scientific, Inc), MEM Vitamin Solution (Life Technologies; Thermo Fisher Scientific, Inc.) and 10% inactivated fetal calf serum (FCS; Life Technologies) in T75 TC flask (Sarstedt Inc.; Thermo Fisher Scientific, Inc.). H6C7 cells were grown in KBM™ Gold Keratinocyte Basal Medium with supplement (Lonza) in T75 TC flask (Sarstedt Inc.; Thermo Fisher Scientific, Inc.). Both PDAC cell lines and H6C7 cells were dissociated using Trypsin-EDTA (0.05%), phenol red (Thermo Fisher Scientific, Inc.) when reaching >90% confluency. 10 times amount of fresh complete medium was added to neutralise the trypsin and 1:10 suspending cells were reseeded. H9 ESC cells were grown in 5 µg⁄ml vitronectin (Thermo Fisher Scientific, Inc.) pre-coated non-treated 6-well plate (Thermo Fisher Scientific, Inc.) in Essential 8 Flex medium with Flex supplement (Life Technologies; Thermo Fisher Scientific, Inc.). H9 ESC cells were passaged every 3-4 days using 0.5 mM EDTA/DPBS (Life Technologies; Thermo Fisher Scientific, Inc.) at room temperature for 4.5 minutes when reaching recommended confluency. The dissociated H9 colonies were resuspended in 1 ml fresh complete

medium and about 1:6 cells were reseeded into newly vitronectin pre-coated well. For 3D cultures, A13A-CD133^{+} cells were selected using CD133 MicroBead Kit (Miltenyi Biotec) and grown in ultra-low attachment plates (Thermo Fisher Scientific, Inc.) in Dulbecco's Modified Eagle Medium/F12 (Sigma-Aldrich) supplemented with 2.5mM L-Glutamine (Thermo Fisher Scientific, Inc.), 1% B27 supplement (Life Technologies; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (Thermo Fisher Scientific, Inc.), and 20ng/ml basic thermostable fibroblast growth factor (FGF-Basic TS, Proteintech) in Corning 96 Well Clear Flat Bottom Ultra Low Attachment Microplate Wrapped with Lid Sterile (Sigma-Aldrich Ltd.). To dissociate sphere cells from 3D culture, Trypsin-EDTA (0.05%), phenol red (Thermo Fisher Scientific, Inc.) was added for incubation at 37°C for 4 minutes and then neutralised in Trypsin inhibitor from Glycine max (Sigma-Aldrich Ltd.). The dissociated cells were pelleted at 200xg, room temperature for 4 minutes.

SILAC-labelling for mass spectrometry analysis

The A13A, A13A-CD133^{+}, and H9 ESC cells were cultured in the same condition where the basal medium was replaced with SILAC DMEM: F12 basal medium (Thermo Fisher Scientific, Inc.) with separate supplements accordingly. Three SILAC-labels including light labelled with L-Arginine and L-Lysine, medium labelled with 13C6-L-Arginine-HCl and D4-L-Lysine, and heavy labelled with 13C615N4-L-Arginine-HCl and 13C615N4-L-Lysine, provided by the kit or purchased separately form the same company were added into the culture medium to generate light, medium and heavy labelled cells. For complete incorporation the cells were cultured in such SILAC condition for over five doubling times before proceeding experiments. The SILAC incorporation efficiency was checked and showed >97% in secretome and >99% in total lysate.

Secretome collection and preparation for mass spectrometry analysis

The SILAC labelled cells were collected and washed with DPBS buffer (Life Technologies; Thermo Fisher Scientific, Inc.) for 4 times before seeding into fresh B27-depleted or FCS-depleted SILAC medium for 48 hours. The condition medium was collected at 200 xg for 4 minutes at room temperature to pellet the cells, followed by adding cOmplete EDTA-free protease inhibitor cocktail (Roche Molecular Systems, Inc.) and PhosStop™ phosphoatase inhibitor (Sigma-Aldrich Ltd.) and a subsequent centrifugation at 2000 xg, 30 minutes at 4°C to remove cell debris. Total secretome was precipitated in 6% trichloroacetic acid (Sigma-Aldrich Ltd.) at 4°C overnight and centrifugated at 16,500 xg at 4°C for 30 minutes. The secretome pellet was washed once with pre-cold 80% acetone (Fisher Scientific

UK Ltd.) at 16,500 xg at 4°C for 30 minutes and resolved in 200 µl SDS solubilisation buffer (5% SDS, Sigma-Aldrich Ltd; 50 mM triethylammonium bicarbonate buffer pH 7.5, Sigma-Aldrich Ltd.). The protein concentration was determined using Pierce BCA protein assay (Thermo Fisher Scientific, Inc.) or Qubit protein assay (Thermo Fisher Scientific, Inc.). Equal amount secretome from three labelled cells was pooled together for reduction in Bond-Breaker™ TCEP Solution (Thermo Fisher Scientific, Inc.) and alkylation in 20 mM iodoacetamide (Sigma-Aldrich Ltd.) at room temperature for 30 minutes. The pooled secretome was then acidified and applied to S-Trap™ micro spin column (Protifi™) for trypsin digestion (Promega UK) at 37°C overnight according to the S-Trap™ digestion protocol. The digested secretome was eluded in 60 µl of 25% acetonitrile (Thermo Fisher Scientific, Inc.) and 0.1% trifluoroacetic acid (Thermo Fisher Scientific, Inc.) for MS proteome analysis.

LC-MS/MS secretomic data search and analysis

Dried peptides were reconstituted in 5%DMSO and 5% formic acid and were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using Ultimate 3000 UHPLC (Thermo Fisher Scientific, Inc.) connected to an Orbitrap Fusion Lumos Tribrid (Thermo Fisher Scientific, Inc.). Briefly, peptides were loaded onto a trap column (PepMapC18; 300µm x 5mm, 5µm particle size, Thermo Fisher) and separated on a 50cm-long EasySpray column (ES803, Thermo Fisher) with a gradient of 2-35% acetonitrile in 5% dimethyl sulfoxide, 0.1% formic acid at 250 nl/min flow rate over 120 min. Eluted peptides were then analysed on an Orbitrap Fusion Lumos Tribrid platform (instrument control software v3.3). Data were acquired in data-dependent mode, with the advance peak detection (APD) enabled. Survey scans were acquired in the Orbitrap at 120 k resolution over a m/z range 400 -1500, AGC target of 4e5 and S-lens RF of 30. Fragment ion spectra (MS/MS) were obtained in the Ion trap (rapid scan mode) with a Quad isolation window of 1.6, 40% AGC target and a maximum injection time of 35 ms, with HCD activation and 28% collision energy. The raw data files generated were processed using MaxQuant (Version 1.5.0.35), integrated with the Andromeda search engine. For protein groups identification, peak lists were searched against human database (UPR_Homo sapiens_9606_UP000005640_20200803.fasta) as well as list of common contaminants by Andromeda. Trypsin with a maximum number of missed cleavages of 2 was chosen. Oxidation (M) and Deamidiation (N, Q) were used as variable modifications while Carbamidomethylation (C) was set as a fixed modification. For quantitation SILAC labels K(4)R(6) and K(8)R(10) were selected. Protein and PSM false discovery rate (FDR) were set at 0.01. Match between runs was applied. The Quantile normalisation was then applied, and the data was visualised in Perseus 1.6.15.0 [81].

Cytosol protein extraction

Cells were collected and washed with DPBS buffer (Life Technologies; Thermo Fisher Scientific, Inc.) once at 200 xg at room temperature for 4 minutes. The cells were lysed in RIPA buffer (Sigma-Aldrich Ltd.) with cOmplete EDTA-free protease inhibitor cocktail (Roche Molecular Systems, Inc.) and PhosStop™ phosphoatase inhibitor (Sigma-Aldrich Ltd.) on ice for 20 minutes then span down at 14,000 rpm at 4°C for 20 minutes. The protein concentration was determined using Pierce BCA protein assay (Thermo Fisher Scientific, Inc.) or Qubit protein assay (Thermo Fisher Scientific, Inc.).

Western Blot

Equal protein amount was boiled in NuPAGE™ LDS Sample Buffer and reducing agent (Thermo Fisher Scientific, Inc.) at 70°C for 10 minutes. The boiled protein was separated in NuPAGE 4-12% Bis-Tris gel (Thermo Fisher Scientific, Inc.) at 130 voltage and transferred to PVDF membrane using iBlot™ 2 transfer stack mini and program 0 in iBlot™ 2 Gel Transfer Device (Thermo Fisher Scientific, Inc.). The membrane was blocking in 5% non-fat milk (Santa Cruz Biotechnology, Inc.) of PBST buffer (Sera Care, USA) at room temperature for 1 hour, followed by first antibody hybridisation at 4°C overnight. The membrane was washed in PBST buffer for 10 minutes, 3 times before hybridising with HRP-conjugated secondary antibody at room temperature for 1 hour. The membrane was washed in PBST buffer for 10 minutes, 3 times and the signals were developed using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). The antibodies used are 1:1000 GDF15 (G5, sc-377195; Santa Cruz Biotechnology, Inc.), 1:1000 VGF (B-8, sc-365397; Santa Cruz Biotechnology, Inc.), 1:5000 β-actin (AC-74, A2228; Sigma-Aldrich Ltd.), 1:1000 α-actinin (H-2, sc-17829; Santa Cruz Biotechnology, Inc.), 1:1000 phospho Smad3 (Ser423/425, C25A9, #9520; Cell Signaling Technology), 1:1000 Smad2/3 (#3102; Cell Signaling Technology), 1:10,000 HRP-conjugated mouse anti-rabbit IgG antibody (L27A9, #5127; Cell Signaling Technology), 1:10,000 HRP-conjugated anti-mouse IgG (A9044; Sigma-Aldrich Ltd.), 1:2000 GCN2 (#Ab134053, GR3312997-1, Abcam), 1:2000 p-GCN2 (#94668, Cell Signalling), 1:2000 p-EIF2α (#Ab32157, GR32542851-2, Abcam), 1:2000 EIF2α (#5324, D7D3, Cell Signalling), 1:2000 ATF4 (#11815, D4B8, Cell Signalling).

CRISPR-mediated gRNA knockdown

The gRNAs targeting GDF15 or VGF were predesigned by IDT (Integrated DNA Technologies, Inc.) and was co-transfected with Cas 9 nuclease (Integrated DNA Technologies, Inc.) using Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the

manufacturer's guideline. The transfected cells were collected at 48 hours post transfection for the following experiments as described in other sections. Three gRNAs targeting GDF15 are gGDF15-1: 5'-GTTACTGTATGGCATACTTC-3', gGDF15-2: 5'- GGCATTTACACCTCAATTCC-3', and gGDF15-3: 5'-GCTTAATAGCACCAGGCAAT-3'. Four gRNAs targeting VGF are gVGF-1: 5'- CAAGTACCTGCGATTAGAAA-3', gVGF-2: 5'- GGTACTTTGCAAGAAATGCG-3', gVGF-3: 5'- CTGGACTGATCGCAGACAAT-3', and gVGF-4: 5'- AAAACTATTTCCTCCGTGAG-3'.

RNA extraction and concentration measurement

The cells were lysed in 100 ul TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and RNA was extracted using Direct-zol™ RNA extraction kit (Zymo Research co.). The RNA was eluted in 50 μl nuclease free water and the concentration was measured in Nanodrop.

Reverse transcription and Real-time PCR

100-500 ng of RNA was used for reverse transcription using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.). The cDNA product was diluted 3-30 times in nuclease free water and 4.5 μl of diluted cDNA was used per reaction. The tested primers and Applied Biosystems Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc.) were added, and the program was set according to the manufacturer's guideline and the samples were ran in the CFX Opus 384 Real-Time PCR Systems (Bio-Rad Laboratories, Inc.). The primers were customer designed and synthesised by Sigma-Aldrich Ltd., including GDF15 primers: Forward 5'-ATACTCACGCCAGAAGTGCGG-3' and Reverse 5'-CTTGCAAGGCTGAGCTGACG-3', VGF primers: Forward 5'-GTGACACCAGCTGTCTCCG-3' and Reverse 5'-ACCCGTTGATCAGCAGAAGG-3', β-actin Forward 5'- CACCAGGGCGTGATGGTG-3 and Reverse 5'-GAGCCACACGCAGCTCAT-3'.

Sphere formation assay

The cells were dissected into single cell and seeded at the density of 800 cells per 100 μl per well in 3D culture condition as described in the previous session. The image of sphere formation was taken by Celigo Image Cytometry (Nexcelom Bioscience LLC.) on the Day 1, 3 6, 9 and 12. The numbers of spheres per well and the average diameter of spheres were measured using the compatible Celigo software. The medium was changed every 3 days after Day 3 after photo being taken.

Cell viability determination

The cell viability was determined using PrestoBlue assay. In brief, the cells were dissected into single cell and seeded at the density of 800 cells per 100 μl per well of Corning® Primaria™ 96 Well Clear Flat Bottom Microtest Microplate (Scientific Laboratory Supplies Limited) or Corning 96 Well Clear Flat Bottom Ultra Low Attachment Microplate (Sigma-Aldrich Ltd.). On the Day 0, 1, 3 6, 9 and 12 post cells seeding, 11 μl of PrestoBlue reagent (Life Technologies; Thermo Fisher Scientific, Inc.) was added to each well and incubated at 37°C incubator for 30 minutes for PDAC cell lines or overnight for A13A-$CD133^+$ cells. The 570 nm absorbance was measured by FLUOstar Omega Multidetection Microplate Reader (Labcompare).

Immunostaining

The immunostaining method has been described previously (Bertero et al., 2015; Pauklin et al., 2016; Pauklin and Vallier, 2013). Cells were fixed for 20 minutes at 4°C in PBS 4% PFA (electron microscopy grade), rinsed three times with PBS, and blocked and permeabilized at the same time for 30 minutes at room temperature using PBS with 10% Donkey Serum (Biorad) and 0.1% Triton X-100 (Sigma). Incubation with primary antibodies diluted in PBS 1% Donkey Serum 0.1% Triton X-100 was performed overnight at 4°C. Samples were washed three times with PBS, and then incubated with AlexaFluor secondary antibodies for 1 hour at room temperature protected from light. Cells were finally washed three times with PBS, and Hoechst (Sigma) was added to the first wash to stain nuclei. Images were acquired using a LSM 700 confocal microscope (Leica).

Transwell assays

Cancer cell invasiveness was analysed by using a modified Boyden chamber based assay, CultureCoat 96 Well High BME Cell Invasion Assay (Trevigen, Cat. No: 3483-096-K) or 24-well tanswell inserts with 8μm pores (Sarstedt, Cat no. 83.3932.800) with EHS Matrix Extract as basal membrane (Merck) according to manufacturer's guidelines. 25,000 cells were used per well and incubated for 24h before analysis.

Cell incubation with conditioned media

Media was incubated with 70-80% confluent cells for 24h, before collection. The media collected from cells, aliquoted, stored at -80 °C, and thawed freshly just before use. Cells were cultured in the collected media not more than 24h before substituting for a fresh aliquot. To avoid possible autocrine effects via WNT signalling due to inconsistent cell density (the more cells the higher levels of WNT in media), particular care should be taken for plating cells at the same density across the experimental samples.

Patient blood samples

Patient samples were obtained with consent and ethics permission (OCHRe ref: 21/A126, REC reference: 19/SC/0173; OCHRe ref: 19/A176, REC reference: 19/SC/0173) from OxCODE Biobank, and from Queen Marry University London Biobank.

ELISA

The plasma levels of GDF15 and VGF were detected using GDF15 Human ELISA Kit (Thermo Fisher Scientific, Inc.) and Human VGF nerve growth factor inducible (VGF) ELISA kit (Cusabio Biotech Co.) separately according to their manufacture's guidelines. The 450 nm absorbance was measured by FLUOstar Omega Multidetection Microplate Reader (Labcompare).

Chromatin Immunoprecipitation (ChIP)

All steps were performed on ice or at 4°C and ice-cold buffers and PBS were supplemented with 1mg/ml Leupeptin, 0.2mM PMSF, and 10mM NaButyrate were used unless otherwise stated. Approximately $5x10^6$ cells were used per sample and cross-linked with 1% formaldehyde for 15 minutes. Cross-linking was stopped by incubating samples with glycine at a final concentration of 0.125M for 5 minutes at room temperature, and the cells were washed with PBS followed by pelleting at 250g for 5 minutes. The pellet was re-suspended in 2ml ChIP Cell Lysis Buffer (CLB: 10 mM Tris pH8, 10 mM NaCl, 0.2% NP-40) and incubated for 10 minutes to lyse the plasma membranes. Nuclei were pelleted at 600g for 5 min, lysed in 1.25ml of ChIP Nuclear Lysis Buffer (NLB: 50 mM Tris pH8, 10mM EDTA, 1% SDS) for 10 minutes, and then 0.75ml of ChIP Dilution Buffer (DB: 20 mM Tris pH8, 2mM EDTA, 150mM NaCl, 0.01% SDS, 1% Triton X-100) was added to the samples. Chromatin was sonicated in 15ml Diagenode Bioruptor Pico water bath sonicator with an automated water cooling system, by performing 30 cycles of 30 seconds ON, 45 seconds OFF. This protocol resulted in the homogeneous

generation of fragments of 100-400bp. Samples were clarified by centrifugation at 16000g for 10 minutes and diluted with 3.5ml of DB. After pre-clearing with 10µg of non-immune IgG for 1h and 50µl of Protein G-Agarose for 2h, ChIP was performed overnight in rotation using specific antibodies (Table S2) or non-immune IgG as a control. After incubation for 1 hour with 30µl of Protein G-Agarose, beads were washed twice with ChIP Washing Buffer 1 (WB1: 20mM Tris pH8, 2mM EDTA, 50mM NaCl, 0.1% SDS, 1% Triton X-100), once with ChIP Washing Buffer 2 (WB2: 10mM Tris pH8, 1mM EDTA, 0.25M LiCl, 1% NP-40, 1% Deoxycholic acid), and twice with Tris-EDTA (TE: 10mM Tris pH8, 1mM EDTA). Precipitated DNA was eluted with 150µl of ChIP Elution Buffer (EB: 100mM $NaHCO_3$) twice for 15 minutes at room temperature in rotation and processed as follows in parallel with 300 µl of sonicated chromatin non-used for ChIP (Input). Cross-linking was reverted by adding NaCl to a final concentration of 300mM for protein-DNA de-crosslinking and incubated at 65°C for 5 hours and 1 µg RNase A (Sigma) to digest contaminating RNA. Finally, 60 µg of Proteinase K (Sigma) were added overnight at 45°C. DNA was extracted by sequential phenol-chloroform and chloroform extractions and precipitated overnight at -80°C in 100mM NaAcetate, 66% ethanol and 50µg of glycogen (Ambion) as a carrier. After centrifugation at 16,000g for 1 hour at 4°C, DNA pellets were washed once with ice-cold 70% ethanol, and finally air dried. ChIP samples were resuspended in 30µl and 1:10 of the samples were used in qPCR for verifying the ChIP samples.

ScRNA-seq analysis of tumor tissues

Single cell RNA sequencing data of 20 treatment naïve PDAC tumor tissues were down from GEO (GSE205013) as expression matrix. Cells with low-quality profiles were excluded based on the percentage of mitochondrial RNA among total UMIs (<15%), and the total number of UMIs (>600). Red cells were further removed based on HBA1 expression. The raw read counts were normalized and scaled using the NormalizeData and ScaleDatafunction. 2000 variable genes were identified for each sample. Data across different samples were then integrated using the FastMNN algorithm.

To identify cell types, we performed principal component analysis (PCA) on integrated data with the RunPCA function to generate principal components (PCs) used for dimensionality reduction. Top 15 PCs were used for Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction. The FindNeighbors and FindCluster functions were used to cluster cells based on global transcriptional profile. Clusters in the 2-dimensional UMAP were used to identify cell types based on marker genes. To better characterize the heterogeneity of PDAC cells, the epithelial compartment was extracted and a new set of 10,000 variable genes were identified for dimensional reduction analysis. CCAIntegration was chosen for a re-integration as it better present the subset difference while removing batch effects.

For cell interaction network inference, we use the statistical analysis method from cellphonedb 4.0. With a focus on the interactions mediated by unique secretome of CSC cells, we curated the interaction pairs from published studies and built a customized database.

Statistical analysis

GraphPad Prism was used for statistical analysis. Unless otherwise indicated in the figure legends, at least three biological replicates were analysed for the graphs, and the level of significance was determined by unpaired t-test and shown as $p< 0.05$ (*), $p< 0.01$ (**), and $p< 0.001$ (***).

Supplementary Figure legends

Supplementary Figure 1. Quantitative secretome analysis of PDAC CSCs, PDAC non-CSCs, H9 hPSCs and non-cancerous pancreatic duct epithelium cells H6C7. (A) CSC markers elevated in CSC spheres compared to non-CSCs based on flow cytometry analyses in A13A cells. **(B)** Heat maps of secreted factors specific for H6C7 non-cancerous pancreatic duct epithelial cells. **(C)** Heat maps of secreted factors specific for hESCs. **(D)** Heat maps of secreted factors specific for non-CSCs.

Supplementary Figure 2. Ligand-receptor identification shown as circos plots. (A) Common ligand-receptor pairs between all four cell types. **(B)** Ligand-receptor signalling pairs shared by CSC, H9 and H6C7. **(C)** Ligand-receptors shared by CSC, non-CSCs and H6C7. **(D)** Ligand-receptors shared by CSC and H6C7. Ligands are marked with pink and receptors with grey colour.

Supplementary Figure 3. Ligand-receptor pairs specific to PDAC non-CSCs shown as circos plots. Ligands are marked with pink and receptors with grey colour.

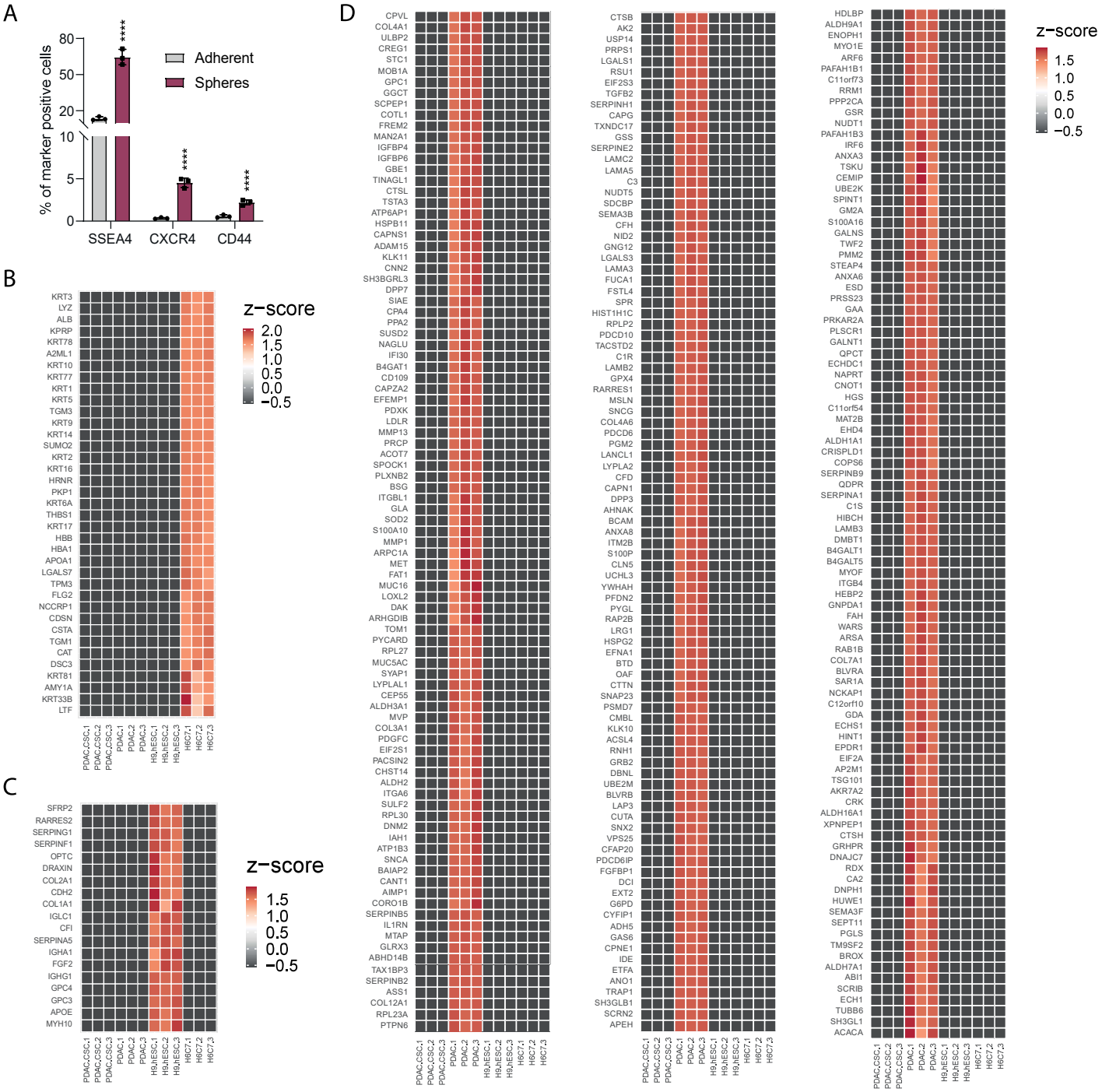
Supplementary Figure 4. Comparison of ligand-receptor pairs in CSCs, non-CSCs, H6C7 cells and hPSCs shown as circos plots. (A) Additionally, our analyses found ligand-receptor pairs present differentially in in CSCs, non-CSCs, H6C7 cells and hPSCs. **(B)** Ligand-receptor pairs shared by H9 and H6C7. **(C)** Ligand-receptor pairs specific for H6C7. **(D)** Ligand-receptor pairs shared by non-CSC and H9. Ligands are marked with pink and receptors with grey colour.

Supplementary Figure 5. Ligand-receptor pairs specific to H9 hESCs shown as circos plots. (A) Ligands are marked with pink and receptors with grey colour. **(B)** Clustering of cancer cell subpopulations in PDAC patient tumour sample RNA-sequencing data. **(C)** Expression of CSC markers ALDH1A1, CD133, KLF4 and CD44 in cancer cell subpopulations in patient tumours. **(D)** GDF15 and VGF expression in cancer cell subpopulations in patient tumours. **(E)** VGF ligand expression in cancer cell subpopulations in patient tumours. **(F)** GDF15 ligand expression in cancer cell subpopulations in patient tumours. **(G)** GDF15 and VGF ligand and receptor protein expression in PDAC lines analysed by western blot.

Supplementary Figure 6. Single-cell RNA-sequencing analyses of PDAC patient data indicate the expression of factors identified by secretome analyses. (A) Cell type annotation in PDAC patient tumour sample RNA-sequencing data. Epithelial cells mark cancer cells. **(B)** Basal-like and EMT-enriched gene signatures in the PDAC patient tumours. **(C)** Expression of VGF receptors in PDAC tumours. **(D)** Expression of GDF15 receptors in PDAC tumours. **(E)** Expression of secretome factors in PDAC patient tumours.

Supplementary Figure 7. Crosstalk between CSC and other cells in PDAC patient tumours. Paracrine signalling between CSCs (C1) and other cancer cells (C2-C8) in PDAC patient primary tumours.

Supplementary Figure 8. Effect of VGF, GDF15 and Substance P-TACR1 signalling in CSC-nociceptor crosstalk. (A-B) SMAD2/3 binding to (A) GDF15 and (B) VGF locus in CSCs. **(C)** GDF15 and VGF knockdown in CSCs. **(D)** Schematic depiction of GDF15 and VGF expression regulation by SMAD2/3-SMAD4-SNON and CREB-ATF4 upon cell stress by gemcitabine. **(E)** VGF and GDF15 impact the neurite numbers and length that grow out from hPSC-derived neurons toward CSCs. **(F)** TACR1 expression in PDAC patient samples. **(G)** Substance P secreted by nociceptors supports PDAC CSC self-renewal whereas its antagonist aprepitant blocks this effect. **(H)** Substance P secreted by nociceptors supports PDAC CSC migration as assessed by transwell assays whereas its antagonist aprepitant blocks this effect. **(I-J)** Overall survival of PDAC patients in relation to (I) VGF and (J) GDF15 expression in PDACs.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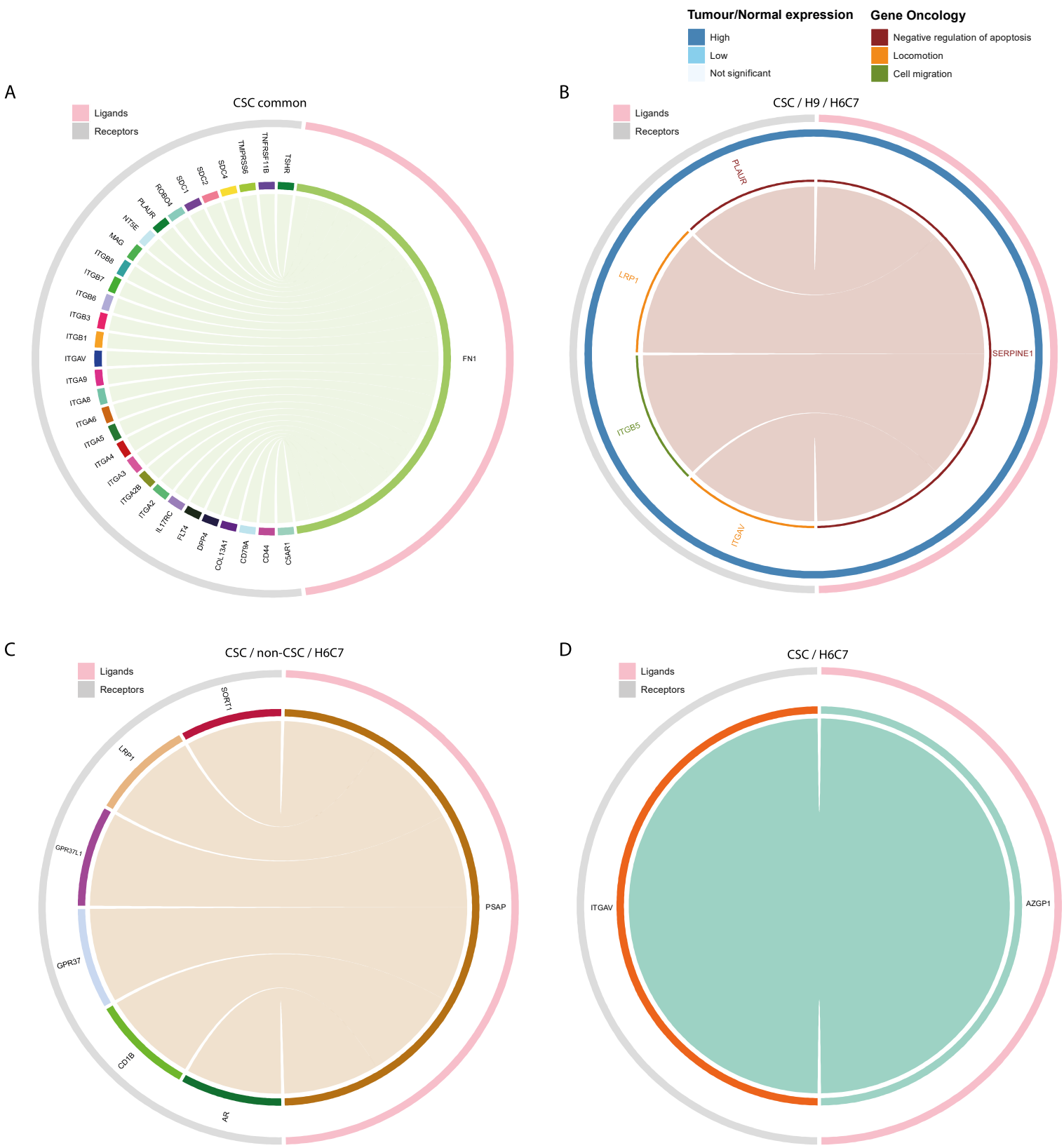
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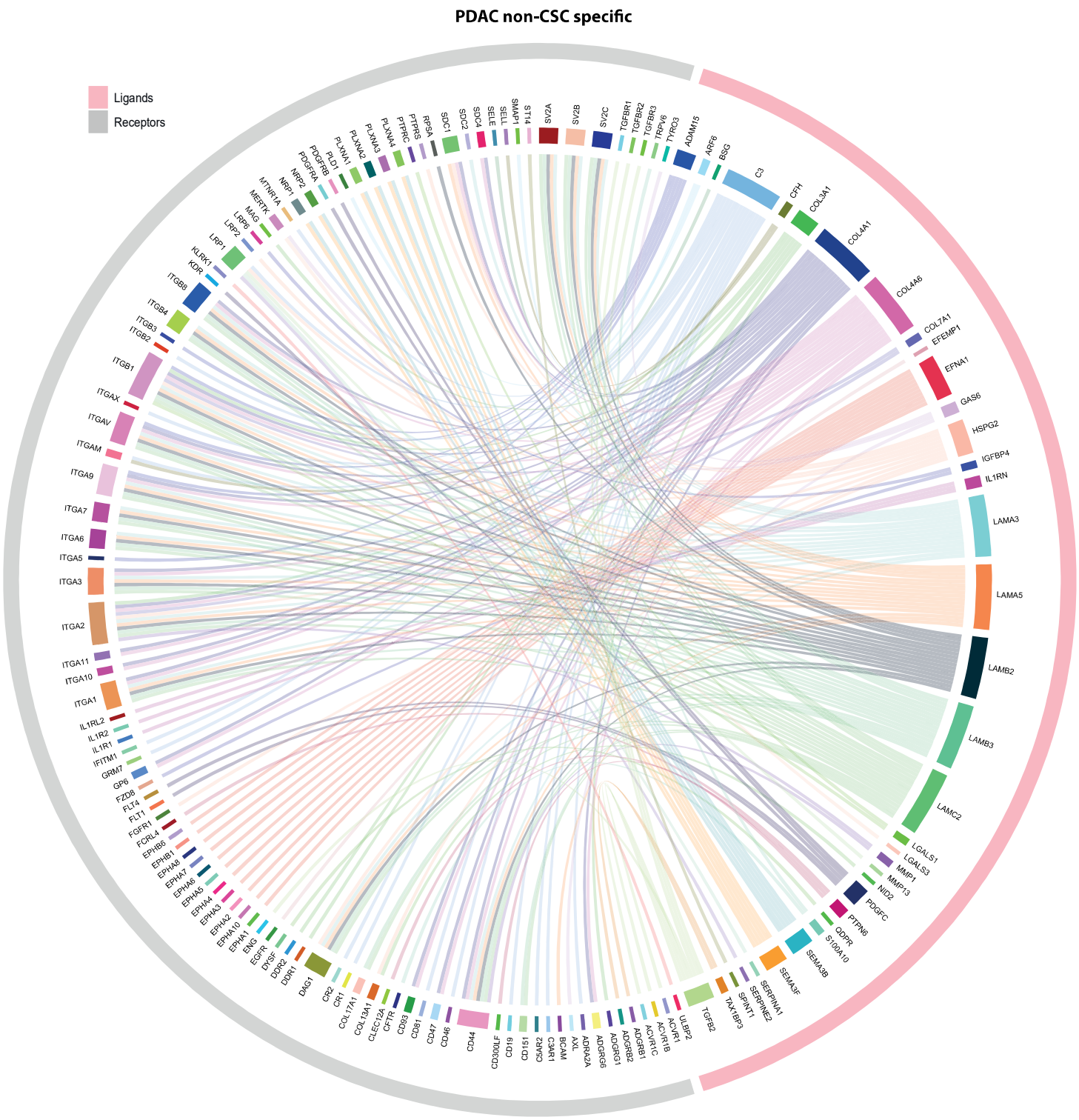
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RPSA
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SDC2
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SELL
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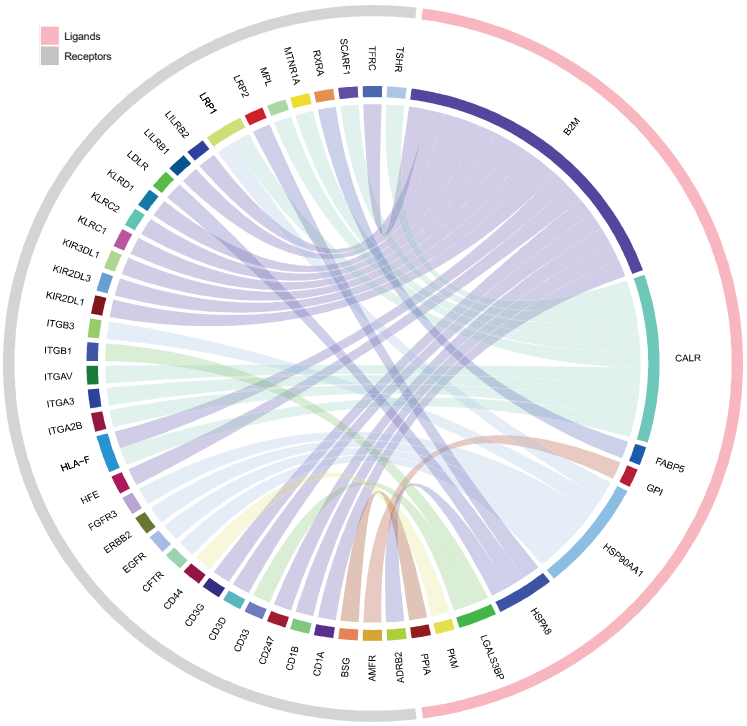
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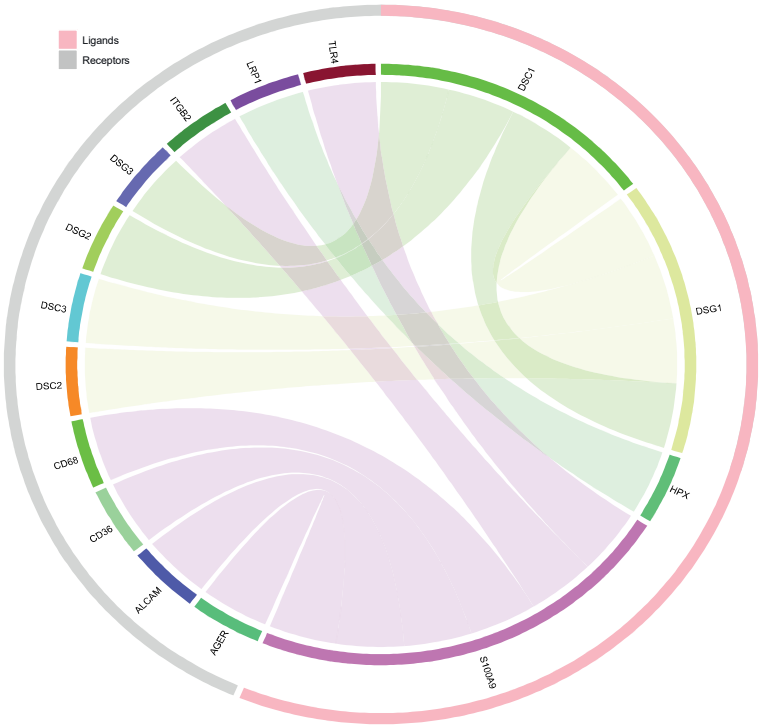
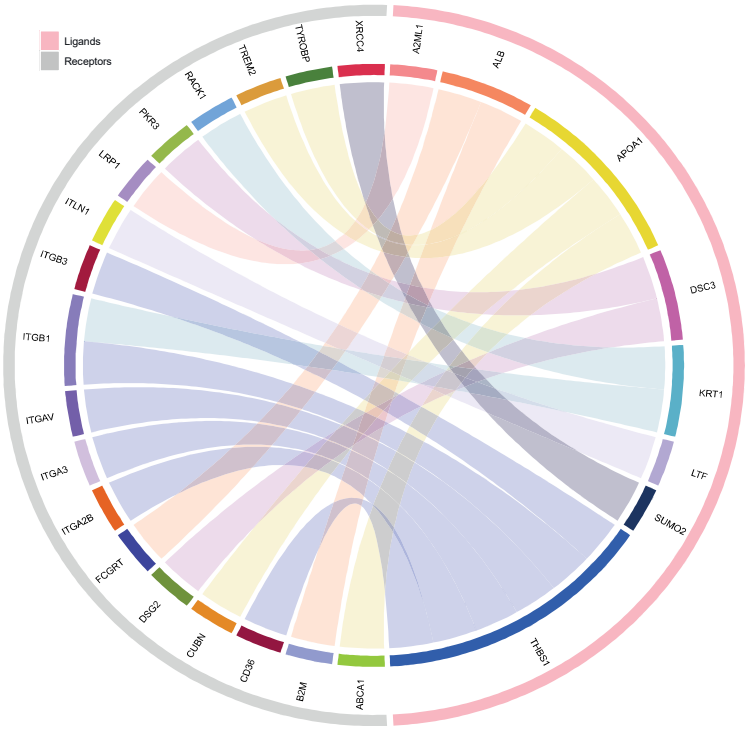
CALR
B2M

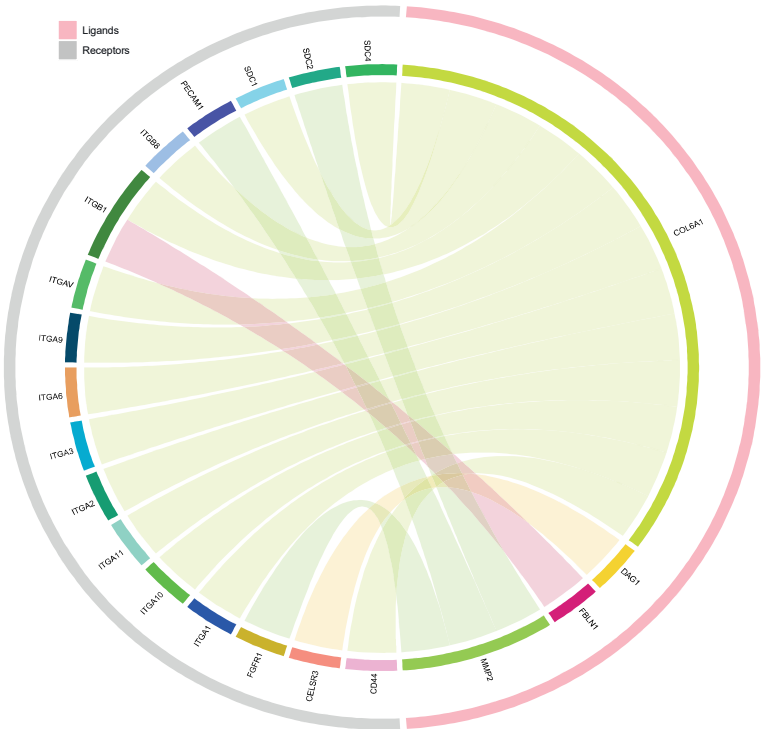
b

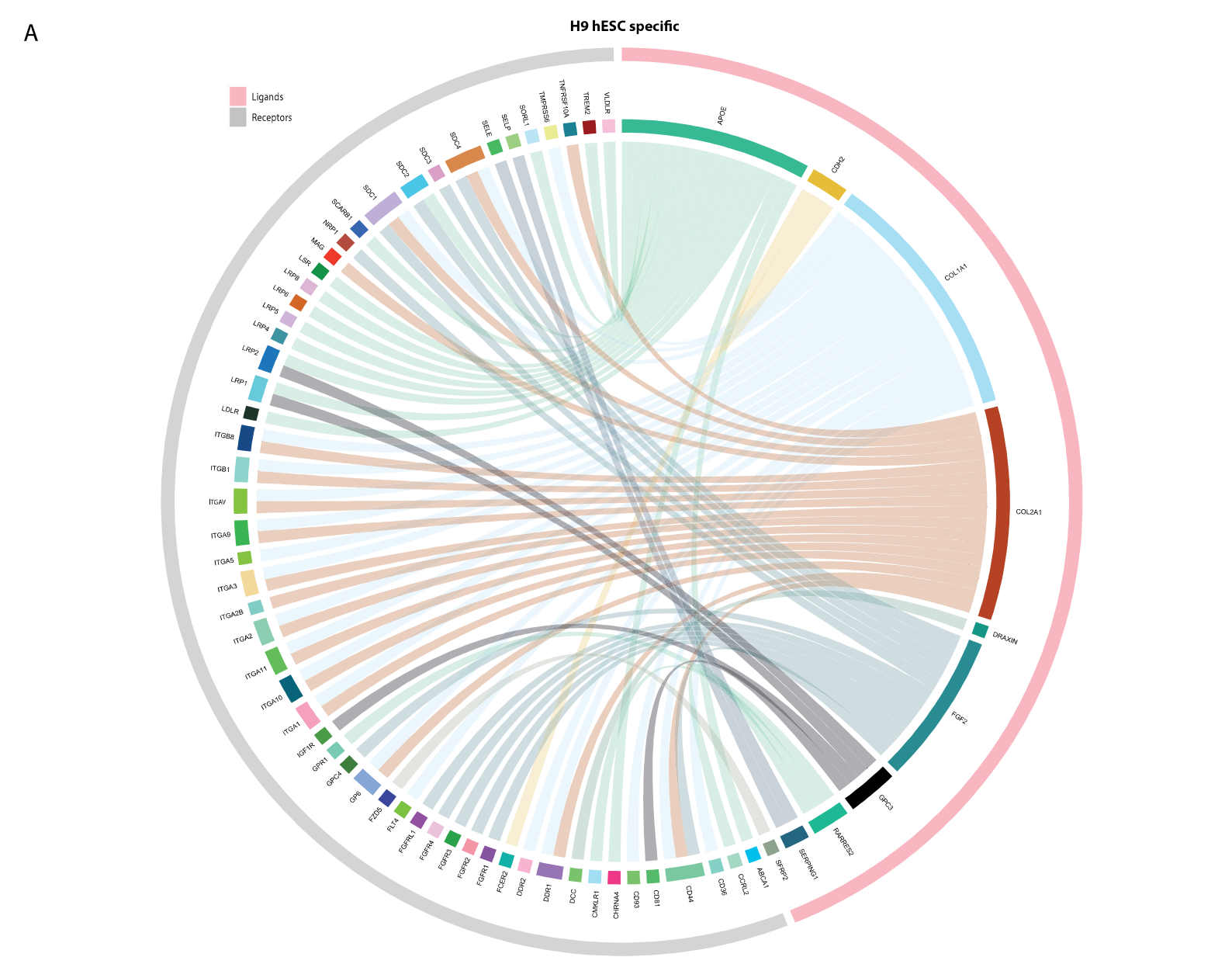

H9 / H6C7
Ligands
Receptors
TLR4
LRP1
ITGB2
DSG3
DSG2
DSC3
DSC2
CD68
CD36
ALCAM
AGER
S100A9
HPX
DSG1
DSC1

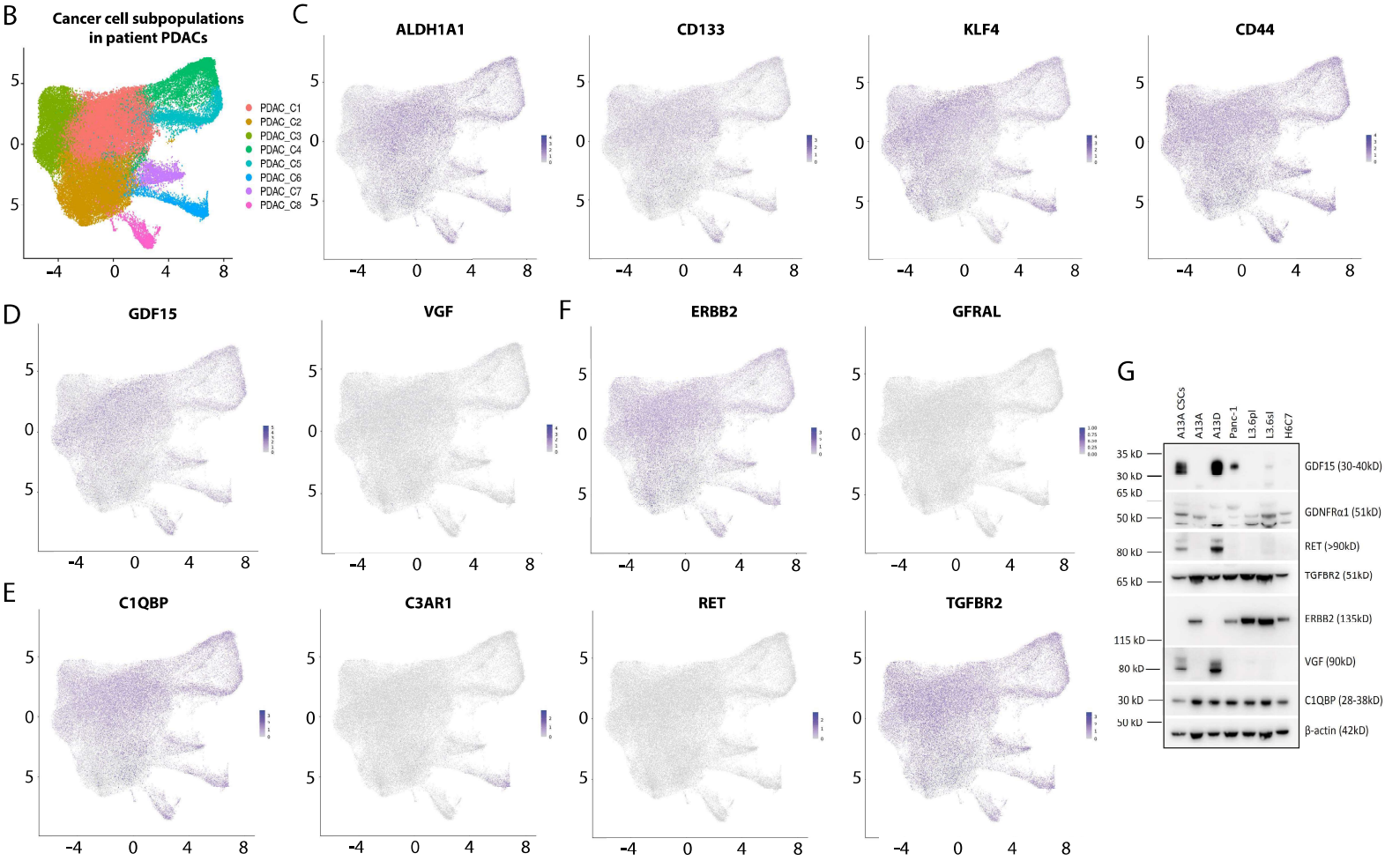
c

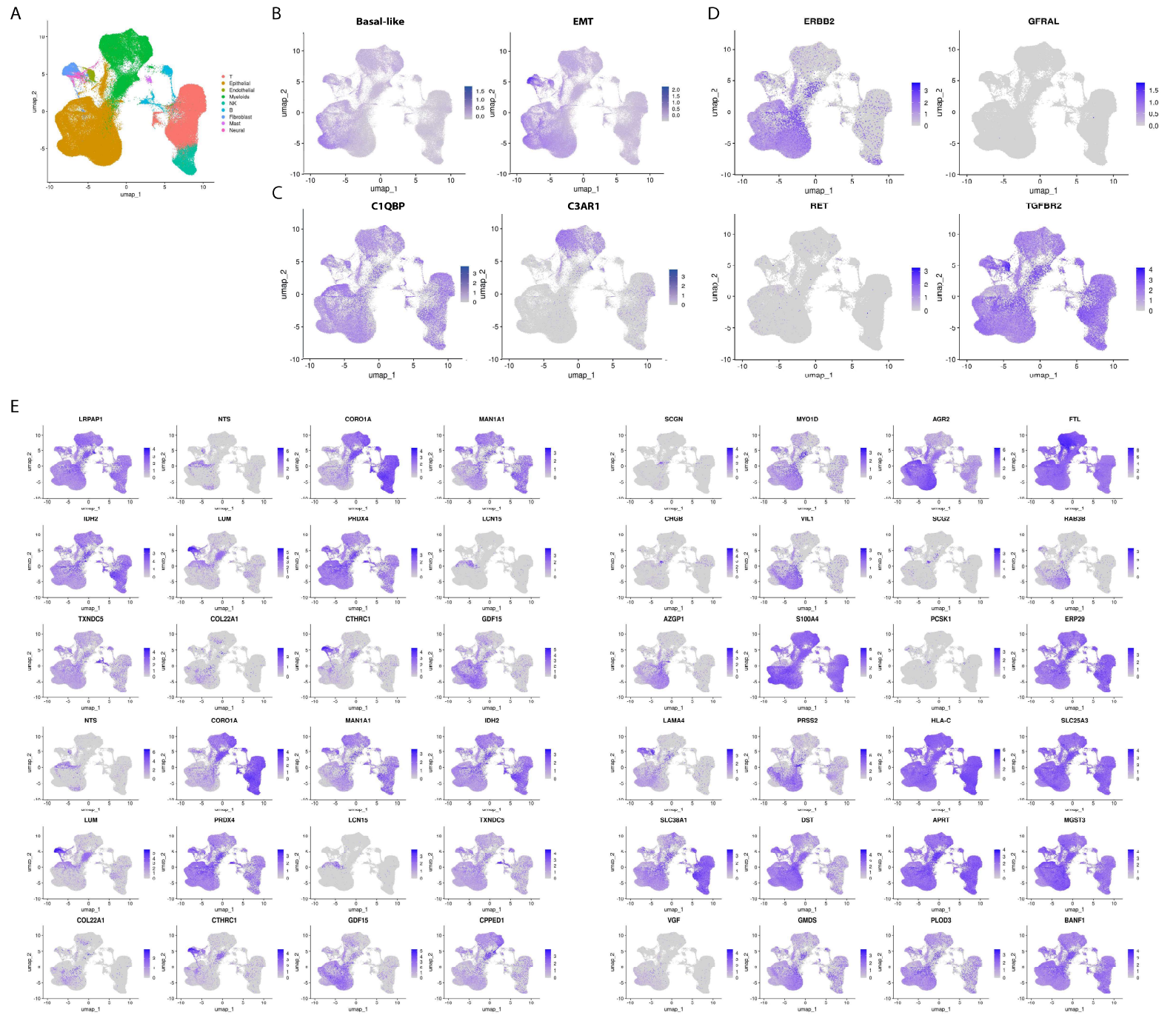

H6C7 specific
Ligands
Receptors
XRCC4
TYROBP
TREM2
RACK1
PKR3
LRP1
ITLN1
ITGB3
ITGB1
ITGAV
ITGA3
ITGA2B
FCGRT
DSC2
CUBN
CD36
B2M
ABCA1
THBS1
SUMO2
LTF
KRT1
DSC3
APOA1
ALB
A2ML1

d

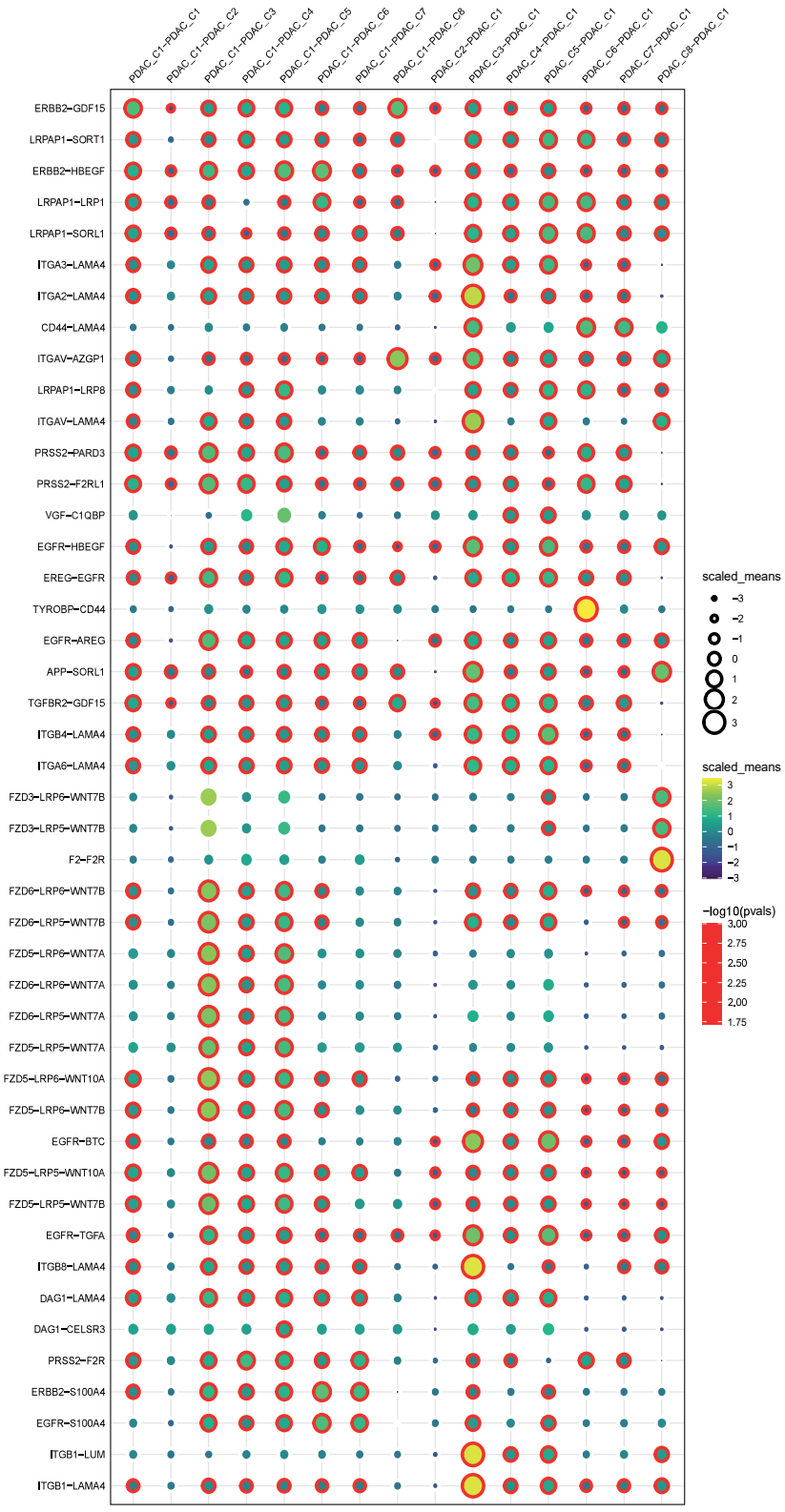

non-CSCs / H9
Ligands
Receptors
SDC4
SDC2
SDC1
PECAM1
ITGB8
ITGB1
ITGAV
ITGA9
ITGA6
ITGA3
ITGA2
ITGA11
ITGA10
ITGA1
FGFR1
CELSR3
CD44
MMP2
FBLN1
DAG1
COL6A1

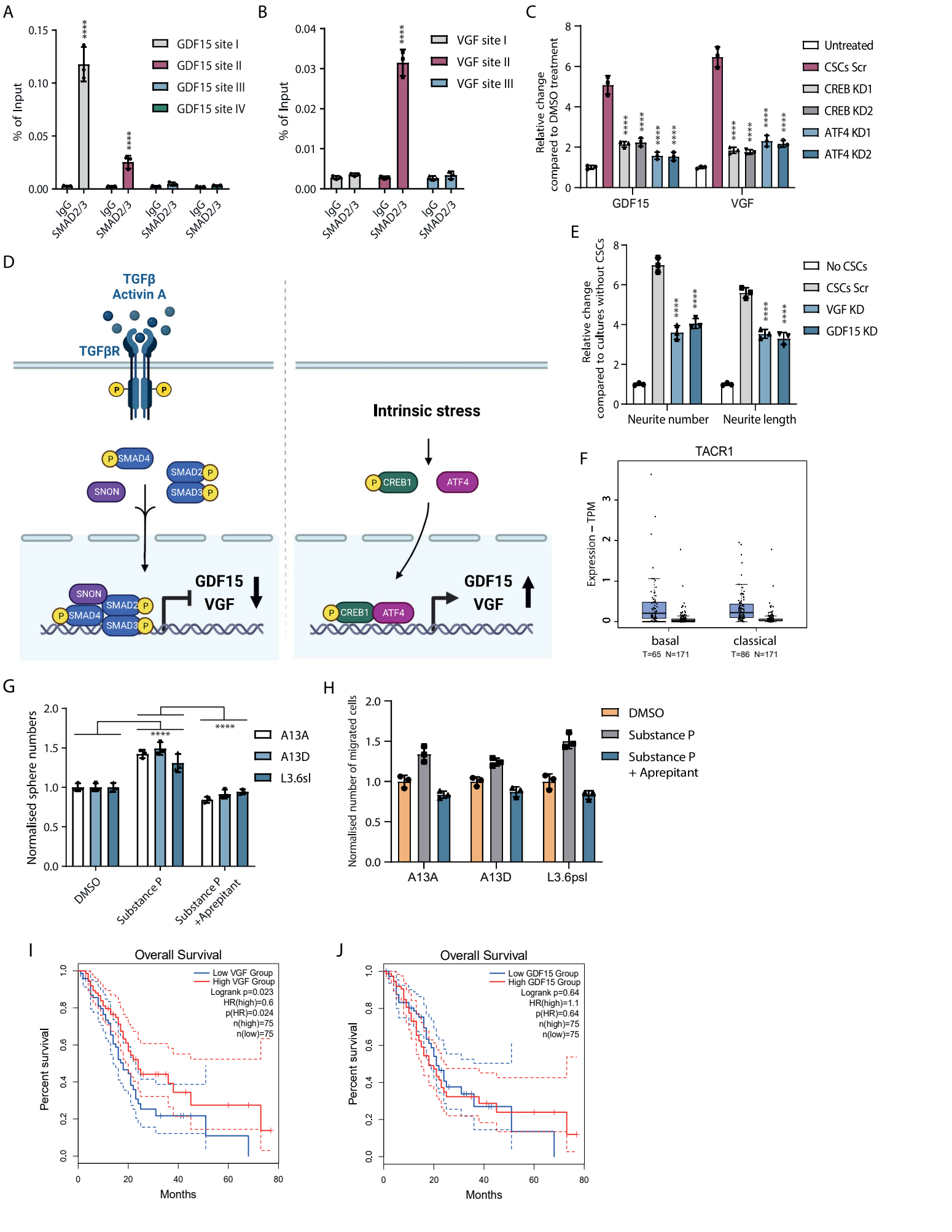

A
H9 hESC specific
Ligands
Receptors
APOE
CDH2
COL1A1
COL2A1
DRAXIN
FGF2
GPC3
RARRES2
SERPING1
SFRP2
ABCA1
CCRL2
CD44
CD36
CD81
CD93
CHRNA4
CMKLR1
DCC
DDR1
DDR2
FCER2
FGFR1
FGFR2
FGFR3
FGFR4
FGFR1.1
FLT4
FZD5
GP6
GPC4
GPR1
IGF1R
ITGA1
ITGA10
ITGA11
ITGA2
ITGA2B
ITGA3
ITGA5
ITGA9
ITGAV
ITGB1
ITGB8
LDLR
LRP1
LRP2
LRP4
LRP5
LRP6
LRP8
LSR
MAG
NRP1
SCARB1
SDC1
SDC2
SDC3
SDC4
SELE
SELP
SORL1
TNFRSF6
TNFRSF10A
TREM2
VLDLR


B
Cancer cell subpopulations in patient PDACs
PDAC_C1
PDAC_C2
PDAC_C3
PDAC_C4
PDAC_C5
PDAC_C6
PDAC_C7
PDAC_C8
C
ALDH1A1
CD133
KLF4
CD44
D
GDF15
VGF
F
ERBB2
GFRAL
E
C1QBP
C3AR1
RET
TGFBR2
G
A13A CSCs
A13A
A13D
Panc-1
L3.6pl
L3.6sl
H6C7
35 kD
30 kD
65 kD
50 kD
80 kD
65 kD
115 kD
80 kD
30 kD
50 kD
GDF15 (30-40kD)
GDNFRα1 (51kD)
RET (>90kD)
TGFBR2 (51kD)
ERBB2 (135kD)
VGF (90kD)
C1QBP (28-38kD)
β-actin (42kD)


A
B
Basal-like
EMT
C
C1QBP
C3AR1
D
ERBB2
GFRAL
RET
TGFBR2
E
LRPAP1
NTS
CORO1A
MAN1A1
SCGN
MYO1D
AGR2
FTL
IDH2
LUM
PRDX4
LCN15
CHGB
VIL1
SCGB2A2
HAMP
TXNDC5
COL22A1
CTHRC1
GDF15
AZGP1
S100A4
PCSK1
ERP29
NTS
CORO1A
MAN1A1
IDH2
LAMA4
PRSS2
HLA-C
SLC25A3
LUM
PRDX4
LCN15
TXNDC5
SLC38A1
DST
APRT
MGST3
COL22A1
CTHRC1
GDF15
CPPED1
VGF
GMDS
PLOD3
BANF1
T
Epithelial
Endothelial
Myeloids
NK
B
Fibroblast
Mast
Neural
umap_1
umap_2

Paracrine signalling between CSCs (C1) and other cancer cells (C2-C8)
in PDAC patient primary tumours




A
% of Input
GDF15 site I
GDF15 site II
GDF15 site III
GDF15 site IV
IgG
SMAD2/3
B
% of Input
VGF site I
VGF site II
VGF site III
C
Relative change compared to DMSO treatment
Untreated
CSCs Scr
CREB KD1
CREB KD2
ATF4 KD1
ATF4 KD2
GDF15
VGF
D
TGFβ
Activin A
TGFβR
SMAD4
SMAD2
SMAD3
SNON
GDF15
VGF
Intrinsic stress
CREB1
ATF4
E
Relative change compared to cultures without CSCs
No CSCs
CSCs Scr
VGF KD
GDF15 KD
Neurite number
Neurite length
F
TACR1
Expression – TPM
basal
T=65 N=171
classical
T=86 N=171
G
Normalised sphere numbers
A13A
A13D
L3.6sl
DMSO
Substance P
Substance P +Aprepitant
H
Normalised number of migrated cells
DMSO
Substance P
Substance P + Aprepitant
A13A
A13D
L3.6psl
I
Overall Survival
Low VGF Group
High VGF Group
Logrank p=0.023
HR(high)=0.6
p(HR)=0.024
n(high)=75
n(low)=75
Percent survival
Months
J
Overall Survival
Low GDF15 Group
High GDF15 Group
Logrank p=0.64
HR(high)=1.1
p(HR)=0.64
n(high)=75
n(low)=75
Percent survival
Months