Loss of Rnf31 and Vps4b sensitizes pancreatic cancer to T cell-mediated killing

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Pancreatic ductal adenocarcinoma (PDA) is an inherently immune cell deprived tumor, characterized by desmoplastic stroma and suppressive immune cells. Here we systematically dissect PDA intrinsic mechanisms of immune evasion by in vitro and in vivo CRISPR screening, and identify Vps4b and Rnf31 as essential factors required for escaping CD8⁺ T cell killing. For Vps4b we find that inactivation impairs autophagy, resulting in increased accumulation of CD8⁺ T cell-derived granzyme B and subsequent tumor cell lysis. For Rnf31 we demonstrate that it protects tumor cells from TNF-mediated caspase 8 cleavage and subsequent apoptosis induction, a mechanism that is conserved in human PDA organoids. Orthotopic transplantation of Vps4b- or Rnf31 deficient pancreatic tumors into immune competent mice, moreover, reveals increased CD8⁺ T cell infiltration and effector function, and markedly reduced tumor growth. Our work uncovers vulnerabilities in PDA that might be exploited to render these tumors more susceptible to the immune system.

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Interferon-γ (IFN-γ) response, TNF-mediated NFκB signaling, and autophagy as core pathways involved in immune evasion across different cancer types. Among the described mechanisms responsible for the highly effective immune evasion of PDA are (i) insufficient antigenicity, (ii) high expression of PD-L1, (iii) exclusion of dendritic cells while attracting T regulatory cells, and (iv) the sequestration of major histocompatibility complex class I (MHC-I).

In order to better understand cell-autonomous mechanisms that protect tumors from immune clearance, genome-wide CRISPR-Cas9 screens have been performed in melanoma, renal-, colorectal- and breast cancer cell lines. Together they identified the interferon-γ (IFN-γ) response, TNF-mediated NFκB signaling, and autophagy as core pathways involved in immune evasion across different cancer types. However, to our knowledge, a comprehensive genetic analysis of anti-tumor immunity in PDA is missing, and it remains unclear which of the identified immune regulators are conserved for PDA or whether there are also genetic factors that are specific to PDA.

Here we use genome-wide in vitro CRISPR screening and targeted in vivo CRISPR screening to systematically reveal positive and negative regulators of cytotoxic T lymphocyte (CTL) sensitivity in PDA. In addition to previously described genes involved in the regulation CTL-mediated tumor cell killing, we identify Rnf31 and Vps4b as central components for PDA immune escape in vitro and in vivo. Rnf31 and Vps4b are classified as the strongest sensitizers to T cell killing in our screen.

Results

A genome-wide CRISPR screen identifies regulators of immune evasion in PDA. To identify genes modulating CTL-mediated killing of PDA we performed a pooled, genome-wide CRISPR knock-out screen in pancreatic cancer cells. We first engineered a PDA cell line derived from the autochthonous KPC mouse model (KrasG12D, Tp53R172H+/−, Pdx-Cre), which stably expresses SpCas9 and chicken ovalbumin (OVA). Cells were subsequently transduced with a murine single-guide (sg)RNA library targeting 19,647 genes at a 500× coverage. To mimic cytotoxic T cell killing, we co-cultured cancer cells for three days with activated, OVA-specific CD8+ T cells (OT-I T cells) at a killing efficiency of approximately 70%, followed by a three-day recovery period prior to DNA isolation for analysis by next-generation sequencing (NGS) from the surviving cell population (Fig. 1a). Validating screening conditions and sufficient library representation, we observed a strong overlap of depleted sgRNAs targeting essential genes in OT-I T cell treated- and untreated KPC cells (Supplementary Fig. 1a, b). Next, we inspected differentially distributed sgRNAs, and defined genes targeted by enriched sgRNAs as resisters and genes targeted by depleted sgRNAs as sensitizers for CTL-mediated killing (FDR < 0.1). We identified several genes with a well-characterized role in CTL-mediated killing in different cancer types, demonstrating that the previously described core cancer intrinsic CTL evasion gene network is also conserved in PDA (Fig. 1b, c). For example, genes associated with the IFN-γ pathway (Jak1, Jak2, Ifngr1, Ifngr2, Stat1) and antigen presentation machinery (B2m, Tap1) conferred resistance to CTL-mediated PDA killing upon inactivation (Fig. 1c, d, Supplementary Fig. 1c). And genes regulating TNF-triggered apoptosis (Cflar, Traf2), NFκB signaling (Nfkbia, Tnfaip3) and autophagy (Atg5, Atg7, Atg10, Atg12, Gabarapl2) sensitized PDA cells to CTL-mediated killing upon inactivation (Fig. 1c, d, Supplementary Fig. 1c). Nevertheless, we also identified genes that have previously not been characterized as modulators of the CTL response, including the two strongest sensitizers Rnf31 and Vps4b. A targeted CRISPR screen validates immune modulators in vivo. To explore whether top candidates from the in vitro screen also affect CTL-mediated PDA killing in vivo we next performed a targeted library screen in mice. We generated a secondary library targeting 63 genes (hits with FDR < 0.1) with ten sgRNAs per gene, and containing 600 non-targeting control sgRNAs as well as seven targeting essential genes in OT-I T cell treated- and untreated PDA cells and control sgRNA treated GFP+ and a non-targeting KPC cells and control sgRNA treated GFP+. We furthermore observed a substantial, albeit not complete overlap between in vitro and in vivo screening results (Fig. 2a, b, c). In line with previous studies, we observed a substantial, albeit not complete overlap between in vitro and in vivo screening results (Fig. 2c, Supplementary Fig. 2a). Resistors of CTL evasion included well-known immune evasion genes, such as Stat1 and Casp8, as well as the positive control ovalbumin (Fig. 2b). Among sensitizers of CTL killing—which are of particular therapeutic interest as they bear the potential to enhance anti-tumor immunity in PDA upon inhibition—were the previously described genes Adar and Cflar22–24, as well as Vps4b and Rnf31 (Fig. 2b, c, d). Since mechanistic insights of how Vps4b and Rnf31 could regulate CTL sensitivity are still missing, and as both genes are transcriptionally upregulated in human PDAC samples compared to normal pancreatic tissue (Supplementary Fig. 2b), we decided to explore their role in PDA immune evasion more closely.

A competition assay confirms the role of Rnf31 and Vps4b in immune evasion. We then performed arrayed validation of Vps4b- and Rnf31-mediated PDA sensitization to CTL killing in a competition assay. KPC-Cas9-OVA cells that express mCherry and carry Vps4b- or Rnf31-targeting sgRNAs were mixed in a 1:1 ratio with KPC-Cas9-OVA cells that express GFP and a non-targeting sgRNA. The formation of insertion- and deletion- (indel) mutations in the targeted loci was confirmed by deep sequencing (Supplementary Fig. 3a), and no changes in cell proliferation were observed upon gene disruption (Supplementary Fig. 3c). Mixtures of targeted and non-targeted KPC cells were co-cultured with CD8+ OT-I T cells and proportions of mCherry+ and GFP+ cells were determined by flow cytometry (Fig. 3a). As expected, unmutated mCherry+ KPC cells and control sgRNA treated GFP+ KPC cells grew equally well in the presence of OT-I T cells (Fig. 3b, c), and targeting Stat1 shifted the ratio towards mCherry+ cells due to a defective IFN-γ response (Fig. 3b, c, Supplementary Fig. 3g). In contrast, KPC cell lines mutant for Vps4b or Rnf31 displayed a strong growth disadvantage under immune attack, leading to an
Fig. 1 Genome-wide CRISPR screen in PDA cells reveals immune evasion mechanisms in vitro. a Schematic of genome-wide in vitro CRISPR screen. b Volcano plot of top ten depleted (blue) and enriched (red) genes. Screening analysis was performed with MaGeCK RRA. c sgRANK of the top (red) and bottom (blue) five depleted genes are represented. Gray bars represent non-targeting sgRNAs. d sgRANK of the enriched (red) and depleted (blue) genes of different immune evasion pathways. Gray bars represent non-targeting sgRNAs. MaGeCK RRA analysis can be found in Supplementary Table 1. The genome-wide CRISPR screening data shown were derived from three independent biological replicates (n = 3).

Fig. 2 A targeted CRISPR library screen validates candidates in vivo. a Schematic of the secondary CRISPR screen in vivo. b Heatmap of normalized read counts of sgRNAs across ten individual mice (M1–M10). c Bar diagram of sublibrary genes in comparison to their predicted phenotype (from in vitro screen). d Volcano plot of depleted (blue dots) genes of the sublibrary in vivo screen when comparing the pool of preinjected KPC cells to OT-I CD8+ T cell treated tumors. Sublibrary screening data were derived from a total of ten individual tumors (transplanted mice n = 10), transplanted in two experiments. Source data are provided as a Source Data file.

outgrowth of GFP+ control cells (Fig. 3b–d). Analyzing Vps4b and Rnf31 in melanoma and breast cancer cell lines, moreover, indicate a partial conservation of this phenotype in other cancer entities, with loss of Rnf31 also sensitizing B16 melanoma cells to CTL killing and loss of Vps4b leading to a trend towards higher sensitivity in EO771 breast cancer cells (Fig. 3d). Since Vps4b is part of endosomal sorting complex III (ESCRT-III), we also tested whether other members of the complex cause a similar phenotype. However, in line with our observation that none of these genes were identified as a hit in our genome-wide CRISPR screen (Supplementary Table 2), arrayed elimination of Vps4a, Vta1, and Chmp4b did not sensitize KPC cells to CTL-mediated killing (Supplementary Fig. 3e).

Functional characterization of the role of Rnf31 and Vps4b in immune evasion. Antigen presentation by major histocompatibility complex I (MHC-I) proteins is necessary for efficient antitumor immunity. As shown in a recent study, pancreatic cancer
Flow cytometry analysis

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co-culture in an alternative KPC cell line, B16-F10 melanoma cells and EO771 breast cancer cells. signaling de parental KPC cells, which, as expected, was perturbed in IFN levels in KPC cells upon exposure to CTLs. Con levels on PDA. To test this hypothesis, we assessed surface MHC-Rnf31

cells commonly sequestrate MHC-I to evade the adaptive immune system, prompting us to suspect that loss of Rnf31 and Vps4b facilitates CD8+ mediated killing by increasing MHC-I levels on PDA. To test this hypothesis, we assessed surface MHC-I levels in KPC cells upon exposure to CTLs. Confirming our assay, we observed a robust induction of MHC-I upregulation in parental KPC cells, which, as expected, was perturbed in IFNγ signaling deficient Stat1KO cells (Fig. 3e, Supplementary Fig. 3h). Next, we analyzed MHC-I induction in Rnf31KO and Vps4bKO KPC cells. However, we observed similar surface MHC-I levels compared to the parental cell line (Fig. 3e), demonstrating that enhanced CTL-mediated killing is not triggered by an increase in antigen presentation.

Fig. 3 Arrayed validation of selected screening hits in vitro. a Schematic of in vitro competition assay. Tumor cell:T cell co-cultures were carried out with an E:T ratio of 1:1 in all experiments. b Representative flow cytometry plots (GFP vs. mCherry) of the arrayed hit validation with and without T cell co-culture. c Log2 Fold change of mCherry+ KPC population before and after OT-I co-culture with different sgRNAs targeting candidates Rnf31 and Vps4b. n = 3 for Rnf31-2; Rnf31-3 and Vps4b-3, all other conditions n = 4. d Log2 Fold change of mCherry+ KPC population before and after OT-I co-culture in an alternative KPC cell line, B16-F10 melanoma cells and EO771 breast cancer cells. n = 4 for KPC-2 Rnf31 and B16-F10 Rnf31, all other conditions n = 3. e Mean fluorescence intensity (MFI) of Pan-H2-Kb. n = 3 independent experiments. Significance was determined with a one-way ANOVA analysis. Non-significant, p > 0.05. Values represent mean±5D, data are derived from three independent experiments. Source data are provided as a Source Data file.

Rnf31 loss sensitizes PDA to TNF-induced apoptosis via caspase 8. Cytotoxic T cells induce death in target cells via different processes, including the release of TNF, secretion of granules filled with granzymes and perforins, and by engaging the Fas-Fasl axis. To systematically explore which of these effector mechanisms are sensitized upon Rnf31 and Vps4b inhibition, we first assessed tumor cell sensitivity to TNF ligands. Interestingly, we found that parental KPC cells and Vps4bKO KPC cells were insensitive to TNF-induced apoptosis, but that Rnf31KO KPC cells rapidly underwent cell death upon TNF treatment (Fig. 4a). Engagement of the TNF receptor triggers several signaling branches, including pro-survival NFκB signaling as well as apoptosis induction via caspase 8 cleavage. Notably, Rnf31 has previously been reported to function as an E3 ubiquitin-protein ligase within the LUBAC, which is involved in regulating NFκB signaling. Therefore, we speculated that the Rnf31 knock-out sensitizes tumor cells to TNF-mediated apoptosis either indirectly, by abrogating NFκB pro-survival signaling, or directly, by facilitating caspase 8 cleavage. When we first assessed TNF-mediated NFκB activation, we found phosphorylation of the inducing PD-L1 (CD274) expression, and low expression of these chemokines is correlated with a better prognosis in human PDA patients (Supplementary Fig. 3i). However, differences in cytokine expression alone could not explain the in vitro phenotypes observed in the KPC-CTL co-culture screen, prompting us to conduct further mechanistic studies on Rnf31 and Vps4b in KPC cells.
NfκB subunit p65/Rela in all genetic backgrounds, including Rnf31KO cells (Fig. 4b), indicating functional NfκB signaling. When we next analyzed caspase 8 cleavage upon TNF treatment, activation was observed in Rnf31KO KPC cells but not in parental KPC- or Vps4bKO KPC cells (Fig. 4b). Hence, our data suggest that in Rnf31-deficient cells intact NfκB signaling is not sufficient to rescue TNF-activated caspase 8 cleavage.

A recent study proposed that Rnf31 is involved in stabilizing the anti-apoptotic protein c-Flip27, prompting us to assess whether loss of Rnf31 sensitized KPC cells to caspase 8-mediated apoptosis via destabilization of c-Flip. In line with this hypothesis, we found that c-Flip was degraded in Rnf31KO KPC cells but not in parental KPC cells upon TNF exposure (Fig. 4c). Moreover, Cflar, the gene encoding for c-Flip, was among the most strongly depleted genes in our genome-wide screen (Fig. 1d), and CflarKO KPC cells mimicked the Rnf31KO phenotype and underwent apoptosis after TNF exposure (Supplementary Fig. 4d).

To next assess whether loss of Rnf31 also sensitizes human PDA to TNF-mediated cell death, we generated patient-derived and engineered human pancreatic cancer organoids (hPDA) with RNF31KO mutations and treated these organoids for four hours with TNF. In line with results from murine PDA tissues, only RNF31KO but not RNF31WT PDA organoids activated apoptotic cell death upon TNF stimulation (Fig. 4d–f, Supplementary Fig. 4a–c). Taken together, our results suggest that loss of the LUBAC subunit Rnf31 sensitizes murine and human pancreatic cancer to CTL killing by rendering cells susceptible to caspase-8-mediated apoptosis upon TNF signaling (Supplementary Fig. 4e).

Vps4b depletion impairs functional autophagy and increases intracellular Granzyme B levels. Vps4b is part of the ESCRT-III complex functions as an AAA-type ATPase involved in diverse processes, including the catalyzation of phagophore closure during autophagy30. Considering that several autophagy-related genes have been identified as sensitizers for CTL-mediated killing, we reasoned that Vps4bKO cells might sensitize PDA to CTL-mediated killing by inhibiting autophagy. To test this hypothesis, we transduced cells with an autophagic flux reporter, and assessed if autophagy is impaired in Vps4b knockout KPC cells31. The reporter consists of a LC3-GFP-LC3 reporter, which encodes for a C-terminal glycine and stably resides in the cytoplasm during autophagy (Fig. 5a). While parental KPC cells showed a strong upregulation of autophagy upon starvation (Fig. 5b), Vps4bKO KPC cells showed an impaired autophagic flux, similar to fully autophagy-deficient Atp5K KO KPC cells (Fig. 5b). These data suggest that loss of Vps4b sensitizes PDA to CTL killing through disrupting autophagy. To test whether this mechanism is also conserved in human cells, we next measured the autophagic flux in HEK293T cells upon VPS4B deletion. However, in contrast to the loss of function mutation in ATG9A, VPS4B depletion did not impair autophagy induction (Supplementary Fig. 5d), and we speculate that in human cells homologs of VPS4B might lead to functional compensation upon gene disruption.

In previous studies, several different mechanisms have been proposed of how autophagy could modulate immune evasion. One study suggested that high autophagy rates in PDA may
contribute to immune evasion by sequestering surface MHC-I levels. In KPC cells, nevertheless, we observed a robust induction of MHC-I surface expression upon CTL exposure, which was also not affected by Vps4b depletion (Fig. 3e). Another study suggested that autophagy inhibition may facilitate CTL-mediated killing by increasing sensitivity to TNF-induced cell death. However, in KPC cells mutant for Vps4b or Atg5 we did not observe apoptosis induction upon TNF treatment (Fig. 4a, b), and pre-treatment of cells with Actinomycin D, a NFκB-mediated sensitizer to TNF-induced apoptosis, revealed similar sensitivity of Vps4bKO, Atg5KO- and parental KPC cells to increasing concentrations of TNF (Fig. 5c, Supplementary Fig. 5b). A third study found that high autophagy levels in breast cancer cells promoted NK cell-derived granzyme B degradation, suggesting that this mechanism could contribute to the resistance to CTL killing.

We, therefore, hypothesized that autophagy deficiency could sensitize PDA cells to CTL killing through insufficient granzyme B clearance, and quantified intracellular granzyme B levels in KPC cells upon OT-I T cell exposure. Indeed, while OT-I T cells produced comparable amounts of granzyme B, autophagy-deficient Atg5KO- and Vps4bKO KPC cells accumulated more granzyme B than parental KPC cells (Fig. 5d, e, Supplementary Fig. 5c). Taken together, our data suggest that Vps4b inhibition perturbs autophagy in KPC cells, which in turn reduces their capability to degrade granzyme B upon CTL-mediated killing.

**Rnf31 and Vps4b inhibition increases CTL infiltration and effector function in vivo.** To further characterize increased CTL susceptibility of Rnf31KO and Vps4bKO KPC cells in vivo, we analyzed the effect of these mutations on PDA progression and the tumor microenvironment in mice. Therefore, orthotopically transplanted KPC-Cas9 cells with different genotypes (wildtype, Rnf31KO and Vps4bKO) into immune-competent C57BL/6 animals, and assessed survival and tumor weight, as well as immune cell composition and effector function using flow cytometry (Fig. 6a, Supplementary Fig. 6d). While remaining Cas9 expression in these lines did not affect tumor growth (Supplementary Fig. 6a), loss of Rnf31 and Vps4b markedly decreased tumor mass and resulted in significantly enhanced survival of tumor-bearing mice (Fig. 6b). In case of Vps4bKO tumors the effect was strongly dependent on adaptive immunity, since tumor mass reduction was not apparent in Rag1−/− mice (Supplementary Fig. 6b). Rnf31KO tumors, however, also showed reduced growth compared to KPCWT tumors in Rag1-deficient hosts, most likely due to the continued expression of TNF and other death receptor ligands by NK cells (Supplementary Fig. 6b).

We next analyzed immune cell infiltration and CD8+ T cell effector function across the different tumor genotypes. While the loss of Vps4b and Rnf31 did not cause significant changes in macrophages, CD11c+ dendritic cells, CD4+ T helper cells, NK cells, and CD4+ Foxp3+ regulatory T cells, we detected a minor increase of neutrophils in Rnf31KO tumors (Supplementary Fig. 6c). In addition, we observed a substantial increase of infiltrating CD8+ T cells in Vps4bKO and Rnf31KO tumors compared to parental KPC tumors (Fig. 6c). Moreover, in Vps4bKO and Rnf31KO tumors infiltrating CD8+ T cells displayed a reduction in PD1 surface levels and intracellular Eomes levels, as well as increased effector cytokine production (i.e., TNF and IFNγ), indicating a reduction in T cell exhaustion (Fig. 6c). Notably, we also observed a decrease in actively proliferating CD8+ Ki67+ tumor-infiltrating T cells in Rnf31KO and Vps4bKO tumors. While
at first sight, this finding seems counterintuitive, it could be explained by the decreased tumor load compared to the parental KPC tumors at the stage of analysis (see reduced tumor mass in Fig. 6b). Finally, we also performed immune checkpoint blockade (ICB) treatment in mice transplanted with Rnf31KO and Vps4bKO tumors, and found that CD8⁺ T cell infiltration was further increased and accompanied by markedly reduced PD1 surface expression (Fig. 6d). In Rnf31KO tumors ICB also potentiated effector cytokine production and led to significant tumor mass reduction (Fig. 6d). Together, our findings from in vivo experiments demonstrate that loss of Rnf31 and Vps4b sensitizes PDA to CTL-mediated killing not only in a cell-autonomous manner, but also in a non-cell-autonomous manner through increasing CTL effector function.

Discussion

Several recent studies performed CRISPR screening in PDA to study metastasis formation, metabolic vulnerabilities, combinatorial drug targeting, and therapy resistance. Here, we applied in vitro and in vivo CRISPR screening in PDA to interrogate tumor intrinsic mechanisms of immune evasion. One of the strongest sensitizers to CTL-mediated killing was Rnf31, for which we show that its inactivation facilitates TNF-induced apoptosis via caspase 8 cleavage in murine and human PDA. Notably, previous work has already linked TNF resistance to immune evasion. However, these studies have been conducted in a TNF-susceptible colorectal cancer cell line (MC38),impeding the identification of TNF sensitizers such as Rnf31. In contrast, KPC pancreatic cancer cells are intrinsically resistant to TNF, which allowed us to unravel a mechanism that abates TNF resistance. We further show that TNF is sufficient to induce apoptosis in Rnf31 deficient pancreatic cancer cells, in contrast to B16 melanoma cells where IFNγ is required for TNF-induced apoptosis after Rnf31 disruption.

Another strong sensitizer to CTL-mediated killing identified in our screen was Vps4b, which could be linked to autophagy. Autophagy has recently been postulated as an important modulator of anti-tumor immunity in several cancer entities. However, in contrast to previous findings, we did not observe increased MHC-I antigen presentation or enhanced TNF-induced apoptosis upon autophagy inhibition. Instead, we provide evidence that impaired autophagy leads to reduced granzyme B clearance, suggesting that Vps4b or Atg5 depletion facilitates tumor cell lysis by CD8⁺ T cells through enhanced granzyme B accumulation. While Vps4b depletion did not perturb autophagy in human cells, in the future it would be interesting to test whether autophagy inhibition also leads to elevated granzyme B accumulation in human cancers. Moreover, since the administration of autophagy inhibitors alone or in combination with conventional chemotherapy did not improve progression-free- or overall survival in previous clinical studies, it will be interesting to see if more recent trials in which autophagy inhibition is combined with immunotherapy will report higher efficacy.

We identified Rnf31 and Vps4b in our in vitro and in vivo CRISPR screens using the OVA/OT-I tumor model, which...
ensured sufficient antigenicity of tumor cells. Importantly, arrayed in vivo validation using orthotopic transplantation of Rnf31 KO and Vps4b KO KPC tumors into immune-competent C57BL/6 showed that the observed phenotype was conserved without employing the OVA/OT-I system, strengthen potential translatability of our findings to the clinics. However, as T cell immunity is generally dependent on sufficient tumor antigenicity, future studies should determine whether Rnf31 and Vps4b depletion will show similar phenotypes in other PDA tumor models. Nevertheless, Rnf31 is a promising candidate in this context, as it acts through TNF-mediated apoptosis that is known to be an important determinant for PDA prognosis and treatment41,42. Therefore, we exclusively focused on tumor cell–immune cell interactions and did not analyze potential changes in the tumor stroma in this study. As the tumor stroma is known to be an important determinant for PDA prognosis and treatment41,42, addressing the effect of Rnf31 and Vps4b depletion in the context of the tumor stroma should be highly valuable. Taken together, we used functional genomic approaches to identify mechanisms for circumventing immune evasion in PDA. Analysis of two of the strongest hits, Vps4b and Rnf31, demonstrated that their inhibition sensitizes tumor cell clearance directly, via cell-autonomous mechanisms, and indirectly, by increasing the number and functionality of intratumoral CD8+ T cells. Our insights in sensitizing pancreatic cancer to the host immune system could open up promising strategies to enhance the efficacy of T cell-mediated tumor killing, potentially allowing PDA patients to benefit from the vast advances made in the field of cancer immunotherapy in the future.

Methods

Animals. Wildtype C57BL/6 mice were obtained from Charles River Laboratories. Rag1−/− (NOD:129S7(B6)-Rag1tm1Mom/J) and OT-I (C57BL/6-Tg(Thy1.2-TcraTcrb)2100Mjb/J) were obtained from Jackson Laboratories and bred in-house. For all experiments, male mice between 8 and 12 weeks were used. All animals were housed in a pathogen-free animal facility in cages with up to five animals at the Institute of Molecular Health Sciences at ETH Zurich and kept in a temperature- and humidity-controlled room on a 12 h light-dark cycle. All animal experiments were performed in accordance with protocols approved by the Kantonale Veterinäramt Zurich in compliance with all relevant ethical regulations.

Cell culture. The KPC-1 cell line (C57BL/6 background) was generated by Dr. Jen Morton (Beatson Institute) and purchased at Ximbio (Cat# 153474). KPC cells were derived from primary PDA tumor obtained from Pdx-Cre; KrasG12D/+; Trp53fl/fl mice. The KPC-2 cell line (C57BL/6 background) was generated in-house from p48-Cre; KrasG12D/+; Trp53fl/fl mice. The melanoma cell line B16, the breast cancer cell line E0771, and HEK293T cells were obtained from ATCC. All cell lines used in this study were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM, 31980030, Gibco) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Gibco) and 50 µM β-Mercaptoethanol (Gibco). Cells were incubated at 37 °C in 5% CO2. The parental cancer cell lines were engineered with LentiCas9-Hygromycin and Lenti-Ovalbumin-m-Cherry-Blasticidin constructs (for details see “Plasmids”) in order to express Cas9 and full-length Ovalbumin (KPC-Cas9-OVA). KPC cell line (KPC-1) was received from Ximbio (Cat# 13474), including a materials transfer agreement.

Plasmids. For generation of Lentiviral vectors we replaced Blasticidin from the original vector (Addgene #52962) by HygromycinR by Gibson Assembly. For generation of Lentiviral vectors we replaced Blasticidin from the original vector (Addgene #52962) by Oovalbumin-P2A-mCherry derived from pKids-OVA (Addgene #11340) with Gibson Assembly. The LC3-GFP-LC3A-RFP construct was obtained from Addgene (#84572) and cloned into Lentiviral vectors by replacing Cas9-P2A-BlasticidinR by Oovalbumin-P2A-mCherry. The following vectors LentiGuide-Puro (#52963), LentiCRISPRv2-puro (#98290) and pLenti-PGK-Hygro-KRAS(G12V) (#35635) were obtained from Addgene.

Lentivirus production. For lentivirus production of CRISPR libraries, transgenes were amplified from Didier Trono. The genome-wide Brie CRISPR-KO library (4 sgRNAs per gene; ~90,000 sgRNAs) was purchased from Addgene (#73632) and amplified according to the supplier’s protocol. For each sample, total DNA obtained from 4 × 107 cells was used as input with 10 µg DNA per 100 µl PCR reaction. DNA was amplified using Hercule II Fusion DNA Polymerase (Agilent) according to the manufacturer’s conditions with 2 µl Hercule II and 2.5 mM MgCl2 and was performed in a total of 24 cycles. PCR reactions were cleaned up using 0.8X AMPure beads (Beckman Coulter). NGS libraries were run on the Illumina NovaSeq 6000 System generating 100 bp single-end reads.

Data analysis. Demultiplexed reads were trimmed to exact 20 bp (sgRNA) using cutadapt. Subsequently, read counts were assessed using MAGeCK (v0.5.6) as well as sgRNA enrichment/depletion with “marpeck -k readcounts --t OT-I -c CTRL --norm-method control --control-sgrna none-targeting.txt”. For MAGeCK analysis three biological screening replicates were pooled. For pathway analysis of gene enrichment/depletion the cut-off was set at FDR < 0.1 and GO term analysis for candidates was performed using the Molecular Signature Database (MSigDB). The screening data set can be found in Supplementary Tables 1 and 2 and via the GEO accession number GSE180834.

Isolation and activation of CD8 T-cells. CD8+ OT-I T-cells were isolated from spleen, axillary, and inguinal lymph nodes from OT-I mice. CD8+ cells were enriched using magnetic beads for MACS (130-104-075, Miltenyi Biotec). T-cells were cultured in IMDM supplemented with 10% FBS, 1% penicillin/streptomycin (Gibco), 100 µM β-Mercaptoethanol (Gibco), and 100 ng/mL IL-2 (PeproTech). Cells were kept at 37 °C in 5% CO2. After T-cell isolation, cells were activated for 24 h using 2 µg/ml of anti-CD28 and anti-CD3 antibodies (101162 and anti-CD3e antibodies (100340, BioLegend; 1:500 each).

In vitro cytotoxicity assays and CRISPR-KO generation. One day prior to OT-I co-culture, a total of 5 × 105 Ovalemum-expressing KPC cells were plated into 24-well plates. For competition assays, KPC-Cas9-OVA-nMCherry (with CRISPR-KO of the indicated gene) and KPC-Cas9-OVA were plated at a 1:1 ratio. Activated OT-I T-cells were added in the presence of 100 ng/mL IL-2 for one to two days, depending on downstream analysis. EGFP:nMCherry ratio was assessed using flow cytometry. In brief, cells were trypsinized, spun down, and washed in FACS buffer (2% FBS, 2 mM EDTA in PBS). For MHC class I assessment, cells were stained using 5 mM EDTA. The following antibodies were used: CD8a-APC (1:600; 53-6.7; eBioscience), CD3e-APC (1:600; 53-6.7; eBioscience), H-2Kb-APC (1:600; 53-6.7; eBioscience), and H-2Dd-APC (1:600; 53-6.7; eBioscience). CD11c-APC (1:600; 53-6.7; eBioscience) was used as a negative control.

SIF. All data generated or analyzed during this study are included in this article.

ARTICLE NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-022-29412-3 | www.nature.com/naturecommunications

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Vps4b-1: 5’ GGTCGACCCAGAATAGAAG 3’
Vps4b-2: 5’ GGAACAGGCGCTTCTAGAG 3’
Chmp4b-1: 5’ TGGTTTATCTGTCATGAC 3’
Chmp4b-2: 5’ ACTACATTATGGTGTCGGG 3’
Chmp4b-3: 5’ CTAACTACGGTTAAGGATGT 3’
Vps4b-4: 5’ CACAGATCAATGTGTGCTG 3’
Sta-1: 5’ GGTAGATCCGGCCACGCACTG 3’
Atg5: 5’ AAGAGGACTGCTATTGTGAG 3’

For Lenti-illation both sGRNAS were transduced in a pool of sGRNA1-3 targeting Rfn31 or Vps4b. For alternative cell line validation KPC-2, B16, and EO771 cells were transduced with a pool of sGRNA1-3 targeting Rfn31 or Vps4b. Cells were selected with 2 µg/ml Puromycin for 3-5 days.

**Autophagy flux assay.** LC3-GFP-LC3ΔG-RFP construct was stably integrated into KPC-Cas9-OVA cells or HEK293T cells and GFP+/RFP− single cell clones were sorted and expanded. Cells were starved for 8 h or overnight (HEK293T cells) and depleted; FDR < 10%) were selected (Supplementary Table 3). If there were

**Human pancreatic cancer organoids.** Human pancreatic tissue was obtained from surgical tumor resections at the University Hospital Zurich. The Clinical Ethics Committee of the University Hospital Zurich approved the use of the samples for the generation of tumor organoids lines, and informed consent was provided from all patients. For Normal human pancreatic tissue was obtained from surgical tumor resections at the University Hospital Zürich; human PDA tissue was obtained from surgical tumor resections at the University Hospital Zurich. The Clinical Ethics Committee of the University Hospital Zurich approved the use of the samples for the generation of tumor organoids lines, and informed consent was provided from all patients. For Normal human pancreatic islet isolation procedures from the University Hospital Zürich; from healthy donors were lentivirally transduced to express oncogenic TP53 (Peprotech), 100 ng/ml FGF10 (Peprotech), 10 nM Gastrin (Tocris Bioscience), and incubated for 100 cycles at 5 min 37˚C. Subcutaneous tumors were collected for flow cytometry analysis in order to assess GFP and RFP expression. The autophagic flux was assessed by calculating the GFP/RFP ratio and comparison to the non-starved control condition. HEK293T cells were engineered for ATG9A-RFP (Peptide1), RNF31-1, and streaked out onto LB agar plates. Library integrity was confirmed by next generation sequencing (Illumina platform). The screening data set can be found in Supplementary Table 3 and in GEO accession number GSE180834.

**Transplantation.** Mice were anesthetized using isoflurane at a constant flow rate. The abdomen was shaved and sterilized before a small incision in the upper left quadrant was made. The pancreas was carefully put onto a cotton-swab and 1.5 x 10^6 KPC cells were injected in 50 µl of PBSMatrigel (1:1) using a 29 G needle. Successful infection was confirmed when a liquid bled formed and no leakage could be observed. Peritoneum and skin were subsequently sutured with Vicryl violet sutures (N383H, Ethicon) and secured with wound clips (IST). Approximately three post transplantations were animals were sacrificed and tumors were harvested. Tumor DNA was isolated using the Qiagen Blood and Tissue Kit and sGRNA cassette was amplified similarly to the in vitro screen and analyzed by Illumina sequencing. Single guide RNA representation was assessed using MAGeCK (v0.5.6) by comparison to the plasmid sublibrary. The screening data set can be found in Supplementary Table 3 and in GEO accession number GSE180834.

**Flow cytometry for tumor microenvironment analysis.** For flow cytometry analysis orthotopic tumors were collected and minced into small pieces before digestion in Collagenase IV (6000 U/ml) and DNase I (200 U/ml) for one hour at 37˚C. Cell suspension was acquired by FACS with 2 mM EDTA, 2% FBS, and 10 µM RhoKinase inhibitor. For sublibrary generation, the top candidates (enriched in the dark) and water up to 50 µl. The reaction was

**Antibodies used for flow cytometry.** PE-1 FITC (1:300; J43; eBioscience), NKI.1 PE (1:300; PK136; eBioscience), CD3e PE-Dazzle594 (1:300; 145-2C11; BioLe-
gend), CD3e PE (1:200; 145-2C11; eBioscience), FoxP3 PE-Cy5.5 (1:200; FJ-
K-1628; eBioscience), CD4 PE-Cy7 (1:2000; 53-6.7; eBioscience), CD8a APC (1:600; 53-6.7; eBioscience), CD8a PE-Cy5.5 (1:400; 53-6.7; eBioscience), CD122 PE-Cy7.5 (1:400; 53-6.7; eBioscience), CD11c BV605 (1:400; N418; BioLegend), Sgp3-
E C (1:300; E50-2440; BD Bioscience), CD68 APC (1:200; BM8; BioLegend), CD8 BV605 (1:4000; 55-11.15; BioLegend), CD64 BV421 (1:200; 55-11.15; BioLegend), TNF FITC (1:300; MP6-
XT22; BioLegend), INFγ PE-Cy7 (1:1000; XMG1.2; BD Biosciences), CD3e PE (1:200; 145-2C11; BioLegend), CD3e PE (1:200; 145-2C11; eBioscience), FoxP3 PE-Cy5.5 (1:200; 53-6.7; eBioscience), CD8a APC (1:600; 53-6.7; eBioscience), CD8a PE-Cy5.5 (1:400; 53-6.7; eBioscience), CD122 PE-Cy7.5 (1:400; 53-6.7; eBioscience), CD11c BV605 (1:400; N418; BioLegend), Sgp3-
E C (1:300; E50-2440; BD Bioscience), CD68 APC (1:200; BM8; BioLegend), CD8 BV605 (1:4000; 55-11.15; BioLegend), CD64 BV421 (1:200; 55-11.15; BioLegend), TNF FITC (1:300; MP6-
XT22; BioLegend), INFγ PE-Cy7 (1:1000; XMG1.2; BD Bioscience), Eomes PE (1:200; W17001A; BioLegend), Ki-67 BV65 (1:200, 11F6; Biolegend), GzmB FITC (1:100; GB11; BioLegend). Gating strategy is depicted in Supplementary Fig. 6d.

**RNA-Seq.** After 6 h of co-culture, OT-I T-cells and KPC-Cas9-OVA cancer cells were sorted using the BD Aria cell sorter. KPC cells expressed mCherry, T-cells were stained with CD6a-PE (clone 33-3-7; BioLegend 100711; 1:600) to separate both populations. RNA was isolated with the QIAGEN RNeasy Mini Kit and sent to the Genomic and Translational Genomic Center at the University of Zurich for standard library preparation and Illumina sequencing. Raw quality was checked using FastQC. Reads were trimmed using cutadapt and mapped to the mouse genome GRCm38 with HISAT2 followed by sorting using samtools. The raw count matrix was generated in RStudio using Rubread. Differential gene (DE) expression analysis was performed with EdgeR and differentially expressed genes (LFC ≥ 1, FDR < 0.1) were used as input for GO term analysis using the Molecular Signature Database (MsigDB). RNA-Seq data can be accessed via GEO.

**TNF treatment of KC cells.** KC cells were treated 24 h with the indicated TNF concentration and stained subsequently for immunofluorescence (see below) or...
with crystal violet to assess cell viability (here in the presence of 1 μg/mL Actinomycin D). Crystal violet dye was reconstituted in 10% acetic acid and absorbance was measured at 595 nm.

**Immunofluorescence of KPC cells.** Cells were grown on glass cover slips, treated with TNF (100 ng/ml), and fixed for 10 min at room temperature in 4% PFA. Cells were permeabilized and blocked in 0.5% Triton-X, 5% normal donkey serum in PBS. Cleaved caspase 3 antibody (1:400, Cell signaling Technology, #9664) was diluted in blocking solution and incubated overnight at 4 °C. Coverslips were washed PBS and incubated 2 h at room temperature with secondary antibody (Donkey anti-rabbit–568, Thermofisher Scientific, 1:400), Alexa Fluor 647 Phalloidin (1:1000, Cell signaling technology, #9490) and DAPI. Coverslips were mounted with Prolong Gold (Thermofisher Scientific) and imaged with a Zeiss LSM 880 Airyscan.

**Whole-mount staining of human pancreatic cancer organoids.** Wildtype or RNF31 KO lip2/iPDA organoids were treated for 4 h with 100 ng/ml human TNF (PeproTech) in 8-well μ-slides (Ibidi). After fixation in 4% PFA, organoids were blocked and permeabilized in blocking solution (10% normal donkey serum; 0.5% Triton-X in PBS). All antibody incubations were performed overnight at 4 °C on a rocking platform. Primary antibodies: E-Cadherin (1:500, R&D Systems, AF748), cleaved Caspase 3 (1:400, Cell Signaling Technology, #9664). Donkey-anti-goat 488 and donkey-anti-rabbit secondary antibodies were used as secondary antibodies and counterstained with DAPI (always 1:400 for secondary antibodies). Organoids were mounted with ProLong Gold. Confocal Images were taken with a Zeiss LSM 880 Airyscan. Organoids were quantified manually using Fiji Image J by counting three individual images from independent experiments per condition.

**Western blot.** Whole-cell lysates were prepared in RIPA buffer (50mMTris-HCl pH 8.0, 150mMNaCl, 0.1% SDS, 0.5% Na-Deoxycholate, 1%IGEPA L CA-630) supplemented with PhosSTOP phosphatase inhibitors and cOmplete protease inhibitor cocktail (both Roche). BCA protein assay (Thermofisher Scientific) was used for protein quantification. Samples were loaded on 4–15% precast polyacrylamide gels (Bio-Rad) and transferred to PVDF (Bio-Rad) membranes in Towbin buffer. Membranes were blocked in 5% bovine serum albumin (Applichem) and incubated overnight in primary antibodies phospho–p65 (1:1000; CST#3033), Caspase 8 (1:1000; CST#8592), FLIP (1:100, CST#563343) and Gadh (1:3000; CST#14C10). IRDye800CW and 680RD donkey anti-rabbit secondary antibodies were used for detection at a 1:10,000 dilution (LI-COR). Protein bands were visualized with the ODYSSEY CLx imaging system (LI-COR).

**Genomic DNA isolation and next-generation sequencing.** Genomic DNA from murine KPC cells was extracted using direct cell lysis buffer: 10 µl of 4× lysis buffer overnight in primary antibodies phospho–p65 (1:1000; CST#3033), Caspase 8 (1:1000; CST#8592), FLIP (1:100, CST#563343) and Gadh (1:3000; CST#14C10). IRDye800CW and 680RD donkey anti-rabbit secondary antibodies were used for detection at a 1:10,000 dilution (LI-COR). Protein bands were visualized with the ODYSSEY CLx imaging system (LI-COR).

**Primers used to amplify target loci.** Rnf31-1: 5’ GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNTTT 3’
Rnf31-2: 5’ GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNC 3’
Rnf31-3: 5’ CTCTGGTCTACAGCAGGACGGC 3’
Rnf31-4: 5’ GTGGTCTACAGCAGGACGGC 3’
Rnf31-5: 5’ CTCTGGTCTACAGCAGGACGGC 3’
Rnf31-6: 5’ GTGGTCTACAGCAGGACGGC 3’
Rnf31-7: 5’ CTCTGGTCTACAGCAGGACGGC 3’
Rnf31-8: 5’ GTGGTCTACAGCAGGACGGC 3’
Rnf31-9: 5’ CTCTGGTCTACAGCAGGACGGC 3’
Rnf31-10: 5’ GTGGTCTACAGCAGGACGGC 3’.

**Human expression data.** Human expression data were retrieved from CTGA and GTEx data bases and visualized using the online platform GEPIA2 (http://gepia2.cancer-pku.cn/#index).

**Data availability**
RNA-seq data and all CRISPR screening data have been made accessible via GEO: GSE180834. Mouse genome GRCm38 was used as reference. Human patient data in Supplementary Fig. 2b can be accessed via GEPIA2 [http://gepia2.cancer-pku.cn/#index]. The remaining data are available within the Article, Supplementary Information or Source Data file. Source data are provided with this paper.

Received: 19 July 2021; Accepted: 10 March 2022; Published online: 04 April 2022

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Acknowledgements

We thank the Flow Cytometry Facilities of the University of Zurich and the ETH Zurich. Also, we thank the Functional Genomics Center Zurich and the ETH Phenomics Center for their support and infrastructure. We also thank Chantal Pauli and Daniela Lenggenhager as well as the University Hospital Zurich Organoid Biobank for their help in providing patient samples. This work was supported by the Swiss National Science Foundation grant 310030_185293 (G.S.), the Swiss National Science Foundation grant 310030B_182829 (M.K.), ETH PhD Fellowship (N.F.), PHRT iDoc Fellowship PHRT_324 (K.M.), and EMBO Long Term 499 Fellowship ALTF 873-2019 (S.J.).

Author contributions

N.F. and G.S. conceptualized the study. N.F. performed experiments, analyzed the data, and wrote the manuscript. L.T. designed, supervised, and helped analyzing flow cytometry experiments and gave valuable input throughout the course of the project. S.J. helped analyzing RNA-Seq data. D.E., K.F.M., T.R., and F.A. performed experiments. N.F. and G.S. wrote the manuscript, L.T. and M.K. reviewed and edited the manuscript. G.S. supervised the study. G.S. and M.K. acquired funding. All authors approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-29412-3.

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Peer review information Nature Communications thanks Thomas Mace, Shashank Patel and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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