

Tumor-intrinsic Ist1 restricts IFN γ responsiveness to drive immune evasion in pancreatic cancer

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Abstract

Tumour-cell-intrinsic mechanisms that determine whether immune encounter culminates in effective killing or immune escape remain incompletely understood, particularly in immune-refractory solid tumours such as pancreatic cancer. Here, through integrative analysis of CRISPR-based genetic screening datasets, we identify the endosomal sorting complex required for transport (ESCRT)-associated factor Ist1 as a key determinant of tumour-cell susceptibility to cytotoxic CD8⁺T-cell attack. Loss of Ist1 enhanced CD8⁺T-cell-mediated killing in vitro and increased tumour clearance in an *in vivo* zebrafish xenograft model. Ist1 deficiency also sensitized tumour cells to exogenous interferon- γ (IFN γ), amplified IFN γ -driven JAK-STAT1 signalling, and upregulated antigen-presentation programs, accompanied by increased surface major histocompatibility complex class I (MHC-I) expression. Mechanistically, Ist1 regulated the membrane stability of the IFN γ receptor, and its loss increased surface availability and persistence, thereby restoring tumour-cell responsiveness to IFN γ . Consistent with this model, IFN γ blockade rescued the increased killing phenotype caused by Ist1 inhibition, indicating that Ist1-dependent membrane regulation controls tumour-cell responsiveness to immune-derived signals. Pharmacological disruption of the Ist1- Charged multivesicular body protein 1 (Chmp1) interaction similarly sensitized tumour cells to cytotoxic CD8⁺T-cell killing, highlighting a tractable vulnerability in cytokine-resistant tumours. Clinically, high Ist1 expression was associated with immune-evasive features and poorer outcomes, whereas low Ist1 expression in the context of high CD8⁺T-cell infiltration correlated with improved prognosis. Together, these findings identify Ist1, for the first time, as a membrane-regulatory determinant of tumour immune susceptibility and suggest that ESCRT-linked control of IFN γ receptor availability may provide a targetable entry point for immune sensitization in pancreatic cancer.

Introduction

The development of cancer immunotherapy has revealed that antitumor immune responses can be therapeutically mobilized, but it has also made clear that their successful execution remains highly context dependent. In many solid tumors, immune activation alone is insufficient to achieve durable clinical benefit, suggesting that the critical limitation often lies not simply in whether antitumor immunity is initiated, but in whether it can be effectively sustained and completed within the tumor bed[1]. This challenge is imposed in large part by the complex architecture of the tumor microenvironment (TME), which shapes immune-cell access, function, persistence, and therapeutic responsiveness[2]. These limitations are especially evident in immune-refractory malignancies such as pancreatic cancer, in which current immunotherapeutic strategies have thus far shown limited and often non-durable benefit[3].

Another important element driving unsuccessful anti-tumour immunity are cancer cell intrinsic mechanisms of immune evasion. Tumor cells can escape immune elimination through multiple autonomous mechanisms operating at different levels of the tumour-immunity cycle[4–5]. One well-established example is defective antigen processing and presentation of tumor antigen, including reduced Major histocompatibility complex (MHC) class I (MHC-I) surface expression, which directly

impairs recognition and killing of tumor cells by primed CD8⁺T cells[6]. Tumor-intrinsic oncogenic signaling can also drive unfavourable immune states. Representative examples include the WNT/ β -catenin[7], cyclin-dependent kinase 4(CDK4)-CDK6[8] and mitogen-activated protein kinase (MAPK)[9] pathways, all of which have been linked to immune exclusion and implicated as potential targets for improving immunotherapy responsiveness[10]. Another major route is impaired responsiveness to immune-derived signals, especially through disruption of the interferon (IFN) γ -JAK-STAT axis, which has been established as a mechanism of resistance to immune checkpoint blockade[11]. Beyond recognition and signaling defects, tumor cells can also withstand immune pressure by engaging stress-adaptation programs such as autophagy and by acquiring resistance to cell death, thereby preserving fitness under cytotoxic attack[12]. Furthermore, pancreatic cancer cells can undergo epithelial-to-mesenchymal transition (EMT) to reshape their sensitivity to T-cells[13–14]. Collectively, these examples indicate that tumor immune escape is not shaped solely by immune-cell abundance, but also by autonomous tumor programs that regulate antigen visibility, immune exclusion, and responsiveness to cytotoxic pressure. However, whether there are other cellular processes involved that can be therapeutically targeted is not known.

To identify additional regulators of tumour-intrinsic resistance in pancreatic cancer, we integratively analyzed previously generated whole genome clustered regularly interspaced short palindromic repeats (CRISPR) screening data from a T cell-tumor co-culture system[14–15]. This analysis highlighted the IST1 factor associated with ESCRT-III (IST1) and prompted us to test whether membrane regulation contributes to tumor-cell resistance to T-cell-mediated killing.

Materials and methods

Cell lines and culture conditions

Cell lines in this study included Human embryonic kidney (HEK)293T cells [CRL-1573, obtained from American Type Culture Collection (ATCC)], KPC3 parental and KPC3-ovalbumin (OVA)-green fluorescent protein (GFP) cells (provided by F. Ossendorp's laboratory, the derivation of this line is described in previous work[14]). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, 41965062), supplied with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, 16000044) and penicillin-streptomycin (100 U/ml; Thermo Fisher Scientific, 15140163), and grown at 37°C in a humidified incubator containing 5% CO₂. All cell lines were regularly tested for mycoplasma contamination and human cell lines were checked for authenticity by short tandem repeat (STR) profiling.

Lentiviral Production and Genetic Perturbation

Lentiviral particles were produced in HEK293T cells by co-transfection of lentiCRISPR-v2 sgRNA vectors (Addgene, #52961) or the indicated overexpression or knockdown constructs together with the packaging plasmids pCMV-VSVG, pMDLg-RRE (gag/pol), and pRSV-REV using polyethylenimine (PEI) as

the transfection reagent. Virus-containing supernatants were collected 48 h after transfection, filtered through a 0.45-µm membrane, and either used immediately for transduction or stored at -80 °C.

For lentiviral transduction, target cells were seeded at approximately 60% confluence and incubated with viral supernatant in complete growth medium supplemented with polybrene (8 µg/mL; Sigma-Aldrich) for 24h. Cells were then allowed to recover for 48 h before antibiotic selection for at least 1 week, followed by further expansion prior to use in downstream experiments.

For gene knockout, sgRNAs targeting the indicated genes were cloned into a puromycin-selectable lentiCRISPR-v2 vector according to the SAM target sgRNA cloning protocol[16]. Stable knockout cell lines were generated by lentiviral transduction followed by puromycin selection. For stable knockdown, shRNA sequences targeting the indicated genes were cloned into pLKO.1 puro Vector (Addgene, #8453) and introduced by lentiviral transduction, followed by puromycin selection. For overexpression, the indicated full-length cDNAs were cloned into pLX304-V5 blast vector (Addgene, #25890) and used to generate stable cell lines by lentiviral transduction and antibiotic selection. Knockout, knockdown, and overexpression efficiency were confirmed by western blot before use in functional assays. The sgRNA and shRNA used in this study were as follows: sgCtrl: 5'-GCGAGGTATTTCGGCTCCGCG-3', sgIst1#1: 5'-CACCGTGAGCGAGCACGCATCCGTG-3', sgIst1#2: 5'-CACCGATTACAGTCCCGATCTGGT-3', shCtrl (Merck Millipore, #SHC001), shIst1#1 (Merck Millipore, #TRCN0000216630), shIst1#2 (Merck Millipore, #TRCN0000266801), shIst1#3 (Merck Millipore, #TRCN0000266802), shIst1#4 (Merck Millipore, #TRCN0000266803), shIst1#5 (Merck Millipore, #TRCN0000266805).

Isolation and in vitro activation of primary CD8⁺ T cells

OT-I CD8⁺T cells were isolated from the spleens of B6.Cg-Ptprc^aTg(TcraTcrb)1100Mjb/Lumc mice, which recognize OVA₂₅₇₋₂₆₄ presented by Histocompatibility-2 (H-2) locus K, haplotype b (H2K^b). Both female and male mice aged 8-14 weeks were used. Mice were bred and maintained under specific pathogen-free conditions at the Leiden University Medical Center (LUMC) animal facility. All animal experiments were conducted in accordance with the Code of Practice of the Dutch Animal Ethical Commission and approved under project AVD11600202013796 ("Immunotherapy and vaccination against cancer").

Spleens were mechanically dissociated, filtered through a 70-µm cell strainer, and subjected to red blood cell lysis for 5 min. OT-I CD8⁺T cells were enriched using the Mouse CD8⁺T Lymphocyte Enrichment Set-DM (BD IMagTM, #558471) according to the manufacturer's instructions. OT-I CD8⁺T cells were directly activated by 10ng/ml OVA(257–264) peptide (Anaspec Cat AS-60193) for 48-72 h by culturing in Iscove's Modified Dulbecco's Medium (IMDM) supplied with GlutaMAX(Gibco, #31980030), 10%FBS, 100 U/ml penicillin-streptomycin, 50 µM β-mercaptoethanol(Gibco, #21985), 100 ng/mL interleukin (IL)2 (Immunotools,#12340026), 10 ng/mL IL-7 (Immunotools, #12340075), and 10 ng/mL IL15 (Immunotools, #12340155).

Cytokine treatment and cell viability assay

A total of 3×10^3 tumour cells per well were seeded in 96-well plates and treated with recombinant murine IFN γ (Immunotool, #12343536), TNF α (Immunotools, #12343016), TNF-Related Apoptosis-Inducing Ligand (TRAIL) (PeproTech, #315-19-10UG), Granzyme B (PeproTech, 140-03-10UG), Perforin (Cusabio, #CSB-YP018668MO) or a combination of them at the indicated concentrations. Cell viability were measured using CellTiter-Blue (Promega, #G8080) or MTS cell proliferation assays (Abcam, #ab197010) according to the manufacturer's instructions. Fluorescence or absorbance was measured on a spectrophotometer.

T cell co-culture assay

KPC3-OVA-GFP cells were seeded in 12-well plates at 5×10^4 cells per well or in 96-well plates at 3×10^3 cells per well and allowed to adhere overnight. Activated OT-I CD8 $^+$ T cells were then added at the indicated effector-to-target (E:T) ratios. Live tumor-cell GFP signal was monitored using the IncuCyte live-cell imaging system.

For end-point crystal violet staining, co-cultures were maintained for 72 h, after which CD8 $^+$ T cells and detached dead tumor cells were gently removed by washing with 1X phosphate buffered saline (PBS). The remaining adherent cells were fixed and stained with a solution containing 0.1% crystal violet (Sigma-Aldrich, C0775) and 50% methanol (Honeywell, 15651630).

For viability measurements, culture medium was removed, and tumor-cell viability was quantified using the CellTiter-Blue Viability Assay or MTS Assay. In all assays, viability was normalized to the untreated control group. Each condition was analyzed with at least three replicate wells.

In vivo zebrafish xenograft model

All zebrafish experiments were conducted in a licensed establishment for the breeding and use of experimental animals (Leiden University) and were performed in accordance with institutional regulations and guidelines. In all procedures, advice from the local animal welfare body was taken into account to minimize potential animal suffering at the facility. The zebrafish assays described in this study were conducted using non-feeding larvae and, therefore, were not classified as animal experiments under the Dutch Experiments on Animals Act (Wet op de dierproeven, Wod; effective 2014), which implements European Directive 2010/63/EU on the protection of animals used for scientific purposes. Accordingly, no additional license was required for experiments involving zebrafish larvae younger than 6 days post-fertilization.

To evaluate the effect of Ist1 perturbation on tumour-cell susceptibility to T cell killing *in vivo*, 2 days postfertilization (dpf), activated CD8 $^+$ T cells were labelled with vibrant DiD fluorescent dye (Invitrogen, #V22887) immediately before transplantation as per the manufacturer's protocol. Briefly, cells were harvested and dispersed in 1xPBS after one wash at a density of 1×10^6 cells/mL. 5 μ L of Vibrant DiD

dye was added to stain $1-5 \times 10^6$ cells and incubated at 37°C for 10 mins after gentle mixing followed by 5 mins incubation at 4°C. Cells were washed once with 1xPBS by centrifuging at 500g for 5 mins and then finally suspended to have a final concentration of 200 cells/nl. Further, KPC3-OVA-GFP tumour cells were harvested and filtered using a 40µm filter before resuspending in a density of 200 cells/nl. ~5-6µL of the tumor cells alone or mixed with labelled CD8⁺T cells (1:4 E:T ratio) loaded into a borosilicate glass capillary needle (Harvard Apparatus, Holliston, MA, USA) using a microloader, and the injections were performed using a Pneumatic Picopump and a manipulator (WPI, Stevenage, UK). Approximately 400 cells were injected into the perivitelline space in dechorionated zebra fish embryos at 2 days post fertilization (dpf) stage, anesthetized with 0.003% tricaine methane-sulfonate 2 min prior to injection and placed on a wet 1.5% agarose layer in a petri dish. Following transplantation, the zebrafish embryos were maintained at 33°C and monitored for 72 h. At 5 dpf the embryos were anaesthetized and image acquisition and cell quantification were performed using a Leica SP5 Stimulated Emission Depletion (STED) confocal microscope. Vibrant TM DiD is a lipidic fluorescent stain that allows CD8⁺ T cells visualization at a wavelength of 647 nm while tumor cells with constitutive GFP expression visualized at a wavelength of 488nm. Confocal stacks were processed for maximum intensity projections with LEICA software. Fluorescence images from zebrafish xeno-grafts were analysed using the QUANTIFISH software. Impact of Ist1 on T cell-mediated tumour cell killing was assessed by comparing residual integrated fluorescence intensity of GFP between KPC3-GFP only and KPC3-GFP + OT-1 T cell in IST1 positive and KO groups under matched conditions. At least 10 fish were analyzed per condition in each experiment, all experiments were repeated at least 3 times independently, and representative results are shown.

Transcriptional reporter assays

To assess cytokine-responsive transcriptional activity, KPC3 tumour cells were transduced with the IFN γ -responsive reporter pGF-GAS4X (Addgene, #225751), which is used to monitor Signal transducer and activator of transcription 1 (STAT1) pathway activation. Reporter-expressing KPC3 cells were then infected with lentiviral particles with shRNAs to stably knock down Ist1. Cells were seeded in 96-well plates overnight before stimulation and treated with recombinant murine IFN γ at the indicated concentrations and durations. Reporter activity was longitudinally measured using the IncuCyte live-cell imaging system. Reporter positive confluent area was quantified at the indicated time points and normalized to the baseline signal.

RNA extraction and Quantitative RT-PCR

Total RNA was extracted from cells using the NucleoSpin RNA kit (Macherey-Nagel, #740955) according to the manufacturer's instructions. For each sample, 1µg of total RNA was reverse transcribed into cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, #K1632). Quantitative PCR was performed using GoTaq qPCR Master Mix (Promega, #A600X) on a CFX Connect Real-Time PCR Detection System (Bio-Rad). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$

method with *GAPDH* (encoding glyceraldehyde 3-phosphate dehydrogenase) as the housekeeping gene. Primer sequences used for RT-qPCR are listed in Table S1.

Western blot Analysis

Cells were washed with PBS and lysed in lysis buffer (10% [w/v] glycerol, 2% [w/v] sodium dodecyl sulphate (SDS), and 60 mM Tris-HCl [pH 6.8]). Equal amounts of protein were separated by SDS-PAGE on 8–15% polyacrylamide gels and transferred onto 0.45- μ m PVDF membranes (Merck Millipore, #IPVH00010). Membranes were blocked in 5% non-fat dry milk in Tris-HCl buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. After washing in TBST, membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Signals were detected using Clarity Western enhanced chemoluminescence (ECL) Substrate (Bio-Rad, #1705060) and visualized on a ChemiDoc Imaging System (Bio-Rad, #17001402). Primary antibodies used in this study are listed in Table S2. Secondary antibodies included anti-mouse IgG (Sigma-Aldrich, #NA931V) and anti-rabbit Immunoglobulin G (IgG) (Cell Signaling Technology, #7074S).

Flow cytometry

Tumor cells were seeded in 6-well plates with or without OT-I T cells and treated with recombinant IFN γ , recombinant TNF α , or IFN γ +TNF α as indicated. After indicated durations, conditioned medium was removed and the remaining cells were harvested and washed three times in Fluorescence-Activated Cell Sorting (FACS) buffer (PBS containing 1% bovine serum albumin [BSA]).

For cell surface/intracellular staining, harvested cells were stained with fluorochrome-conjugated antibodies against H2Kb (BioLegend, #116518), SIINFEKL-H2Kb (BioLegend, #141606), Ifngr1 (Miltenyi Biotec, #130-132-427), Ifngr2 (Miltenyi Biotec, #130-105-671), and Annexin V (BioLegend, #640926). Flow-cytometry outputs were reported as percentage positive cells and geometric mean fluorescence intensity (gMFI), with unstained controls used to define background. Surface-to-total receptor ratios were calculated from matched surface and total staining measurements.

For the antibody feeding assay, cells were labelled with a primary antibody against the extracellular domain of IFN γ receptor 1 (Ifngr1) (Proteintech, #31951-1-AP) at 4°C to restrict staining to surface-accessible receptor. After removal of unbound antibody, cells were shifted to 37°C for 30, 60, or 90 min, with or without IFN γ to permit receptor internalization and turnover. Cells were then returned to 4°C and stained with fluorescent secondary antibody (ThermoFisher, A11014) to quantify residual surface-accessible receptor by flow cytometry. Relative residual surface Ifngr1 gMFI was normalized to the 0-min condition within each genotype.

Pharmacologic Perturbation of Ist1-Chmp1 interaction

For the pharmacological experiment, Vector and Ist1-KO1 KPC3-OVA-GFP cancer cells were seeded on a 96-well plate at 3000 cells/well. DMSO or Tantalosin (4 μ M) [17] was added to the cancer cells and 4h

later, medium was removed and cells were washed three times with 1x PBS. Activated OT-I T cells were added to each group at different E:T ratios. Relative killing activity was longitudinally measured using the IncuCyte live-cell imaging system.

Data resources and bioinformatics analysis

Integrative CRISPR Screen Data Collection and Preprocessing

To systematically dissect the core tumour-intrinsic regulators of immune evasion, we aggregated publicly available genome-wide CRISPR knockout screen datasets performed under CD8⁺T-cell (OT-I) killing pressure. The integration incorporated two independent studies encompassing four distinct screening contexts: an in-house screen using epithelial-like (Epi) and mesenchymal-like (Mes) KPC3-OVA-GFP cell models [14], and an external screening pipeline using KPC-OVA cells in both in vitro and focused *in vivo* settings [15]. Raw sgRNA read counts were previously processed via the Model-based Analysis of Genome-wide CRISPR-Cas9 Knockout (MAGeCK) algorithm [18]. Gene-level negative log₂ fold-change (neg_lfc) and false discovery rate (neg_fdr) were extracted, and mouse gene symbols were standardized across all datasets to enable cross-context integration.

Gene Essentiality Scoring and sgRNA-level Validation

To depict the global landscape of immune-selection phenotypes, we generated rank plots by ordering genes in ascending order of their neg_lfc. Thresholds (LFC < 0 and FDR < 0.05) were used to identify significant sensitizing hits, whereas resistance-conferring genes were defined by LFC > 0 and FDR < 0.05. Additionally, gene-level volcano plots were generated by mapping statistical significance (-log₁₀ p-value) against phenotypic effect size (log₂ fold-change) with the available in-house screening data. To validate the phenotypic consistency of critical protein complexes (e.g., the ESCRT-III complex) and exclude off-target artifacts, sgRNA-level barcode ridgeline plots were generated to visualize the log₂ fold-change distribution of individual sgRNAs targeting specific candidate genes.

Cross-Context Similarity and Overlap Analysis

To evaluate the functional concordance between disparate tumour states and screening environments, pairwise Pearson Correlation Coefficients (PCC) were calculated using the genome-wide log₂ fold-change values across all intersecting genes, yielding a similarity heatmap. For hit-overlapping analysis, UpSet plots and four-way Venn diagrams were used. Specifically, the top 100 essential hits from each context were rigorously extracted by ranking all genes first by statistical significance (ascending FDR) and subsequently by effect size (ascending neg_lfc), identifying a small core consensus of context-agnostic immune-evasion regulators.

Robust Rank Aggregation (RRA) and Pathway Enrichment

To mathematically derive a global consensus of essentiality unaffected by single-screen biases, we applied the Robust Rank Aggregation (RRA) algorithm[19]. Context-specific rankings were integrated by calculating the geometric mean of the fractional ranks, and the resulting integration scores (-log₁₀ of the

geometric mean) identified the top consensus sensitizing genes. Finally, functional over-representation analysis of the core consensus hits was performed using the gseapy Python package[20] via the Enrichr[21], querying gene ontology (GO) Biological Process, Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome, and Molecular Signatures Database (MsigDB) Hallmark ontologies[22]. To assess the specific contextual reliance of these biological processes, we projected the pathway members onto proportional pie charts, with each sector's angular area proportional to the enrichment significance ($-\log_{10}$ adjusted P) in the Epi, Mes, in vitro, or in vivo screening contexts. All bioinformatics integrations and visualizations were performed in Python using the pandas, matplotlib, and seaborn libraries.

Cancer Dependency Map (DepMap) Essentiality Analysis

To distinguish context-specific immune evasion regulators from general pan-essential survival genes, we interrogated the DepMap CRISPR knockout database[23]. For the identified core immune-evasion hits, the baseline tumor-intrinsic essentiality was evaluated using the mean Chronos scores[24] retrieved from pancreatic adenocarcinoma (PAAD) cell lines. A resulting mean Chronos score of less than -1.0 indicates a pan-essential gene required for intrinsic tumour survival, whereas a score approaching 0 indicates non-essentiality in the absence of immune pressure. A dual-metric scatterplot was subsequently generated to contrast this intrinsic PAAD dependency (x-axis) against the integrated immune screening score ($-\log_{10}$ FDR, y-axis), highlighting ideal therapeutic targets that exhibit robust immune sensitization without conferring general cytotoxic dependencies.

Protein-Protein Interaction (PPI) Network Construction

To map the functional topography and physical interactomes of the robust immune-evasion candidates, a protein-protein interaction (PPI) network was constructed. The gene list comprising the core consensus targets and the top-ranked integration hits (derived from the RRA analysis) was submitted to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database API (v12.0, Mus musculus)[25]. Interactions were filtered using a medium-to-high confidence score threshold (>0.40). The resulting interaction network was imported into Python utilizing the NetworkX library[26]. Network topology was visualized, and nodes were partitioned into seven distinct functional modules based on biological ontologies.

Single-cell RNA-sequencing (scRNA-seq) data integration

To construct a comprehensive cross-cohort single-cell atlas, we aggregated scRNA-seq data from eight independent cohorts (six human and two mouse: GSE155698[27], GSE205013[28], GSE291124[29], GSE212966[30], GSE278694[31], GSE205049[32], GSE125588[33], GSE129455[34]). Count matrices were uniformly processed via total-count normalization and log-transformation. Highly variable genes (HVGs) were selected utilizing a batch-aware dispersion stabilization strategy to preserve biological variance while suppressing platform-specific noise. Principal Component Analysis (PCA) was performed on the scaled HVG matrix. To overcome the profound inter-cohort batch effects, we employed the Harmony algorithm[35]. By embedding the cohort identifier as a strictly controlled covariate, Harmony iteratively projected the cells into a shared latent space. The batch-corrected principal components were subsequently used to construct the k-nearest neighbors (KNN) graph and compute Uniform Manifold

Approximation and Projection (UMAP) embeddings[36]. To prevent the introduction of de novo clustering biases and over-stratification artifacts across datasets, base cellular identities were curated from original meta-annotations. To harmonize varying annotation resolutions, discrepancies were systematically reconciled using a consensus scoring matrix based on canonical lineage anchors. The global fidelity of these harmonized compartments was orthogonally validated by projecting established gene signatures onto the integrated latent space. For feature visualization, single-cell expression intensities were robustly scaled, intrinsically mitigating visual artifacts driven by expression outliers.

IST1 Expression Profiling and Linear Mixed-Effects Modeling (LMM)

The distribution of IST1 expression across distinct cellular compartments was visualized using split-violin plots generated via Gaussian kernel density estimation. To assess the enrichment of IST1 within specific cell lineages while strictly controlling for inter-cohort technical variation and baseline biases, we employed a sample-level pseudobulk approach coupled with a linear mixed-effects model (LMM). The IST1-positive fraction per patient sample was aggregated and fitted using a deviation coding scheme. Cell type was modeled as a fixed effect to estimate deviations from the grand mean, while the distinct sequencing cohort was modeled as a random intercept. This approach robustly estimates the effect size and 95% confidence intervals (CI) for each cell lineage. Cross-species conservation of IST1 expression was verified by mapping human IST1 to its 1:1 mouse ortholog (*Ist1*).

ESCRT Complex Scoring and IST1 co-expression

A composite "ESCRT module score" was generated by averaging the expression of core ESCRT components (VPS4A, CHMP1A/B, TSG101, PDCD6IP, etc.), explicitly excluding IST1 to prevent autocorrelation biases. Sample-level (pseudobulk) association between mean IST1 and the mean IST1-excluded ESCRT-III score across Ductal/Tumor samples was assessed by Spearman correlation. To quantify the spatial and cell-type-specific co-regulation between IST1 and specific ESCRT-III components (e.g., VPS4A, CHMP1B, CHMP4B), co-expression was evaluated within four major microenvironmental compartments (Ductal/Tumor, cancer associated fibroblast (CAF), T cell, and Myeloid cell). The relationship was reported at two levels: a pseudobulk Spearman correlation across samples, and a conditional single-cell Spearman correlation restricted to cells co-expressing both genes, which mitigates zero inflation.

Pathway Activity Scoring and Immune Cytotoxicity Correlation

Functional pathway activities were evaluated within ductal/tumor cells using the `score_genes` function in Scanpy[37]. For categorical comparisons, Ductal/Tumor cells were stratified into IST1-High and IST1-Low subpopulations, and differential pathway activity was assessed using the Mann-Whitney U test. Continuous relationships were evaluated by aggregating single-cell pathway scores to sample-level means and calculating Spearman's rank correlation with sample-level IST1 expression. Furthermore, anti-tumor cytotoxic tone was quantified using a core six-gene module (PRF1, GZMB, GZMA, IFNG, GNLY, NKG7), and its association with malignant IST1 expression was similarly evaluated at the sample level using Spearman correlation.

Cell-Cell Communication

Directed cell-cell communication from T cells to malignant cells was quantified across 14 ligand-receptor (L-R) pairs involving key immune effector, checkpoint, and suppressive programs. Interaction scores were defined as the product of the mean ligand expression in the T cell subset and the mean receptor expression in the malignant subpopulation (Simple L×R model). The differential communication potential was expressed as the log2FC of interaction scores directed toward IST1-High versus IST1-Low receivers. Statistical significance was established via a label-permutation null model, randomly shuffling the IST1-High/Low labels 1,000 times to compute empirical P-values (FDR-adjusted). Orthogonal validation was performed using the LIANA framework[38], and its resulting log2FC estimates were correlated with the Simple L×R model (Spearman ρ) to confirm method concordance. To further dissect ligand origins, T cells were sub-clustered into CD4⁺ conventional T cells, CD8⁺ T cells, and Regulatory T cells (Treg). Subtype-specific baseline ligand heterogeneity was evaluated via the Kruskal-Wallis test. Subtype-specific absolute communication effect sizes (Δ score) were computed by multiplying the subtype's mean ligand expression by the differential receptor expression, with significance bounds derived from a dedicated 2,000-shuffle permutation test over the receiver compartment.

In silico IST1 perturbation

To predict the transcriptional consequences of IST1 alterations in malignant cells, we applied an in silico perturbation framework based on covariate-adjusted partial regression co-expression modeling in Ductal/Tumor cells across scRNA-seq cohorts. Using a Frisch-Waugh-Lovell residualized ordinary-least-squares scheme, each highly variable gene and IST1 were residualized against a design matrix containing an intercept, the first 10 principal components, standardized log total counts, standardized mitochondrial percentage, and a standardized IFN-tone score; residual gene expression was then regressed on residual IST1 to estimate beta coefficients, t statistics, two-sided p values, and Benjamini-Hochberg FDR values. The IFN-tone covariate decoupled IST1-associated effects from global inflammatory-state variation, yielding conservative (lower-bound) estimates for IFN-related targets, and the linear extrapolated knockout effect was computed as $-\text{beta} \times \text{mean}(\text{IST1})$.

Per-gene t-statistics were combined across cohorts by Stouffer's method ($Z = \sum t_i / \sqrt{k}$) and empirically zero-centered by subtracting the genome-wide mean Z to remove residual cross-cohort inflation. The shifted meta-Z was tested against two orthogonal target networks sharing an identical null: eight TF-anchored MSigDB Hallmark programs, and direct TF→target regulons from Collection of Transcription Regulation Interactions (CollecTRI)[39] (signed +1/-1 edges retrieved through decoupler)[40], restricted to transcription factors with at least 30 expressed targets, of which the ten matched to the Hallmark anchors are shown - the latter providing a direct-target, single-transcription factor (TF)-resolution validation of the curated programs. For each gene set, the statistic was the mean shifted Z across the program's member genes (Hallmark) or the transcription factor's target genes (CollecTRI); a two-sided empirical p value was computed against 10,000 size-matched random gene sets drawn from the genome-wide pool, with 95% confidence intervals from 2,000 bootstrap resamples and Benjamini-Hochberg FDR across all sets tested. Positive enrichment denotes programs that are positively co-coupled to IST1 and thus predicted to decrease upon in silico IST1 loss.

Spatial transcriptomics

Spatial transcriptomics analyses used GSE274103[41], a 10x Visium FFPE cohort of five treatment-naive primary pancreatic ductal adenocarcinoma (PDAC) specimens (PDAC-p1-PDAC-p5). Per-spot counts were library-size normalized to 10,000 and log₁p-transformed. Per-spot tumor purity was defined as the malignant-epithelial content inferred from a matched untreated PDAC single-cell reference (GSE212966) via Seurat anchor-based label transfer[42], using the malignant-epithelial prediction score. An ESCRT-III module score was computed within each section as the mean z-scored expression of the core ESCRT-III genes; IST1 was excluded from this score in every analysis relating ESCRT-III to IST1 to avoid circularity. Spots were classified as tumor-rich or stroma/immune-rich based on the transferred lineage scores, and within tumor-rich spots, IST1-high, IST1-low, and ESCRT-III-high spots were defined per section using the corresponding upper- or lower-quartile expression thresholds. For visualization, IST1 expression, the ESCRT-III score, and tumor purity were each min-max scaled to [0,1] and mapped onto the tissue coordinates.

Spot-level associations among IST1, the ESCRT-III score, and tumor purity were assessed using Spearman's rank correlation within and across the five sections. ESCRT-III module and submodule scores were compared between IST1-high and IST1-low tumor spots, and between tumor-rich and stroma/immune-rich spots, using two-sided Mann-Whitney U tests with Benjamini-Hochberg false discovery rate correction. Spatial co-localization of IST1-high and ESCRT-III-high tumor spots was quantified as the observed-versus-expected frequency of double-high spots under independence and tested by a label-permutation test. Robustness was confirmed using an alternative ESCRT-III signature definition and an alternative IST1-high stratification threshold, and by residualizing ESCRT-III scores against per-spot library size and benchmarking them against expression-matched random gene sets.

Clinical expression, prognosis, and immunotherapy prediction

Gene expression profiles and corresponding clinical data were obtained from three independent cohorts: the combined The Cancer Genome Atlas and Genotype-Tissue Expression (TCGA+GTEx) dataset[43-44], the Clinical Proteomic Tumor Analysis Consortium (CPTAC)[45], and the Gene Expression Omnibus (GEO) dataset GSE183795[46]. After normalization and log₂-transformation, the differential expression of IST1 between tumor and normal (or adjacent non-tumor) tissues was evaluated using the Wilcoxon rank-sum test. To systematically assess the consistency of differential expression across these independent datasets, a meta-analysis was conducted to calculate Cohen's d effect sizes and 95% confidence intervals (CIs), which were visualized using a forest plot. To investigate the prognostic value of IST1 in the context of the tumor immune microenvironment, patients were stratified into four subgroups based on IST1 expression and CD8⁺ T cell infiltration abundance (IST1-low/CD8⁺ T-high, IST1-low/CD8⁺ T-low, IST1-high/CD8⁺ T-high, and IST1-high/CD8⁺ T-low). Survival probabilities were estimated using Kaplan-Meier curves, and statistical differences were evaluated with the log-rank test. Furthermore, the Tumor Immune Dysfunction and Exclusion (TIDE) algorithm[47] was utilized to predict potential clinical responses to immune checkpoint blockade (ICB) therapy. Samples were split into IST1-high and IST1-low and TIDE-score distributions (Wilcoxon rank-sum), and the proportion of predicted responders versus non-responders per group was compared.

Statistical Analysis

Statistical analysis were performed using GraphPad Prism 9.0, R 4.3.0 and Python 3.10. For two-group comparisons, Student's t tests or Mann-Whitney U tests were used according to the experimental design and data distribution. For paired tumor-normal comparisons, Wilcoxon signed-rank tests were used. For three or more groups, one-way ANOVA with appropriate post hoc testing or Kruskal-Wallis tests was used. Multiple testing was controlled with Benjamini-Hochberg FDR correction unless otherwise stated. Statistical significance was annotated as ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$.

Grammar check

Grammarly was used to check grammar and spelling. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

Results

An integrative, multi-context CRISPR screen nominates *Ist1* as a consensus tumour-intrinsic regulator of CD8⁺T-cell-mediated immune evasion

Pancreatic ductal adenocarcinoma (PDAC) responds poorly to T cell-based immunotherapy, and the tumor-cell-intrinsic programs that blunt CD8⁺T cell attack remain only partly mapped. Reasoning that critical determinants that are shared across genetic and microenvironmental contexts would be the most dependable, we integrated two genome-wide CRISPR-knockout screens performed in OT-I co-culture systems, in which PDAC cells presenting the ovalbumin peptide SIINFEKL on H-2Kb are eliminated by antigen-specific OT-I cytotoxic T lymphocytes (Fig. 1A). One screen, generated in-house, profiled epithelial-like (Epi) and mesenchymal-like (Mes) KPC3-OVA-GFP cells, capturing the two states of PDAC plasticity [14]. The other, drawn from published work, captures the two conditions of *in vitro* culture and *in vivo* modeling [15]. MAGeCK-processed gene-level data were retrieved from respective analyses and uniformly standardized across contexts for cross-screen integration. Internal benchmarking confirmed expected biology: canonical sensitizers of T cell killing, including the antigen-presentation gene *B2m*, encoding beta-2 microglobulin and the Janus kinase (JAK)-STAT pathway component *Jak1*, ranked among the most robustly depleted gene hits (Fig. 1B).

Pairwise correlation of standardized gene-level effects across all context pairs was uniformly positive (Fig. 1C), indicating a context-independent core of immune-evasion regulators shared across cellular state and assay format. Intersection of the top-100 ranked hits from each context identified 12 consensus genes common to all four contexts (Fig. 1D and Fig. S1A): *Adar*, *Aph1a*, *Cflar*, *Gpaa1*, *Ist1*, *Otulin*, *Rnf31*, *Socs1*, *Table 2*, *Tbk1*, *Tnfaip3*, and *Traf2*. The majority of this set mapped onto two established immune-regulatory axes: ubiquitin-modifying enzymes (*Otulin*, *Rnf31*, *Tnfaip3/A20*, *Table 2*) and nuclear factor (NF)- κ B/innate immune signaling components (*Cflar*, *Tbk1*, *Traf2*, *Socs1*, *Adar*). Both

categories have well-documented roles in tumor immune evasion. Against this background, *Ist1* emerged as a functionally discordant hit: an ESCRT-III membrane-scission subunit in the consensus set, belonging to a class of endosomal regulators with no established precedent in tumor-intrinsic immune control. Robust rank aggregation (RRA), applied without hit-list threshold assumptions across all four contexts, placed *Ist1* among the highest-ranked genes by both FDR and effect size (Fig. S1B), corroborating its statistical prominence by an orthogonal analytical framework.

Pathway enrichment of the shared hits across GO Biological Process, KEGG, Reactome, and MSigDB Hallmark gene sets recovered a coherent immune-evasion landscape: interferon/cytokine and JAK-STAT signaling, NF- κ B/TNF and innate-immune signaling, protein modification (Fig. S1C). A module-level network restricted to programs enriched in at least three of four contexts reinforced these as cross-context immune-evasion mechanisms (Fig. S2). Detailed characterization of the in-house screen-resolved negative-selection sensitizer hits and multiple ESCRT-III subunits, alongside positive-selection resistance hits in both epithelial and mesenchymal cells. Guide-level profiles confirmed concordant depletion of *Ist1* and genes encoding core ESCRT-III subunits across both cellular states at single-guide resolution (Fig. S3). Thus, beyond recovering canonical antigen-presentation and interferon-pathway sensitizers, the integrated multi-context analysis identified *Ist1*, a gene encoding ESCRT-III membrane-scission subunit, as a high-confidence tumor-intrinsic determinant of PDAC susceptibility to CD8⁺T cell-mediated killing, belonging to a functional class not previously well-explored in cancer immune evasion.

***Ist1* loss sensitizes pancreatic cancer cells to CD8⁺T cell killing without compromising intrinsic tumor-cell fitness**

To determine whether *Ist1* represents a tractable immune-evasion target rather than a broadly essential fitness gene, we evaluated the 12 consensus hits by cross-referencing the screen output with Cancer Dependency Map (DepMap) data. *Ist1* satisfied a favorable target criterium, being dispensable for intrinsic tumor-cell fitness, yet consistently required for immune evasion, making it a therapeutically tractable candidate (Fig. 2A). Interaction network analysis of the top shared hits further positioned *Ist1* within genes encoding components of an ESCRT/endosomal-trafficking module that bridged to a cytokine regulation signaling module (Fig. 2B), implicating ESCRT/endosomal-trafficking as a candidate mechanism underlying the immune-evasion phenotype.

Ist1 was therefore selected as the lead candidate for experimental validation. We generated two independent *Ist1*-knockout cell lines (*Ist1*-KO1, *Ist1*-KO2) in KPC3-OVA-GFP cells and verified the loss of protein by western blot analysis (Fig. 2C). In kinetic live-cell imaging IncuCyte co-culture assays, these KPC3-OVA-GFP cells were co-cultured with antigen-specific OT-I CD8⁺T cells across effector-to-target (E:T) ratios from 1:8 to 2:1, with tumor cell viability monitored by longitudinal GFP imaging (Fig. 2D). Endpoint quantification at 48 h or 72 h showed significantly lower residual GFP⁺ tumor signal in both knockout clones compared to Vector control (Fig. 2E-F, Fig. S4B), and area-under-the-killing-curve (AUC) analysis of the full time course confirmed enhanced clearance of cancer cells upon OT-I CD8⁺T cells challenge after *Ist1* loss (Fig. 2G, Fig. S4A). The phenotype was reproduced by crystal violet staining and

independent CellTiter-Blue and MTS viability assays, thereby excluding a GFP-reporter-specific artifact (Fig. 2H, Fig. S4C-S4E).

On-target specificity was supported by complementary genetic perturbations: five independent *Ist1* shRNA-mediated depletion lines phenocopied the *Ist1* knockout, whereas over-expression of *Ist1* reversed the killing phenotype (Fig. 2I, Fig. S4F-S4K). Furthermore, these results were confirmed in an *in vivo* zebrafish perivitelline-space xenograft assay, where KPC3-OVA-GFP cells, co-injected with activated OT-I T cells were cleared more efficiently at 3 dpi when *Ist1* was disrupted (Fig. 2J-K; Fig. S4L-M). Together, these data establish *Ist1* as a tumor-cell-intrinsic immune-evasion factor that protects pancreatic cancer cells from CD8⁺T cell-mediated killing, while remaining largely dispensable for basal tumor-cell fitness.

***Ist1* deficiency amplifies IFN γ -JAK-STAT1 signaling and antigen presentation**

To understand how *Ist1* restrains T-cell killing and to identify the conserved mechanism in human and mouse pancreatic cancer, we constructed a Harmony-integrated single-cell atlas from eight PDAC cohorts (six human, two murine), annotated into 12 major lineages by canonical marker expression, with malignant epithelial identity independently confirmed by inferred copy-number variation profiles (Fig. S5-S6, Fig. S7H). *Ist1* was detectable across all major lineages (Fig. S7A-C), indicating that its expression is not confined to a single cellular compartment within the PDAC microenvironment. The fraction of *Ist1*-positive cells varied by lineage identity; to partition this variation while controlling for inter-cohort heterogeneity, we analyzed per-sample *Ist1*⁺ frequencies using a linear mixed model with cell-type identity as a fixed effect and cohort of origin as a random intercept. Ductal/tumour epithelial cells and myeloid cells ranked among the highest *Ist1*⁺ lineages across cohorts, while acinar cells showed the lowest; immune-compartment lineages, including T cells and B cells, consistently ranked below the malignant epithelial compartment (Fig. S7D-G), suggesting that a high *Ist1*⁺ cell fraction is a feature preferentially shared by the malignant epithelial and myeloid cell compartments rather than a pan-cellular phenomenon.

Given its enrichment in the malignant compartment, we focused on ductal/tumour cells and asked what pathway activities marked by high *Ist1* there. Across the integrated atlas at the sample level, malignant-cell *Ist1* expression was inversely associated with immune-effector programs while positively with stress programs (Fig. S8A-B): *Ist1*-high tumours showed lower IFN- γ -response, MHC-I antigen-presentation and complement activity, alongside higher KRAS-signalling and IL-6-JAK-STAT3 activity. Consistent with reduced immune engagement, malignant-cell *Ist1* was also inversely correlated with a cytotoxic-lymphocyte score across samples (Fig. S8C). Thus, these associations indicated that high *Ist1* expression within the malignant compartment is associated with a transcriptionally immune-cold, IFN- γ /MHC-I-low state. To resolve the regulators underlying this state and to control for confounders in cross-sectional single-cell data, we modelled the transcriptional effect of *Ist1* within ductal/tumour cells using a covariate-adjusted virtual-perturbation regression and meta-analysed across the eight cohorts as a globally mean-centered Stouffer Z (Fig. 3A). After adjustment, high *Ist1* remained significantly coupled

to suppression of the STAT1- and Interferon regulatory factor 1 (IRF1)-anchored interferon target programs and of MYC target program, whereas the transforming growth factor- β (TGF- β)/SMAD target program shifted in the opposite direction. The concordant direction of the unadjusted sample-level correlation (Fig. S8B) and the covariate-adjusted regression supports a robust inverse association between *IST1* expression and the IFN- γ /STAT1 program, rather than a co-expression patterns artifact. To test whether these program-level associations also hold at the level of individual transcription-factor regulons, we repeated the analysis using CollecTRI for the ten focal factors shown in Fig. 3A (Fig. S10A). The direction of the *IST1* association was reproduced for the IFN-STAT/IRF1 and MYC factors, but at the stringent direct-regulon level only the MYC regulon reached significance; the STAT1 and IRF1 regulons were directionally concordant yet did not reach significance. Together, these multi-cohort analyses nominated the potential interferon-STAT1-MHC-I axis as the potential mechanism coupled to tumour-cell *Ist1*.

Guided by these atlas-derived observations, we next examined whether genetic *Ist1* perturbation altered cytokine-induced JAK-STAT signalling and antigen-presentation machinery in KPC3 cells. We first monitored IFN γ -induced STAT1 transcriptional activity using a (GAS4)X-driven STAT1 transcriptional GFP-reporter in the five independent sh/*st1* lines (Fig. S10B). After IFN γ stimulation, all five sh/*st1* lines showed stronger reporter activity than vector controls. This increase was evident during both short-term and long-term response kinetics, indicating that *Ist1* loss enhances not only the initial transcriptional response to IFN γ , but also the sustained phase of STAT1-dependent signaling (Fig. 3B-C). We next tested whether this transcriptional reporter phenotype was accompanied by enhanced activation of the endogenous JAK-STAT1 pathway. In two *Ist1*-knockout cell lines, IFN γ stimulation induced higher phosphorylation of Stat1 at Tyr701 than in vector controls, together with stronger induction of *Irf1*, a canonical Stat1-dependent transcription factor downstream of IFN γ signalling (Fig. 3D and Fig. S10C). Total Stat1 was also upregulated after cytokine exposure, in line with the feed-forward nature of IFN γ -JAK-STAT signalling. Consistent with this biochemical amplification, *Ist1*-deficient cells were more sensitive to IFN γ -induced loss of viability across a dose-response range (Fig. 3E and Fig. S11A). Extended cytokine profiling showed that this increased vulnerability was most apparent under IFN γ -containing conditions, including combined IFN γ and TNF α exposure, whereas TNF-related apoptosis-inducing ligand (TRAIL) sensitivity was not broadly increased (Fig. S11B-J). These results indicate that *Ist1* loss does not simply create a nonspecific death-prone state, but preferentially heightens tumour-cell sensitivity to cytokine programs involving IFN γ .

Because IFN γ -JAK-STAT1 signalling is a principal inducer of the antigen-presentation machinery, we then determined whether amplified cytokine signaling translated into increased antigen presentation at the cell surface. Flow-cytometric analysis showed that IFN γ increased surface H2-K $\emptyset$ in all genotypes in a dose-dependent manner, but the induction was stronger in *Ist1*-KO1 and *Ist1*-KO2 cells than in vector controls across the tested cytokine doses (Fig. 3F). This genotype-dependent increase was also observed under OT-I challenge, where *Ist1*-deficient cells displayed higher surface H2-K $\emptyset$, as measured by both the fraction of H2-K $\emptyset$ -positive cells and gMFI (Fig. S12A-B). To further define the cytokine context of this effect, we profiled surface H2-K $\emptyset$ across a broader panel of cytokine conditions. The *Ist1*-dependent

increase was most evident under IFN γ -containing conditions, including IFN γ alone and combined IFN γ /TNF α stimulation, whereas non-IFN γ conditions produced weaker or less consistent genotype-dependent differences (Fig. S12C-D). To determine whether elevated surface H2-K β was accompanied by increased cognate antigen display, we directly quantified the H2-K β -SIINFEKL complex recognized by OT-I cells. Flow cytometry for the H2-K β -SIINFEKL complex showed higher antigenic complex abundance in *Ist1*-deficient cells after cytokine treatment, indicating that *Ist1* loss augments not only generic MHC-I expression but also presentation of the cognate OVA-derived antigen recognized by OT-I T cells (Fig. S12E-F). In parallel, apoptosis profiling after cytokine challenge showed greater vulnerability of *Ist1*-knockout cells under cytotoxic cytokine pressure, consistent with the enhanced IFN γ sensitivity observed in the dose-response assays (Fig. S12G), linking amplified STAT1 signaling to improved recognition and elimination by antigen-specific CD8⁺T cells. At the transcriptional level, *Ist1* knockout amplified the induction of IFN γ -responsive and antigen-processing genes (Fig. 3G and Fig. S10D-F). Although individual genes differed in the magnitude and timing of induction, the overall pattern was consistent across two knockout cell clones and supported the conclusion that *Ist1* loss amplifies the IFN γ -induced STAT1 signaling pathway and thereby promotes antigen-presentation-associated transcription.

***Ist1* restrains surface IFN γ R1 abundance and turnover to limit cytokine-driven killing**

The preceding results established that *Ist1* loss amplifies cytokine-induced JAK-STAT signaling and antigen-presentation output. Because IFN γ signaling is initiated at the cell surface through the IFN γ receptor complex, we next examined whether *Ist1* regulates the receptor-level input that precedes STAT1 activation. We first interrogated this possibility in the integrated human and murine single-cell atlas by analyzing inferred ligand-receptor communication from T cells to ductal/tumour receivers. Among a set of T cell-to-tumour ligand-receptor pairs related to cytotoxic effector function and immune regulation from T cell senders to ductal/tumor receivers, IFNG-IFNGR1/2 interactions were reduced toward *Ist1*-high ductal/tumour receivers relative to *Ist1*-low receivers (Fig. S8D-E). Orthogonal inference using Ligand-receptor ANalysis Amework (LIANA) reproduced the direction of the IFNG-IFNGR interaction shift, supporting the robustness of this communication-level pattern (Fig. S8F-G). Subtype-resolved analysis further localized this communication phenotype. The ligand-receptor pairs examined showed the expected cell-type distribution, with immune effector ligands enriched in T/natural killer (NK) cell compartments and their cognate receptors detectable in ductal/tumour receivers (Fig. S9A-B). When T cells were separated into CD4 conventional, CD8 and Treg subsets, IFNG-IFNGR communication from CD8 T cells to ductal/tumour receivers was lower toward *Ist1*-high malignant cells than toward *Ist1*-low malignant cells (Fig. S9C). Baseline ligand-expression analysis identified CD8 T cells as a principal IFNG-expressing subset, and subtype-specific Δ interaction scores supported a receptor-side contribution from the *Ist1*-defined tumour receiver state (Fig. S9D-E). Together, these atlas-derived observations localized the *Ist1*-associated reduction in inferred IFNG-IFNGR communication to a CD8 T-cell-to-malignant-cell axis, suggesting altered IFN γ receptor-axis signaling at the tumour-cell interface and providing a single-cell rationale for testing IFN γ dependence in the OT-I co-culture system.

We therefore examined the IFN γ receptors in *Ist1*-deficient KPC3-OVA-GFP cells. Flow-cytometric analysis showed that *Ist1*-KO1 and *Ist1*-KO2 cells had higher surface *Ifngr1* than vector controls. In contrast, total *Ifngr1* abundance was not comparably increased (Fig. 4A-B), resulting in an elevated surface-to-total *Ifngr1* ratio in *Ist1*-deficient cells (Fig. 4C). An identical pattern was observed for the cognate signaling subunit IFN γ R2 (Fig. S13A-C). An orthogonal knockdown panel supported this surface-biased receptor phenotype: across three independent *sh/st1* lines, both *Ifngr1* and *Ifngr2* showed increased surface abundance without a comparable increase in total receptor levels (Fig. S13D-G). These orthogonal perturbations indicated that enhanced IFN γ receptor surface availability follows *Ist1* suppression. The discordance between increased surface receptor abundance and relatively stable total receptor levels suggested that *Ist1* might regulate receptor trafficking. Because *Ifngr1* is the ligand-binding receptor chain required for initiating IFN γ signaling, we next focused on *Ifngr1* receptor dynamics. To determine whether the increased surface pool reflected altered receptor turnover, we used an antibody-feeding assay to label the pre-existing surface pool of *Ifngr1* and followed residual surface-accessible receptor during a chase over time (Fig. S13H). *Ist1*-deficient cells retained more surface *Ifngr1* during the chase, indicating slower clearance of *Ifngr1* from the plasma membrane and increased receptor surface persistence (Fig. 4D). Notably, the rebound of surface *Ifngr1* at later time points in *Ist1*-deficient cells suggested that receptor recycling or resurfacing contributes to the enlarged surface *Ifngr1* pool. We then repeated the assay in the presence of recombinant IFN γ to determine whether ligand engagement altered this phenotype. IFN γ stimulation accelerated the clearance of surface-accessible *Ifngr1* in vector cells, whereas *Ist1*-deficient cells maintained higher residual surface *Ifngr1* throughout the chase (Fig. S13I). Together, these data support a trafficking model in which *Ist1* limits the persistence and turnover of surface *Ifngr1*, thereby restricting receptor availability for IFN γ signalling.

The convergence of surface IFN γ R stabilization and amplified JAK-STAT1 signaling in *Ist1*-deficient KPC-OVA-GFP cells predicted that their heightened susceptibility to OT-I T-cell attack would be specifically preventable by intercepting the IFN γ signal. We tested this by supplementing OT-I co-cultures with neutralizing antibodies against IFN γ or TNF α . In *Ist1*-KO cells, isotype-matched control antibodies had no effect on baseline tumour-cell killing (Fig. S14A), while anti-IFN γ treatment selectively restored residual tumour-cell survival in *Ist1*-KO cultures toward vector-control levels, measured using both IncuCyte time-lapse imaging and relative mean AUC analysis. Interaction analysis confirmed a significant IFN γ -neutralization $\times$ tumour-genotype interaction (Fig. 4F). By contrast, TNF α neutralization failed to eliminate the genotype-dependent killing difference, despite TNF α being a physiologically relevant effector cytokine in this co-culture system (Fig. 4E-G). Decomposing the killing time-course into cytokine-preventable and cytokine-independent components attributed the excess clearance of *Ist1*-KO cells to an IFN γ -related preventable fraction, while the TNF α -relative preventable fraction was much weaker between genotypes (Fig. 4H). Similar results were obtained at E:T = 1:4 ratio's, where IFN γ neutralization preferentially reduced this *Ist1*-KO-associated killing phenotype, as supported by AUC heatmaps, endpoint killing, and interaction analyses (Fig. 4I, Fig. S14D-H). Furthermore, in five independent *sh/st1* knockdown cell lines, IFN γ neutralization specifically reversed the killing-enhancing phenotype caused by

Ist1 suppression, whereas TNF α neutralisation had little effect (Fig. S15A-G). Again, at E:T 1:4, this selectivity was preserved with equivalent magnitude (Fig. S15H-M).

Collectively, the atlas-derived communication analysis, receptor-surface measurements, antibody-feeding assays and cytokine-neutralization experiments support a coherent mechanism: *Ist1* limits IFN γ receptor surface abundance by trafficking, thereby restraining IFN γ -dependent JAK-STAT1 signalling and CD8 + T-cell-mediated killing of PDAC cells.

The IST1-ESCRT-III axis is spatially coordinated in PDAC and pharmacologically targetable

Given that *Ist1* loss sensitized PDAC cells to IFN γ -dependent CD8⁺T-cell killing by regulating IFN γ -receptor surface availability and that *Ist1* belongs to the ESCRT-associated machinery, we next asked whether the IST1–ESCRT-III module coordinate to affect T-cell susceptibility. In the integrated atlas, an ESCRT-III module score computed from ESCRT-III subunit-encoding genes, with IST1 excluded to avoid self-contribution, co-enriched with IST1-high ductal/tumour cells (Fig. S16A), and pseudobulk IST1 expression positively correlated with the ESCRT-III module score across ductal/tumour samples (Fig. S16B). Cell-type-resolved analysis showed that IST1 and ESCRT-III genes were preferentially co-expressed in the malignant-epithelial compartment relative to stromal and immune lineages in both aggregate cell-type comparisons and per-cell co-expression analysis (Fig. S16C-D, S17A).

To explore whether this transcriptional co-expression reflects coordinate spatial engagement within intact primary tumour tissue, we analysed five primary human PDAC 10 $\times$ Visium sections (GSE274103). Spot-level *Ist1* expression, an *Ist1*-independent ESCRT-III module score and tumour-purity estimates were mapped across tissue sections. *Ist1* and ESCRT-III module scores showed overlapping enrichment in tumour-rich regions across multiple specimens (Fig. 5A). Quantitative spatial analyses supported this visual pattern: spot-level correlations linked *Ist1* expression, ESCRT-III activity and tumour purity, supporting the idea that *Ist1*-high regions are embedded within tumour-rich ESCRT-active niches rather than being randomly distributed across the tissue (Fig. S17B). Consistently, ESCRT-III activity was higher in tumour-rich spots than in stroma/immune-rich spots across sections (Fig. S17C). When the analysis was restricted to tumour spots, *Ist1*-high tumour regions showed higher ESCRT-III activity than *Ist1*-low tumour regions (Fig. S17D). Submodule-level analysis further showed that the higher ESCRT-III activity in *Ist1*-high tumour spots was not driven by a single submodule, but extended across multiple ESCRT-III submodules, with variation in magnitude across tissue sections (Fig. S17E).

To determine whether the co-distribution of IST1 and ESCRT-III module within the tumour compartment was statistically non-random, tumour-rich spots were classified within each section into four mutually exclusive categories (IST1-high, ESCRT-III-high, double-high and other tumour spots) and the observed double-high co-occurrence was compared against an independence null constructed by permuting ESCRT-III-high labels within tumour-rich spots. Spatial class-overlay maps confirmed consistent co-localization of IST1-high and ESCRT-III-high tumour spots across all five Visium sections (Fig. S18A).

Permutation analysis confirmed that double-high tumour spots occurred more often than expected under independence, with observed-to-expected overlap ratios greater than one across sections and significant enrichment in most specimens after FDR correction (Fig. S18B). This non-random spatial co-localisation establishes the IST1-ESCRT-III transcriptional programme as a coordinate feature of the malignant compartment in primary human PDAC, providing mechanistic rationale for IST1-driven restriction of tumour-cell IFN γ R surface availability in intact tumour architecture.

Having established that the IST1-ESCRT-III relationship is present in human PDAC tissue, we next asked whether this axis was pharmacologically targetable in the T-cell killing system. Tantalosin has been described as a chemical inhibitor of the IST1-CHMP1B copolymer, a component of ESCRT-III-associated endosomal remodelling[17]. We therefore pre-treated vector control and Ist1-deficient KPC3-OVA-GFP cells with Tantalosin and repeated the OT-I co-culture assays. In the absence of OT-I T cells, Tantalosin did not significantly affect tumour cell viability (Fig. 5B). By contrast, in the presence of OT-I cells, greater loss of residual cancer cells was detected in Vector+Tantalosin relative to Vector+DMSO conditions at both effector-to-target ratios examined (1:8 and 1:4), approaching the depletion observed in Ist1-KO1 + DMSO cultures (Fig. 5B). Tantalosin produced little additional reduction in Ist1-KO1 cells, consistent with IST1-dependent activity of the compound in this setting (Fig. 5C). The same results were obtained using live-cell imaging of the Tantalosin-treated cancer cells, supporting the notion that pharmacological disruption of the IST1-CHMP1B axis and genetic Ist1 loss converge functionally in sensitizing tumor cells to OT-I killing (Fig. 5D). Thus, Tantalosin rendered IST1-proficient tumour cells more susceptible to OT-I killing, thereby functionally resembling genetic Ist1 loss.

These pharmacological data extend the genetic findings by showing that the IST1-ESCRT-III axis is not only associated with tumour-cell resistance to CD8 T-cell killing, but is also amenable to small-molecule intervention. Collectively, these findings identified IST1-ESCRT-III axis as a tumour-cell-intrinsic trafficking node that restricts IFN γ receptor surface availability, restrains STAT1-driven antigen-presentation programs and protects PDAC cells from antigen-specific CD8 T-cell killing (Fig. 5E).

IST1 marks an immune-evasive, adverse-prognosis state in human pancreatic cancer

Having identified IST1 as a tumour-cell-intrinsic regulator of CD8⁺T-cell killing and a pharmacologically tractable target, we next asked whether IST1 expression is altered in human PDAC at the cohort level and whether it is linked to immune-relevant clinical features. We first compared IST1 expression between tumour and non-tumour pancreatic tissues across three independent bulk transcriptomic resources. IST1 was consistently elevated in pancreatic tumour samples relative to normal pancreas or adjacent non-tumour tissues in TCGA+GTEx, CPTAC-WashU and GSE183795 (Fig. S18C). This pattern was not driven by a single cohort or platform. Effect-size analysis using Cliff's δ confirmed large tumour-versus-normal differences across all three datasets, with estimates exceeding the conventional large-effect threshold in each cohort (Fig. S18D). These data indicate that elevated IST1 expression is a reproducible feature of human PDAC at the tissue level.

We then evaluated whether IST1 expression was linked to the immune context most relevant to the functional phenotype observed in our co-culture assays. As Ist1 loss primarily sensitized tumour cells to CD8⁺T-cell-mediated killing, we stratified patients jointly by tumour IST1 expression and CD8 T-cell infiltration. This combined analysis separated patients into distinct survival groups, with the IST1-high/CD8-low subgroup showing the most adverse overall survival pattern (Fig. S18E). This relationship suggests that elevated IST1 may be most clinically unfavourable in tumours with limited cytotoxic T-cell infiltration, a setting in which tumour-cell-intrinsic resistance programs and insufficient immune pressure could converge to weaken anti-tumour immune control. Notably, patients with IST1-low/CD8-high tumours showed the most favourable overall-survival trajectory, whereas this survival advantage was attenuated in IST1-high tumours despite comparable CD8 T-cell enrichment (Fig. S18E). These data suggest that high CD8⁺T-cell infiltration is the most clinically beneficial when tumour IST1 expression is low, consistent with a model in which effective cytotoxic immune control requires not only T-cell infiltration but also a tumour-cell state permissive to IFN γ -dependent antigen presentation followed by CD8⁺ T-cell-mediated killing.

To further assess whether IST1 expression was associated with an immune-resistant state, we used Transcript Identification and Selection (TIDE), a transcriptome-based framework that infers T-cell dysfunction and exclusion and predicts immune checkpoint blockade responsiveness. IST1-high tumours showed a shift toward higher TIDE scores, consistent with greater inferred immune dysfunction or exclusion, and contained a larger fraction of predicted immune-checkpoint-blockade non-responders than IST1-low tumours (Fig. S18F-G). This computational immune-resistance pattern was concordant with the experimental data, in which IST1 restrained IFN γ -dependent antigen-presentation programs and reduced CD8⁺T-cell-mediated tumour-cell killing. Together, these cohort-level analyses indicate that IST1 is enriched in human PDAC and associated with an adverse immune context in which CD8⁺T-cell infiltration, tumour-cell-intrinsic IST1 state and predicted immunotherapy resistance are interlinked.

Discussion

Pancreatic ductal adenocarcinoma (PDAC) remains among the most refractory malignancies to cancer immunotherapy, in that the induction of a measurable anti-tumour immune response only rarely translates into durable tumour control. The prevailing explanation has centred on the dense, immunosuppressive tumour microenvironment: its fibroblast-rich desmoplastic stroma, myeloid-derived suppression, regulatory lymphocyte activity, restricted effector-cell access and heterogeneous antigenicity [48]. These barriers are real and clinically formidable, yet they do not, by themselves, account for a more fundamental observation: tumour cells that are physically engaged by cytotoxic T cells can nonetheless survive the encounter. Our study focuses on the final step of the anti-tumour response and identifies IST1 as a tumour-cell-intrinsic determinant of cytotoxic-T-cell killing. Combining integrative CRISPR-based genetic screen analysis, genetic perturbation, receptor-trafficking assays, cytokine-neutralization experiments, and human tumor profiling, we define IST1 as an ESCRT-associated regulator

that constrains IFN γ receptor surface availability, attenuates STAT1-driven antigen presentation, and thereby shields PDAC cells from antigen-specific CD8 + T-cell killing.

Genome-wide CRISPR genetic perturbation studies in pancreatic cancer have repeatedly converged on a conserved set of tumour-cell programs that govern cytotoxic T-cell killing, spanning IFN γ signalling, antigen presentation, TNF/NF- κ B regulation, ubiquitin-linked inflammatory control and ESCRT-associated factors such as Vps4b. By integrating several independent immune-selection contexts and then experimentally resolving a single high-confidence candidate, our work identified ESCRT-mediated trafficking of the IFN γ R as an additional pathway driving tumor cell intrinsic resistance. Mechanistically, our data position IST1 upstream of IFN γ -JAK-STAT1 activation, at the level of receptor disposition. Genetic deletion or knockdown of Ist1 increased the surface abundance of the IFN γ receptor without affecting total receptor levels. This redistribution toward the plasma membrane was functionally coupled to amplified downstream signalling: stronger STAT1 phosphorylation, sustained IRF1 induction, heightened GAS-reporter activity and broad transcriptional induction of IFN γ -responsive genes. Critically, neutralising IFN γ preferentially abolished the enhanced killing of Ist1-deficient cells, whereas TNF α neutralisation produced few effects. The selectivity of this epistasis indicates that IST1 acts mainly on the tumour-cell receiver arm of a CD8⁺T-cell-to-tumour IFN γ axis, rather than modulating a generic sensitivity to inflammatory stress or to cell death. Placing IST1 at the receptor itself situates it within an emerging theme: that the surface dwell-time of IFNGR1 is an actively regulated, and hence potentially druggable, immune regulatory node. A similar principle is exemplified by optineurin, whose loss drives palmitoylation-dependent lysosomal sorting and degradation of IFNGR1 and, consequently, immune evasion [49]. IST1 and optineurin converge on the surface availability of the IFN γ receptor but appear to operate through distinct trafficking logics, namely ESCRT-III-linked endosomal handling versus lysosomal targeting. This implies that tumour cells can reach a shared immune-resistant end-state by tuning different arms of receptor traffic: lysosomal degradation in the case of optineurin, and endosomal sorting and surface retention in the case of IST1. For IST1, the trafficking role of itself is selective. At the early/sorting endosome, where, together with CHMP1B, it apportions internalised cargo between onward sorting and return to the surface. Crucially, IST1 makes these choices in a cargo-specific manner, contributing to a subset of recycling routes and acting on different cargoes in distinct ways [50–52]: Disruption of IST1-CHMP1B copolymer by Tantalosin inhibits the recycling of transferrin-receptor [17], which is a canonical constitutive recycling cargo, whereas loss of IST1 increases the surface availability of IFN γ R1, which is cytokine-signaling receptor whose surface abundance may be shaped by multiple trafficking steps. The identification of IFN γ R1 as an IST1-restrained substrate is therefore an empirical result of our screen and functional experiments, not an inference borrowed from other cargoes. Consistent with a cargo- and route- specific mechanism, the increased surface IFN γ R1 with an unchanged total receptor pool suggested that IST1 shapes IFN γ R availability through altered endosomal sorting and turnover, and the precise itinerary of IFN γ R1 remains to be resolved by direct receptor fate-mapping.

The ESCRT connection merits emphasis because it broadens the conceptual role of membrane trafficking in tumour immunity. ESCRT proteins are canonically associated with endosomal sorting,

membrane scission, plasma-membrane repair, cytokinesis, autophagy and signal termination[53]. In the setting of immune killing, ESCRT-mediated membrane repair can reseal perforin-induced lesions and so protect tumour-derived cells from cytolysis[54]. Our work identifies a complementary, signalling-level route to ESCRT-linked immune resistance: the control of cytokine-receptor availability before the transcriptional antigen-presentation response is engaged. ESCRT activity can thus influence tumour-cell survival at two distinct interfaces-the physical one of cytotoxic membrane attack, and the signalling one through which the tumour cell senses immune-derived cytokines. This functional duality makes membrane-remodelling systems unusually attractive, but correspondingly complex, candidates for therapeutic immune sensitisation.

The integration of primary cohorts and spatial data supports the disease relevance of this mechanism. IST1 was enriched in malignant epithelial and myeloid compartments in the integrated single-cell atlas, and high IST1 malignant cell states were associated with lower IFN γ -response and MHC-I antigen-presentation programs. The inferred CD8 T-cell-to-tumour IFNG-IFNGR communication deficit localized this association to the same cytokine axis tested experimentally. Spatially, IST1-high tumour regions co-enriched with an IST1-excluded ESCRT-III module across primary human PDAC sections, and double-high tumour spots occurred more frequently than expected under spatial independence. These observations do not prove a mechanism in patient tissue, but they show that the IST1-ESCRT-III program is not an isolated cell-line artifact. It is embedded in tumour-rich regions of human PDAC and aligned with an immune state predicted to be less responsive to cytotoxic T-cell control. The clinical-cohort analyses extend this interpretation, although they remain correlative. Across independent datasets, IST1 expression was higher in pancreatic tumour tissue than in normal or adjacent pancreas. When tumours were stratified jointly by IST1 expression and CD8⁺T-cell infiltration, the most favourable outcomes occurred in the IST1-low+CD8⁺T cell-high group, indicating that the prognostic benefit of CD8⁺ T cell infiltration was greatest when IST1 was low. This interaction follows from the experimental model: cytotoxic T cells confer more benefit when tumour cells remain permissive to IFN γ sensing, antigen presentation and killing, whereas in IST1-high tumours the prognostic value of CD8⁺T cell infiltration is blunted since the malignant cells respond poorly to the effector signals those T cells deliver. Consistent with this, TIDE-based inference linked high IST1 to a transcriptomic immune-resistance profile. Although these associations cannot directly establish that IST1 predicts response to checkpoint blockade, they indicate that IST1 marks an adverse immune contexture in human PDAC.

Several limitations remain in our work and each requires further exploration in the future. The OT-I/OVA system isolates tumour-cell-intrinsic resistance under defined, antigen-specific pressure, but it uses a model antigen and does not reproduce patient-specific antigen repertoires or TCR diversity. The zebrafish xenograft confirms an in vivo killing phenotype, yet cannot reconstitute the mammalian PDAC immune microenvironment. The integrated single-cell, spatial, and bulk data establish relevance across cohorts but remain observational; to confirm the mechanism, it will ultimately need more experimental evidence for a finer dissection of how Ist1 loss alters receptor trafficking. However, these limitations do not undermine the central mechanistic conclusion that IST1 regulates tumour-cell susceptibility to CD8

T-cell killing through a cell-intrinsic trafficking mechanism. Rather, they indicate that translating this mechanism into a therapeutic strategy will require further validation in the complex and heterogeneous immune ecosystems of PDAC.

In summary, this work identifies the IST1-ESCRT-III axis as a tumor-cell-intrinsic resistance mechanism linking membrane trafficking to IFN γ sensing, antigen presentation, and CD8 + T-cell killing, and provides candidate entry points for sensitizing pancreatic cancer to antigen-directed immunotherapy.

Declarations

Ethics approval and consent to participate

OT-I mice (both female and male mice were used, age 8 to 14 weeks), which were bred and housed at the Leiden University Medical Centre (LUMC) animal facility under specific pathogen-free conditions; these cells recognize OVA_{257–264} in the context of H2K^b. The animal experiments were designed according to the Code of Practice of the Dutch Animal Ethical Commission and subjected to the regulations as stated in project AVD11600202013796. The title of the project is Immunotherapy and vaccination against cancer.

The embryonic zebrafish experiments were conducted in a licensed establishment for the breeding and use of experimental animals (at Leiden University), and we adhered to internal regulations and guidelines. Advice from the animal welfare body was followed to minimize suffering within the facility. The zebrafish assays described do not fall under the definition of an animal experiment according to the Experiments on Animals Act (Wod, effective 2014), the applicable legislation in the Netherlands, in compliance with European guidelines (EU directive 2010/63/EU) on the protection of animals used for scientific purposes. Since non-self-feeding larvae were used, no specific license was required for these assays on zebrafish larvae (<5 days post-fertilization).

Consent for publication

No applicable.

Availability of data and materials

Data sharing is not applicable to this article as no new large scale datasets were generated during the current study. The public datasets that support the findings of this study are available under their accessions and the citations for these datasets were included.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WL and GM designed, performed experiments and interpreted the results. PtD and RW supervised the study. WL drafted the manuscript and organized the figures. WL and SH performed bioinformatic analyses and managed data interpretation. MC and FO assisted in animal experiments. YWW provided Tantalosin.

All the authors reviewed, edited, and approved of the final manuscript, ensuring the accuracy and integrity of the work.

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Figures

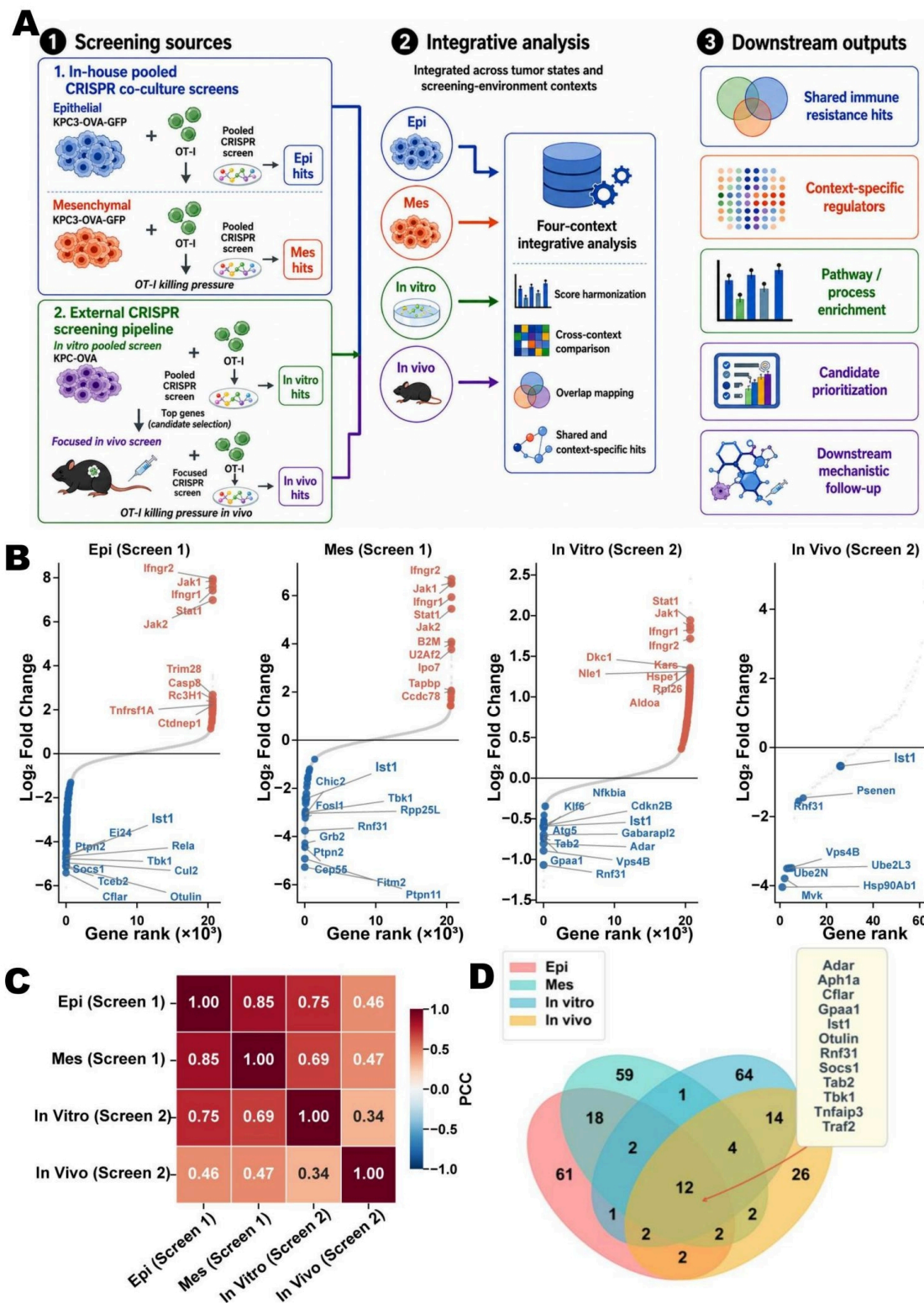


Figure 1

Integrative multi-context CRISPR screening nominates shared tumour-intrinsic regulators of CD8⁺ T-cell-mediated immune evasion in pancreatic cancer.

(A) Schematic overview of the integrative CRISPR-based geneticscreen analysis. Two independent genome-wide CRISPR knockout screens performed in OT-I-co-cultured PDACsystems were combined: an

in-house screen in epithelial-like (Epi) and mesenchymal-like (Mes) KPC3-OVA-GFP cells [14] and an external screen in KPC-OVA cells under in vitro and in vivo settings [15]. sgRNA read counts were processed via MAGeCK to compute gene-level phenotype scores for each of the four contexts. Integrated outputs feed into downstream analyses including pathway enrichment, target prioritization, and mechanistic validation.

(B) Rank-ordered gene-level phenotype scores for each screening context. Genes at the distribution tails represent the strongest immune-selection phenotypes. Significantly enriched and depleted hits are highlighted, and selected benchmark genes in antigen presentation and IFN γ signalling (*Jak1*, *B2m*), together with *Ist1*, are annotated to illustrate concordance of known immune-selection biology with the integrated candidate prioritization.

(C) Cross-context similarity heatmap showing pairwise Pearson correlation coefficients (PCC) computed from genome-wide, standardized log₂ fold-change values across all genes between the four contexts.

(D) Four-way Venn diagram of the top-100 ranked hits from each context. The central intersection identifies a small set of robust consensus immune-evasion regulators (12 genes) shared across all four contexts, defining a context-agnostic core of tumour-intrinsic determinants of CD8⁺T-cell susceptibility in pancreatic cancer.

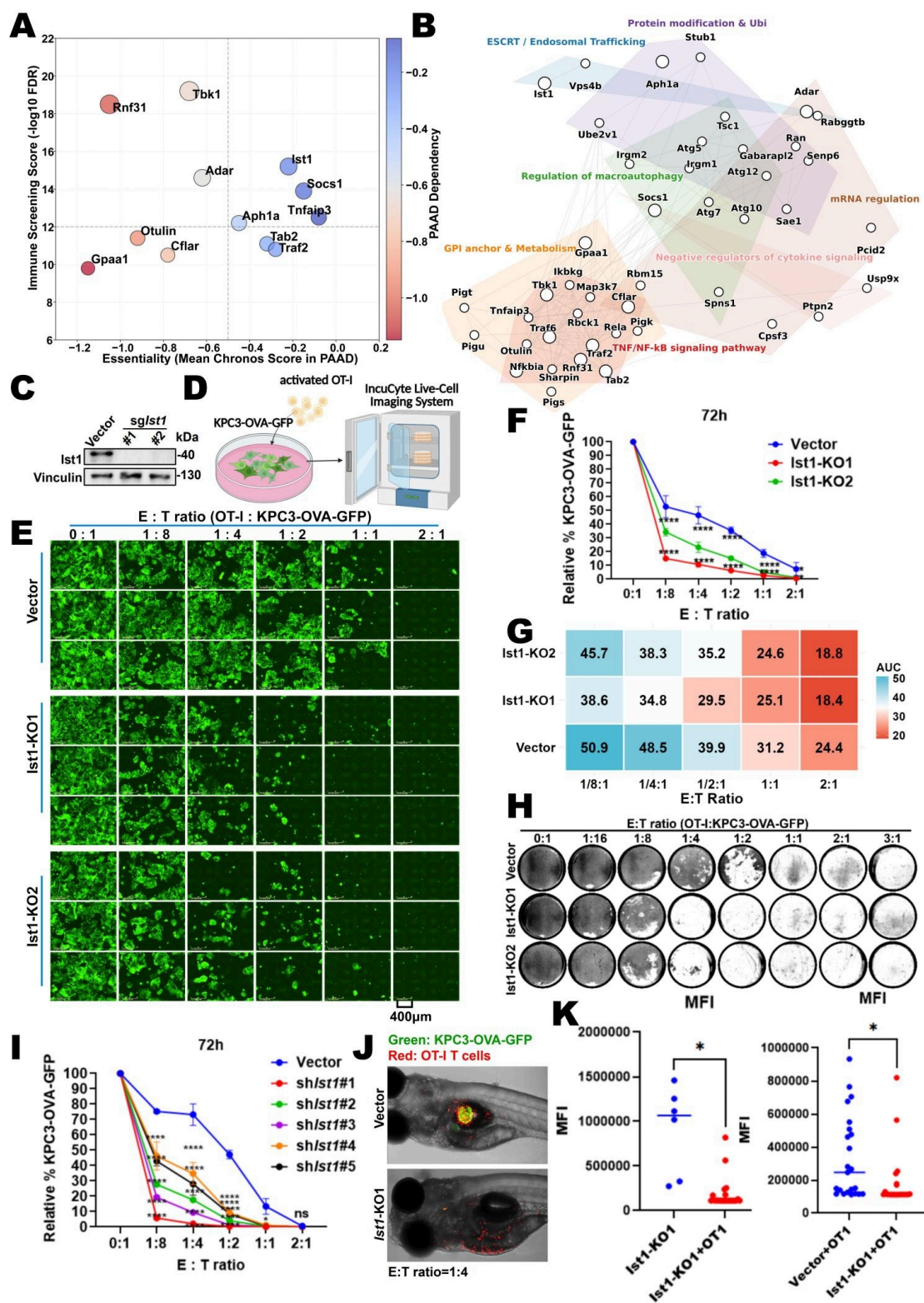


Figure 2

Loss of *Ist1* sensitizes pancreatic cancer cells to CD8⁺ T-cell-mediated killing in vitro and *in vivo*.

(A) Two-dimensional DepMap-based prioritization plot of integrated screen hits. Each gene is plotted by its essentiality in PDAC (mean Chronos score in PAAD, DepMap; x-axis) versus its immune-screening

score ($-\log_{10}$ FDR; y-axis), and colored by PAAD-specific dependency. Genes in the upper-right quadrant were prioritized for mechanistic follow-up. *Ist1* was selected as the lead candidate.

(B) STRING-derived functional interaction network of consensus screen hits. Nodes are coloured by functional module: ESCRT/endosomal trafficking, protein modification and ubiquitin-proteasome system, mRNA regulation, GPI anchor and metabolism, regulation of macroautophagy, NF- κ B and TNF/NF- κ B signalling, and negative regulations of cytokine signalling. *Ist1* sits at the ESCRT/endosomal-trafficking cluster and bridges to antigen-presentation and signalling modules.

(C) Western blot validating *Ist1* knockout in KPC3-OVA-GFP cells. Two independent single-guide RNAs (*Ist1*-KO1, *Ist1*-KO2) were used. Vinculin is shown as a loading control.

(D) Experimental schematic of the OT-I/KPC3-OVA-GFP co-culture assay used for kinetic monitoring of T-cell-mediated killing on the IncuCyte live-cell imaging platform. E:T ratios: effector-to-target ratios.

(E) Representative IncuCyte fluorescence images of Vector, *Ist1*-KO1, and *Ist1*-KO2 KPC3-OVA-GFP cells (green) at the indicated E:T ratios (0:1, 1:8, 1:4, 1:2, 1:1, 2:1) at 72 h.

(F) Endpoint quantification of relative residual KPC3-OVA-GFP signal (% of $t = 0$) at the indicated E:T ratios for Vector, *Ist1*-KO1 and *Ist1*-KO2(72h). Data are shown as mean $\pm$ SD; $n=3$; $*p<0.05$, $****p<0.0001$ by two-way ANOVA.

(G) Heatmap of area-under-the-curve (AUC) values derived from the full killing time-courses, summarizing tumor-cell survival across all E:T ratios for Vector, *Ist1*-KO1 and *Ist1*-KO2. GFP signal was monitored via IncuCyte and normalized to the respective tumor-only controls. Lower AUC = stronger T-cell killing.

(H) Crystal-violet end-point staining of surviving KPC3-OVA-GFP cells after 72h co-culture across the same E:T ratios for Vector, *Ist1*-KO1 and *Ist1*-KO2.

(I) Independent shRNA validation. Relative percentage of KPC3-OVA-GFP⁺ cells at 72 h after OT-I co-culture for Vector vs five independent *Ist1* shRNAs (sh/*Ist1*#1–#5). Data are shown as mean $\pm$ SD; $n=3$; $*p<0.05$, $****p<0.0001$, ns = not significant by two-way ANOVA.

(J) Zebrafish embryo xenograft model. Representative confocal images of KPC3-OVA-GFP tumour cells (green) co-injected with DiD-labelled activated OT-I T cells (red) into the perivitelline space at E:T = 1:4, comparing Vector and *Ist1*-KO1 at 3 days post-injection time point.

(K) Quantification of *in vivo* tumor cell elimination. Mean fluorescence intensity (MFI) of GFP⁺ tumor cells in *Ist1* vs *Ist1*+OT-I (left) and Vector+OT-I vs *Ist1*-KO1+OT-I (right). Each dot represents one zebrafish; horizontal bar = mean. Data are shown as mean $\pm$ SD; $n>3$; $*p<0.05$ by two-tailed Mann-Whitney U test.

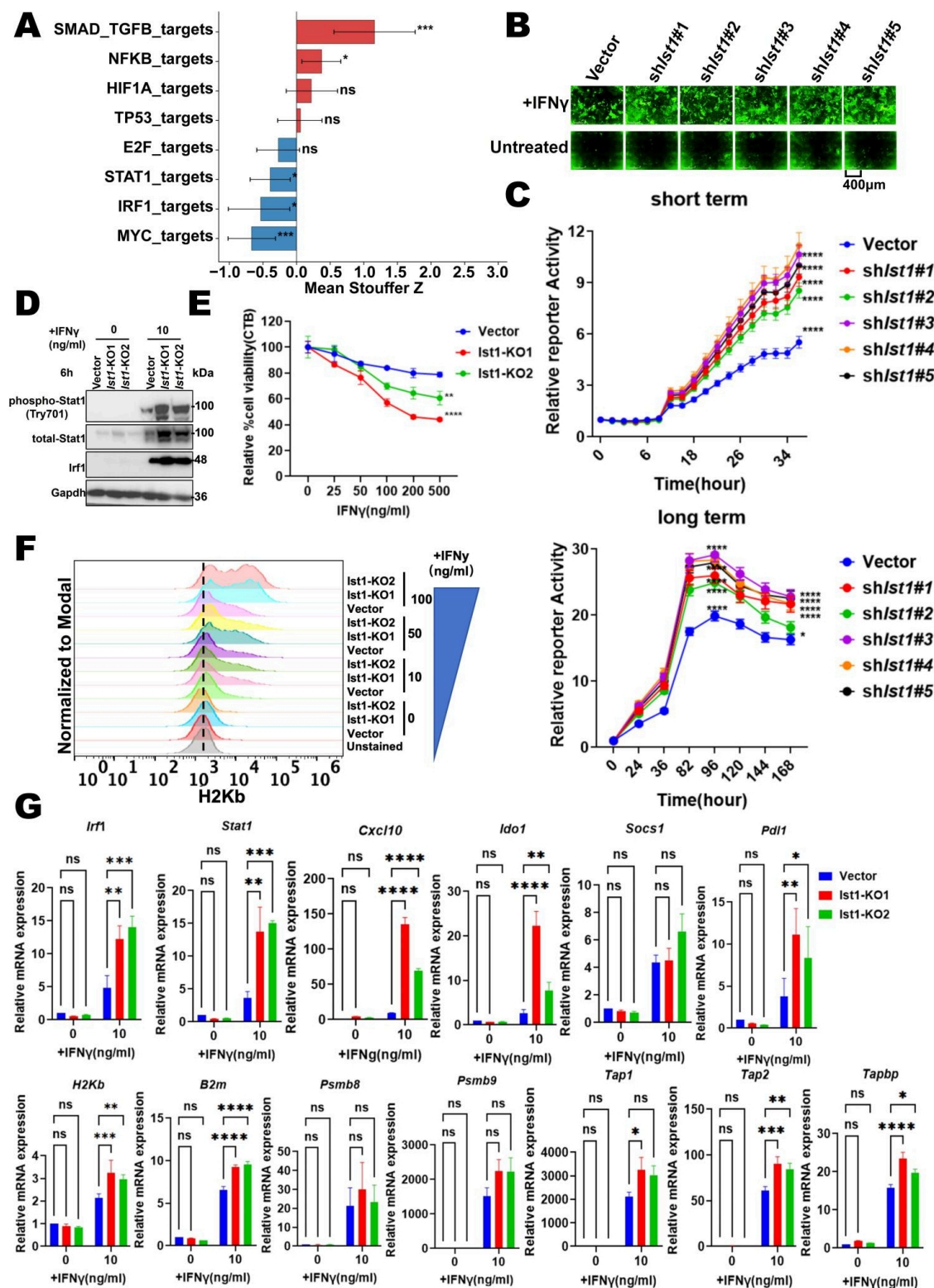


Figure 3

Ist1 deficiency amplifies IFN γ -driven JAK-STAT1 signalling and upregulates antigen presentation in pancreatic cancer cells.

(A) Hallmark transcription-factor target-module enrichment following virtual *IST1* perturbation across 8 PDAC single-cell cohorts (6 human, 2 mouse; ductal/tumor cells). Per-cohort gene effects (covariate-

adjusted regression on *IST1*) were combined as a Stouffer Z and globally mean-centred. Bars show the mean shifted Z for members of each of 8 Hallmark modules; horizontal error bars, 95% bootstrap CI (2,000 resamples); empirical P from 10,000 size-matched random draws, BH-corrected. Red, downregulated after virtual *IST1* perturbation; Blue: upregulated after virtual *IST1* perturbation. *FDR < 0.05; **FDR < 0.01; ***FDR < 0.001.

(B) Representative fluorescence images of Vector and five independent sh/*st1* KPC3-OVA-GFP lines under untreated and IFN γ -stimulated conditions.

(C) GAS4X-driven IFN-g/STAT1-transcriptional reporter (pGF-GAS4X) activity in vector-control and sh/*st1* KPC3-OVA cells following cytokine stimulation, displayed as short-term(top) and long-term(bottom) reporter-response kinetics. Data are shown as mean $\pm$ SD; n=3; *p<0.05, ****p<0.0001 by two-way ANOVA.

(A) Western blot of pSTAT1(Tyr701), total STAT1, *Ist1*, and GAPDH (loading control) in Vector, *Ist1*-KO1, and *Ist1*-KO2 cells at 6 h after stimulation with 0 or 10 ng/ml IFN γ .

(B) IFN γ dose-response cell-fitness assay in vector-control and *Ist1*-knockout cells. Cell viability was normalized to untreated controls across the indicated cytokine concentrations and assessed using CellTiter-Blue (CTB).

(C) Surface H2-Kb (MHC-I) flow-cytometry histograms in Vector, *Ist1*-KO1, and *Ist1*-KO2 cells after stimulation with IFN γ at 0, 10, and 100 ng/mL, with unstained control.

(G) qRT-PCR analysis of IFN γ -responsive and antigen processing and presentation genes in Vector, *Ist1*-KO1, and *Ist1*-KO2 cells after IFN γ stimulation. The upper row shows samples collected 6h after +IFN γ ; the lower row shows samples collected 12h after +IFN γ . Data are shown as mean $\pm$ SD; n=3; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant by two-way ANOVA.

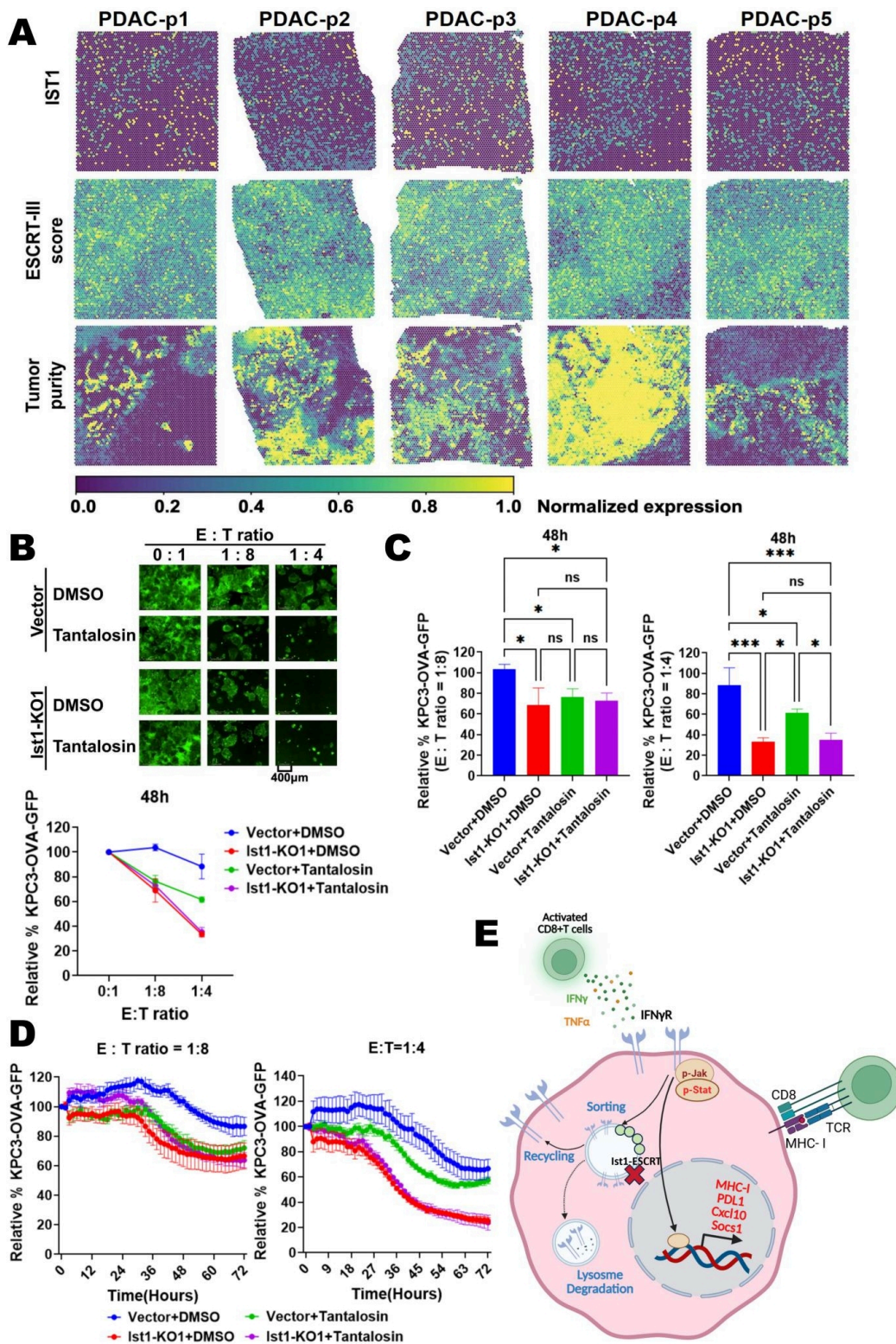


Figure 4

The IST1-ESCRT-III axis is spatially co-expressed in patient PDAC and pharmacologically targetable by a small-molecule inhibitor selectively targeting IST1-CHMP1B copolymer.

(A) Spatial transcriptomics (10x Visium) of five primary human pancreatic ductal adenocarcinoma (PDAC) specimens (PDAC-p1 to PDAC-p5) from GSE274103 dataset. Top row: spot-level IST1

expression. Middle row: ESCRT-III module score. Bottom row: Tumor purity. Color scale = normalized expression 0.0–1.0. The ESCRT-III score represents the average of spot-level z-scores across 13 core ESCRT-III pathway genes (*CHMP1A/B*, *CHMP2A/B*, *CHMP3*, *CHMP4A/B/C*, *CHMP5/6/7*, and *VPS4A/B*). Tumor purity denotes malignant epithelial cell lineage transfer scores derived from cell-type deconvolution.

(B) Top: Representative IncuCyte fluorescence images of OT-I + KPC3-OVA-GFP co-cultures at E:T ratios=0:1, 1:8, and 1:4, comparing Vector vs Ist1-KO1 cells under DMSO vs Tantalosin (small-molecule IST1-CHMP1B inhibitor). Bottom: quantification of relative % KPC3-OVA-GFP⁺ cells at 48 h at different E:T ratios for Vector+DMSO, Ist1-KO1+DMSO, Vector+Tantalosin, and Ist1-KO1+Tantalosin.

(C) Quantification of relative % KPC3-OVA-GFP⁺ cells at 48 h for the four conditions (Vector+DMSO, Ist1-KO1+DMSO, Vector+Tantalosin, Ist1-KO1+Tantalosin) at left: E:T = 1:8 and right: E:T = 1:4. Data are shown as mean ± SD; n=3; *p<0.05, ***p<0.001, ns = not significant by two-way ANOVA

(D) Continuous IncuCyte time-courses (0-72 h) of relative percentage of residual KPC3-OVA-GFP⁺ cells under OT-I co-culture at E:T=1:8 (left) and E:T=1:4 (right) for Vector+DMSO, Ist1-KO1+DMSO, Vector+Tantalosin and Ist1-KO1+Tantalosin. Data are shown as mean ± SD; n=3.

(E) Proposed model showing that Ist1 loss enhances IFN γ signaling by promoting IFN γ R surface abundance. Activated CD8⁺ T cells secrete IFN γ and TNF α in the tumor microenvironment. In tumor cells, Ist1-associated ESCRT machinery regulates endosomal sorting and surface turnover of membrane proteins, including IFN γ R. Loss of *Ist1* alters ESCRT-dependent endosomal sorting, leading to increased IFN γ R surface retention and/or resurfacing, thereby increasing its surface availability at the plasma membrane. Enhanced IFN γ R surface persistence potentiates IFN γ -induced JAK/STAT signaling and promotes the expression of immune-related genes, including *MHC-I*, *PD-L1*, *Cxcl10*, and *Socs1*. This augmented inflammatory signaling increases tumor cell antigen presentation and sensitivity to CD8⁺ T cell-mediated recognition and killing.

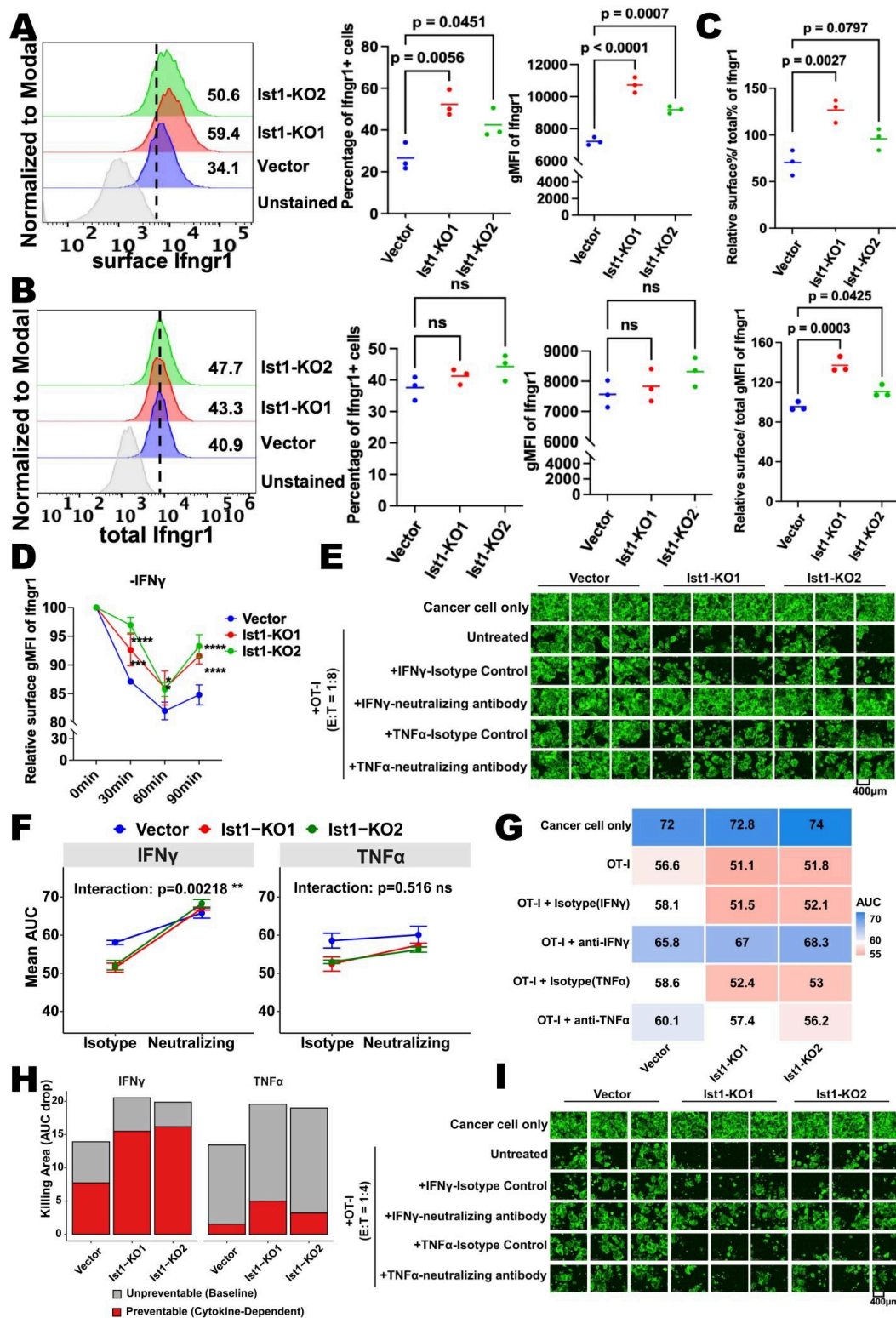


Figure 5

Ist1 deficiency increases surface IFN γ R1 abundance and licenses cytokine-driven tumor killing.

(A) Surface Ifngr1 flow cytometry. Left: representative histograms of surface Ifngr1 staining in Vector, Ist1-KO1, and Ist1-KO2 KPC3-OVA-GFP cells (Unstained control included). gMFI values are inset. Right:

quantification-percentage of surface *Ifngr1*⁺ cells and gMFI of surface *Ifngr1*⁺ cells across n=3 replicates. Data are mean $\pm$ SD; p values by one-way ANOVA.

(B) Total *Ifngr1* flow cytometry in the same genotypes; representative histograms and gMFI quantification. Right: quantification-percentage of total *Ifngr1*⁺ cells and gMFI of total *Ifngr1*⁺ cells across n=3 replicates. Data are shown as mean $\pm$ SD; p values by one-way ANOVA.

(C) Relative surface to total *Ifngr1* levels. Top: ratio of percentage of surface *Ifngr1*⁺ cells to total *Ifngr1*⁺ cells; Bottom: ratio of surface to total *Ifngr1* gMFI. Data are shown as mean $\pm$ SD; n=3; p values by one-way ANOVA.

(D) Antibody feeding assay for tracing basal surface *Ifngr1* internalization and turnover. Relative residual surface *Ifngr1* gMFI was measured over a time course (30, 60, 90 min after antibody feeding) in Vector, *Ist1*-KO1, and *Ist1*-KO2. Data are shown as mean $\pm$ SD; n=3; *p<0.05, ***p<0.001, ****p<0.0001 by two-way ANOVA.

(E) Representative IncuCyte images from OT-I co-culture assays performed with isotype controls, IFN γ -neutralizing antibody, or TNF α -neutralizing antibody at E:T=1:8 (72h) across Vector, *Ist1*-KO1, *Ist1*-KO2 KPC3-OVA-GFP cells.

(F) Interaction plots. Plotted is the mean AUC of relative confluence(normalized to tumour-only controls). Mean AUC of relative residual Vector, *Ist1*-KO1, *Ist1*-KO2 KPC3-OVA-GFP cells after co-culturing with OT-I T cells at E:T=1:8 in the presence of isotype or neutralizing antibodies against IFN γ or TNF α . Data are shown as mean $\pm$ SD; n=3; **p<0.01, ns = not significant by two-way ANOVA.

(G) AUC heatmap summarizing tumor cell survival across all conditions of (E): Tumor only, OT-I, OT-I + Isotype(IFN γ), OT-I + anti-IFN γ , OT-I + Isotype(TNF α), OT-I + anti-TNF α for Vector, *Ist1*-KO1, *Ist1*-KO2 at E:T=1:8 (72h). For +OT-I groups, individual values represent the mean AUC of n=3 replicates normalized to tumor-only controls. The color gradient ranges from red (low AUC, high T-cell killing) to blue (high AUC, low T-cell killing).

(H) Attribution of OT-I mediated killing to IFN γ /TNF α -dependent and independent pathways. Stacked columns representing the partitioning of total T-cell killing of Vector, *Ist1*-KO1, *Ist1*-KO2 KPC3-OVA-GFP cells at E:T=1:8 (72h). Red bars represent the preventable killing fraction rescued by neutralizing antibodies against IFN γ or TNF α . Values are calculated from the mean AUC of n=3 replicates.

(I) Representative IncuCyte images from OT-I co-culture assays performed with isotype controls, IFN γ -neutralizing antibody, or TNF α -neutralizing antibody at E:T=1:4 (72h) across Vector, *Ist1*-KO1, *Ist1*-KO2 KPC3-OVA-GFP cells.

Supplementary Files

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