Supplementary Information: Materials and methods

Fab library construction.
For optimization of Fabs isolated from naïve libraries, oligonucleotide-directed combinatorial mutagenesis was used to simultaneously diversify CDR-H3, -H2, -H1 in a human Fab framework, as described. [1-3] Mutagenic oligonucleotides were designed with mixtures of nucleotides for each diversified codon such that the resulting degenerate codon encoded for approximately 50% wildtype sequence, as described. [1-3]

Selection of FZD-binding Fab-phage.
Phage pools from naïve library F [4] or libraries designed for Fab optimization were cycled through rounds of selection for binding to Fc-tagged FZD4 or FZD7 CRDs (R&D Systems) immobilized in Maxisorp immuno plates (ThermoFisher, catalog number 12-565-135) as described. [3, 5] After 3-5 rounds of selection, specific binding of individual clones was evaluated by phage ELISAs as described below. Clones that exhibited at least 10-fold greater signal for binding to antigen compared with Fc alone were subjected to DNA sequencing to decode the sequences of the phage-displayed Fabs.

Fab and IgG production.
Fabs were produced and purified as described, [2, 6] with modifications. Briefly, Fab expression from constructs based on plasmids LP2.2avi or RH2.2 were performed in *Escherichia coli* BL21-DE3 T1R grown in 2YT media supplemented with 100 µg/ml carbenicillin. Cells were grown at 37°C with shaking until an OD_{600} of 0.8-1 was reached and protein expression was induced by 1 mM IPTG. Cell pellets were frozen, lysed and clarified by centrifugation for 30 min. Fast-flow rProtein A-Sepharose (GE Healthcare) was added to the clarified lysate and allowed to bind for 1-2 hours at 4 °C. The resin was loaded into a 10-ml polyprep column (Bio-Rad) and washed with phosphate-buffered saline (PBS). Bound protein was eluted, buffer exchanged and quantified as described. [6] DNA fragments encoding variable heavy and light chain domains were cloned into mammalian expression vectors designed for production of light chain or IgG_1 heavy chain. IgGs were expressed in HEK293F cells (Invitrogen) and affinity purified on Protein A affinity columns (GE Healthcare).

ELISAs.
Direct binding ELISAs were performed in 384-well Maxisorp plates coated overnight with 1 µg/ml FZD-CRD antigen (R&D Systems). Plates were washed with PBS (405 HT microplate washer, BioTek) and blocked with 0.5% bovine serum albumin (BSA) for 1 hour at room temperature. 200 nM Fab was added and allowed to bind for 30 minutes at room temperature. Plates were washed with PBS and binding was detected with 1:5000 anti-FLAG HRP antibody (catalog number A8592, Sigma) by measuring OD_{450} (Epoch, BioTek).
For Wnt (R&D Systems) and Norrin (R&D Systems) competition ELISAs, 384-well plates were coated with 1 µg/ml Norrin, WNT3A or WNT5A for 1 hour. Plates were washed with PBS (405 HT microplate washer, BioTek) and blocked with 0.5% BSA for 1 hour at room temperature. 150 nM FZD5-His (Sino Biological) or 25 nM FZD4-His (Sino Biological) was mixed with 200 nM Fab or IgG competitor for 1 hour at room temperature and then transferred to plates coated with Norrin,

WNT3A or WNT5A and incubated with shaking for 30 minutes. Plates were washed with PBS and binding of FZD was detected with 1:5000 anti-His HRP antibody (catalog number A7058, Sigma) by measuring OD_{450} (Epoch, BioTek).

Surface plasmon resonance.
Surface plasmon resonance measurements were performed at 25 °C using a ProteOn XPR36 instrument (Bio-Rad). Proteins (70-100 RUs) were immobilized by amine coupling to GLC sensor chip surfaces. Analyte proteins were diluted to 100 nM, and 3-fold dilutions were made with PBS buffer and injected for 1200 seconds at a flow rate of 50 µl/min. Dissociation in PBS buffer was monitored for 1200 seconds, and surfaces were regenerated by injection of 10 mM glycine, pH 1.5. For binding kinetics, sensorgrams were fitted to a 1:1 Langmuir model using ProteOn Manager Software (Bio-Rad). Values for k_a, k_d and K_D were calculated by taking the average of values determined by locally fitting each binding curve of an SPR data set with globally fitted R_{max} to the Langmuir binding model. Errors are standard errors of the mean values determined from fitting each binding curve of an SPR data set.

Bio-Layer Interferometry.
Bio-layer interferometry (BLI) measurements were performed using an Octet HTX instrument (ForteBio). IgG F2.A or OMP-18R5 was immobilized on a BLI sensor (PALL, catalog number 18-5092) by amine-coupling. The sensor with immobilized IgG was dipped for 300 seconds into 100 nM FZD1-Fc, FZD2-Fc, FZD4-Fc, FZD5-his, FZD7-Fc or FZD8-Fc analyte, or analyte controls (IgG F2.A, OMP-18R5 or buffer control, no analyte) in assay reaction buffer (PBS, 1% BSA), 0.05% Tween20). The sensor was transferred for 300 seconds into either 100 nM IgG F2.A or OMP-18R5 in assay reaction buffer, or to the assay reaction buffer alone.

Size-exclusion chromatography.
Size-exclusion chromatography was performed on a BioRad NGC chromatography system with a C96 autosampler. Proteins (50 µg) were injected onto a Tosoh Bioassist G3SWxL column at a flow rate of 0.5 ml/min in a PBS mobile phase. Protein elution was monitored by measuring absorbance at 215 nm and 280 nm.

Cell culture.
HPAF-II, PaTu8988s, PANC-1, HEK293T and mouse L-cell lines were maintained in DMEM containing 4.5 g/L D-glucose, L-glutamine (LifeTechnologies, lot 1812193), 10% fetal bovine serum (FBS; LifeTechnologies, lot 1802782) and 1% penicillin/streptomycin (LifeTechnologies, lot 1780184). AsPC-1, BxPC-3 and YAPC cell lines were maintained in RPMI 1640 with L-glutamine (LifeTechnologies, lot 1810978), 10% FBS and 1% penicillin/streptomycin (LifeTechnologies, lot 1780184). CHO, GP2A and GP3A cell lines were maintained in DMEM/F12 (LifeTechnologies, lot 1798394), 10% FBS and 1% penicillin/streptomycin. Capan-2 cells were cultured in McCoy medium (LifeTechnologies, lot 1809323), 10% FBS and 1% penicillin/streptomycin. Lentiviruses engineered to express FZD CRDs fused to a Myc tag and a GPI anchor were used to establish stable CHO cells, as described. [7] HPAF-II, AsPC-1, Capan-2, PANC-1, BxPC-3 were from ATCC. Patient-derived PDAC cell lines were generously provided by D. Hedley and M. Tsao (University Health Network, Toronto, Canada). The PaTu8988s cell line was a gift from F. Real, Madrid,

Spain. HUVEC cells (Lonza, catalog number C2517A) were maintained in Endothelial Growth Medium (EGM)-2 BulletKit, (Lonza, catalog number CC-3162). Normal human dermal fibroblasts (NHDF) (Adult skin, Lonza, catalog number CC-2511, Lot 0000465422) were maintained in DMEM containing 4.5 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate (ThermoFisher Scientific, catalog number 11965-065) and supplemented with 10% FBS (ThermoFisher Scientific, catalog number 10437-028) and 1% penicillin/streptomycin (ThermoFisher Scientific, catalog number 15140-163). All cell lines were maintained in a humidified environment at 37 °C and 5% CO_2.

TopFlash reporter assay.
Lentivirus engineered to express the TopFlash β-catenin-dependent luciferase reporter (firefly luciferase) and Renilla luciferase were used to establish a stable HEK293T Wnt-reporter cell line. Cells were seeded on 24-well plates at 50% confluency in a final volume of 500 µl. After 24 hours, half of the media was replaced with 250 µl WNT3A or control conditioned media from L cells and 200nM Fabs or IgGs were added. After 15 hours, the cells were assayed in accordance with the dual luciferase protocol (Promega) using an Envision multilabel plate reader (Perkin-Elmer).

Immunofluorescence microscopy.
PDAC cells were seeded and cultured overnight in Cell carrierTM-96 well plates (PerkinElmer). Staining was performed with 200 nM Fab diluted in Dulbecco's PBS (DPBS) containing 100 mg/L Ca^{2+} and 100 mg/L Mg^{2+} (ThermoFisher, catalog number 14040-133) and 5% donkey serum. Alexa Fluor488 AffiniPure F(ab')2 (Jackson ImmunoResearch, catalog number 109-546-097) was used as the secondary antibody. Subsequently, cells were washed and fixed in 4% paraformaldehyde (PFA) and nuclei were stained with Hoechst 33342, trihydrochloride trihydrate solution (Molecular Probes). Cells were imaged on an Opera QEHS (PerkinElmer) and acquired images were processed using Columbus software (Donnelly Centre, University of Toronto).

Flow cytometry.
Primary staining of cells was performed with 200 nM anti-FZD Fab or IgG as indicated. Alexa Fluor 488 AffiniPure F(ab')2 was used as the secondary antibody (Jackson ImmunoResearch, catalog number 109-546-097). Dead cells were excluded by staining with Fixable Viability Dye eFluor 660 (eBioscience, catalog number 65-0864). All reagents were used according to manufacturer's instructions. For cell cycle analysis, cells were dissociated with trypsin and fixed with ethanol. For each sample, 10^6 fixed cells were washed in PBS, 1% FBS and were stained with PBS, 50 µg/ml propidium iodide (ThermoFisher, catalog number P1304MP), 50 µg/ml RNase A (ThermoFisher, catalog number EN0531), 1% FBS. Fixed and stained cells were subjected to flow cytometry on a FACSCanto II (BD Biosciences) and data were analyzed with FlowJo Software (FlowJo, LLC).

Reverse transcription and quantitative real-time PCR.
Cells were lysed and RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, catalog number 74134). RNA concentration was quantified with Nanodrop 1000 and 2 µg RNA was used to make cDNA with SuperScript Vilo (Invitrogen, lot 1790795). Real-time PCR was performed using Power SYBR Green Master Mix on the Applied Biosystems Real-Time PCR system. The following forward and reverse PCR primer pairs were used: *AXIN2*, CTCCCCACCTTGAATGAAGA and TGGCTGGTGCAAAGACATAG; *NKD1*, TGAGAAGATGGAGAGAGTGAGCGA and

GGTGACCTTGCCGTTGTTGTCAAA; *β-actin*, CCTCTATGCCAACACAGTGC and GTACTCCTGCTTGCTGATCC. Analysis was performed with the comparative cycle threshold method (Applied Biosystems) with quantification relative to untreated control. [8] All samples were normalized to *β-actin* expression.

Cell proliferation assays.
Cells were seeded in 96-well plates (HAPF II and Capan-2 at 1000 cells per well, AsPC-1 and BxPC-3 at 2500 cells per well, PaTu8988s and PANC-1 at 5000 cells per well, and GP3A at 2000 cells per well), cultured overnight, and treated with IgG for 6 days. Cell viability was assayed by adding 20 µl Alamar Blue (Invitrogen, catalog number DEL1100) to 200 µl cell culture medium. Plates were incubated at 37 °C for 2-4 hours and fluorescence was measured at 540/25 nm excitation and 620/40 emission on a BioTek Synergy 2 system. GP2A cells were seeded in 48-well plates at 50,000 cells per well, treated with IgG for 6 days, and counted using an automated cell counter (Beckman Coulter).

siRNA cell transfections.
HUVEC cells were transfected with negative control #1 siRNA (Invitrogen, catalog number AM4611), FZD4 Pre-designed siRNA (Invitrogen, catalog number 139764) or ERG Pre-designed siRNA (Invitrogen, catalog number 5271), using Lipofectamine RNAiMAX (Thermofisher Scientific, catalog number 13778075) and Opti-MEM reduced serum medium (ThermoFisher Scientific, catalog number 31985-070), according to manufacturer's instructions. After 24 hours, transfection mix was removed and replaced with fresh medium for 48 hours prior to the fibrin gel bead assay.

Fibrin gel bead assay.
Three-dimensional *in vitro* model of angiogenesis was performed as described, with minor modifications. [9] HUVEC cells were transfected with the indicated siRNAs (control, FZD4 or ERG) or treated with 30 µM Suramin (Northern Biologics), 1 µM LGK974 (Cayman Chemical, catalog number 14072), or 300 nM IgG (F2, F2.A or OMP-18R5) over the course of 12 days. At the indicated time points, endothelial vessel length was imaged and quantified. At day 12, gels were incubated with Calcein AM (1:1000, Molecular Probes, catalog number C1430) for 2 hours, washed twice with DPBS and fixed in 4% PFA for 30 minutes. Endothelial vessel lengths per bead were imaged and analysed by collecting 5 random brightfield images per well (in triplicate) using Zeiss LSM 700. Quantification of vessel length was performed using ImageJ 1.41o. The lengths of vessels were determined by measuring the total length of each vessel from its initial sprouting point from the bead to the tip of the endothelial sprout. Mean endothelial vessel length for each treatment group was determined.

References

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Supplementary Figure Legends

Supplementary Figure S1. Sequences of naïve anti-FZD7 Fabs, and sequences and specificities of F7-optimized Fabs; biophysical characterization of F2-optimized IgG F2.A. (A) The sequences of the CDRs diversified in the Fab library are shown for Fabs selected for binding to the CRD of FZD7. Residues are numbered according to IMGT standards, [1] and dashes indicate gaps in the alignment. (B) Sequences and specificities of F7-optimized Fabs. Residues are numbered according to IMGT standards, [1] and dashes indicate identity with the F7 sequence. (C) SEC profiles of IgG F2.A and OMP-18R5. (D) IgG F2.A binding kinetics measured by BLI response (y-axis) monitored in real-time (x-axis). Each sensorgram shows binding of the indicated analyte (FZDs, IgG F2.A, OMP-18R5 or buffer control, no analyte) to a chip with immobilized IgG F2.A. The vertical line (360 seconds) indicates a switch from solution containing the indicated analyte to a solution containing the reaction buffer.

Supplementary Figure S2. Cell binding of anti-FZD Fabs. (**A**) Specificity profiling of IgGs F2 and F2.A by flow cytometry with various CHO cell lines, each expressing a different myc-tagged FZD CRD anchored on the outside of the plasma membrane by a GPI anchor. Numerical values indicate the fold increase in median fluorescence intensity of 200 nM IgG over background (secondary antibody alone) normalized to the control (Ctrl, CHO-GPI). Filled histograms indicate binding and represent a population of >10,000 cells in a single experiment performed in duplicate. (**B**) Binding of Fab F2.A to PDAC cell lines detected by flow cytometry. Numerical values indicate the fold increase in median fluorescence intensity of 200 nM Fab over background (secondary antibody alone). Filled histograms indicate binding and represent a population of >10,000 cells in a single experiment. (**C-J**) Binding of Fab F2.A to PDAC cells detected by indirect immunofluorescence. Cells were stained with 200 nM Fab and binding was confirmed by indirect immunofluorescence (FZD-green). Nuclear staining was performed with Hoechst (blue). Staining was performed in duplicate, images are representative of ≥15 images for each sample. Scale bar = 20 µm.

Supplementary Figure S3. Images of HUVECs after treatments with FZD Abs. Fluorescent images were taken following Calcein AM staining for 2 hours at day 12 of treatments with IgG F2, F2.A or OMP-18R5. Representative of n = 5 random images per well (in triplicate). Scale = 170 µm. 'B' = cytodex bead.

References

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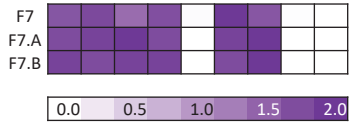
Fig. S1

(A)

Antigen	Fab ID	CDRL3 107	108	109	110	111	113	114	115	116	CDRH1 30	35	36	37	38	39	CDRH2 55	56	57	58	59	62	63	64	65	66	CDRH3 107	108	109	110	111	111.1	111.2	111.3	112.2	112.1	112	113	114	115
	F1	S	S	Y	-	-	-	S	L	I	L	S	Y	Y	S	M	S	I	Y	P	S	Y	G	Y	T	Y	P	S	P	G	S	-	-	-	-	-	Y	H	G	M
	F2	G	V	Y	-	-	-	-	L	F	I	Y	S	S	S	I	S	I	Y	S	S	Y	G	S	T	S	Y	H	Y	P	F	-	-	-	-	-	G	H	A	L
	F3	A	A	Y	H	W	P	P	L	F	L	Y	Y	S	S	M	Y	I	S	P	Y	S	G	Y	T	S	Y	W	-	-	-	-	-	-	-	-	-	-	A	M
	F4	Y	W	A	-	-	-	-	P	I	I	Y	Y	Y	Y	M	S	I	Y	S	Y	S	G	S	T	Y	Y	A	S	Y	V	G	-	-	Y	Y	P	W	A	L
FZD7	F5	V	S	G	G	-	G	G	L	I	I	Y	S	S	Y	I	Y	I	Y	S	Y	S	G	Y	T	Y	W	Y	H	P	Y	W	-	-	-	Y	A	S	A	I
	F6	W	A	Y	-	-	-	G	P	F	I	Y	Y	Y	S	M	S	I	Y	S	S	Y	S	Y	T	S	S	S	P	G	A	-	-	-	-	-	D	Y	G	L
	F7	Y	Y	S	-	-	-	-	L	F	I	Y	Y	Y	S	M	Y	I	S	P	S	Y	G	Y	T	Y	G	Y	F	Y	S	-	-	-	-	-	W	G	G	M
	F8	Y	W	Y	G	-	V	A	P	I	I	S	S	S	Y	I	Y	I	Y	S	S	Y	G	S	T	Y	A	S	W	-	-	-	-	-	-	-	-	Y	A	L
	F9	Y	Y	H	-	-	-	-	P	I	I	S	S	Y	Y	I	S	I	Y	P	Y	Y	S	S	T	Y	V	W	Y	-	-	-	-	-	-	-	-	G	A	M

(B)

Fab ID	CDRL3 107	108	109	115	116	CDRH1 30	35	36	37	38	39	CDRH2 55	56	57	58	59	62	63	64	65	66	CDRH3 107	108	109	110	111	112	113	114	115
F7	Y	Y	S	L	F	I	Y	Y	Y	S	M	Y	I	S	P	S	Y	G	Y	T	Y	G	Y	F	Y	S	W	G	G	M
F7.A	-	-	-	-	-	F	S	S	S	S	I	-	-	Y	-	F	N	-	-	S	-	-	-	-	-	T	-	-	-	-
F7.B	-	-	-	-	-	F	S	S	S	S	I	-	-	Y	-	-	-	D	-	-	-	-	-	-	-	T	-	-	-	-



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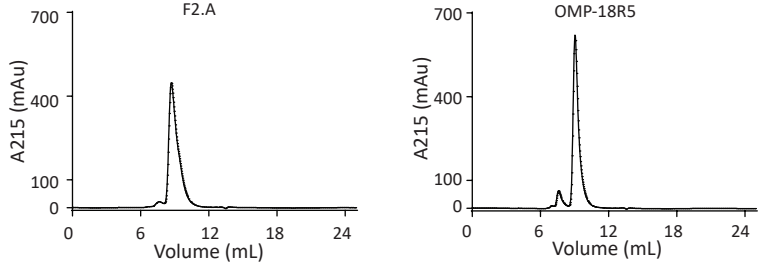

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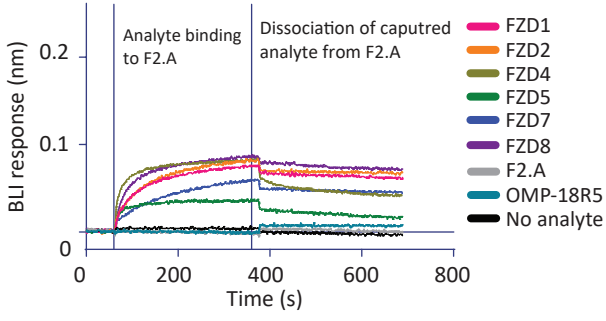

Fig. S2

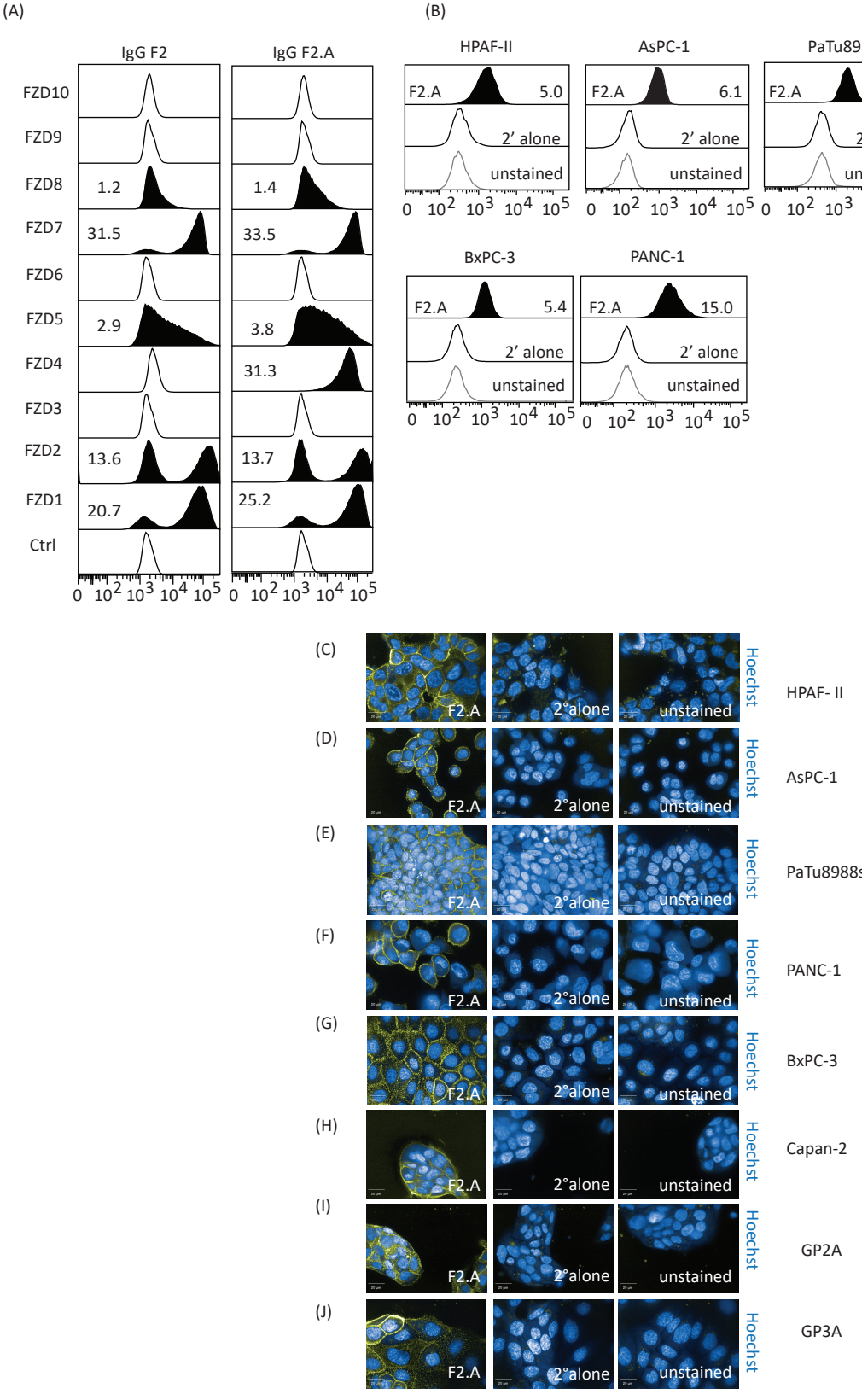
(A)
IgG F2
IgG F2.A
FZD10
FZD9
FZD8
1.2
1.4
FZD7
31.5
33.5
FZD6
FZD5
2.9
3.8
FZD4
31.3
FZD3
FZD2
13.6
13.7
FZD1
20.7
25.2
Ctrl
0 10^2 10^3 10^4 10^5
(B)
HPAF-II
AsPC-1
PaTu8988s
F2.A
5.0
6.1
5.1
2' alone
unstained
BxPC-3
PANC-1
5.4
15.0
(C)
(D)
(E)
(F)
(G)
(H)
(I)
(J)
F2.A
2°alone
unstained
Hoechst
HPAF- II
AsPC-1
PaTu8988s
PANC-1
BxPC-3
Capan-2
GP2A
GP3A

Fig. S3