

DOUTORAMENTO

PATOLOGIA E GENÉTICA MOLECULAR

ExoCanCell: The impact of exosomes biogenesis impairment in pancreatic cancer progression and therapy response

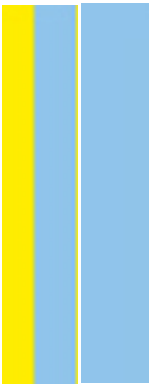
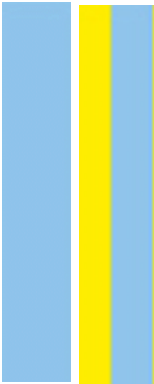
Nuno Bastos

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Nuno Ricardo Alves Bastos



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EXOCANCELL: THE IMPACT OF EXOSOMES BIOGENESIS IMPAIRMENT IN PANCREATIC CANCER PROGRESSION AND THERAPY RESPONSE

Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular; Programa Doutoral do Instituto Ciências Biomédicas Abel Salazar da Universidade do Porto

Orientadora - Professora Doutora Sónia Melo, PhD

Categoria – Investigadora Principal; Professora Afiliada; Afiliação – i3S - Instituto de Investigação e Inovação em Saúde; FMUP - Faculdade Medicina da Universidade do Porto; P.CCC Porto Comprehensive Cancer Center Raquel Seruca, Portugal.

Co-orientador – Professor Doutor Raghu Kalluri, MD, PhD

Categoria – Professor; Afiliação – Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA.

*Started from the bottom,
Now we are here.*

Drake

PREFACE

The work presented in this thesis was performed at the Intercellular Communication and Cancer group, Institute for Research and Innovation in Health (i3S), and Department of Cancer Biology, University of Texas MD Anderson Cancer Center (MDACC), under the supervision of Professor Sónia Melo and co-supervision of Professor Raghu Kalluri.



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DECLARAÇÃO

"Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico".

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ORIGINAL PUBLICATIONS

Original research article

1. **Bastos N**, Castaldo SA, Adem B, Machado JC, Melo CA, Melo SA

SMC3 epigenetic silencing regulates Rab27a expression and drives pancreatic cancer progression. Journal of Translational Medicine, 2023 DOI: 10.1186/s12967-023-04448-1

Complementary publications related to the thesis

Research articles

1. Ruivo CF*, **Bastos N***, Adem B, Batista IA, Duraes C, Melo CA, Castaldo SA, Campos-Laborie FJ, Moutinho-Ribeiro P, Morão B, Costa-Pinto AR, Soraia S, Osorio H, Ciordia S, Costa JL, Goodrich DW, Cavadas B, Pereira L, Kouzarides T, Macedo G, Maio R, Carneiro F, Cravo M, Kalluri R, Machado JC, Melo AS. Extracellular Vesicles from Pancreatic Cancer Stem Cells Lead an Intratumor Communication Network (EVNet) to Fuel Tumor Progression. Gut, 2022 DOI: 10.1136/gutjnl-2021-324994

*These authors contributed equally to this work.

2. Adem B, **Bastos N**, Ruivo CF, Vieira P, Seidler B, Machado JC, Dieter S, Dawen C, Melo SA. ExoBow: A transgenic strategy to study CD63 exosomes in vivo. Biorxiv, 2021. DOI: 10.1101/2021.03.10.434407

Review articles

1. **Bastos N**, Ruivo CF, da Silva S, Melo SA. Exosomes in cancer: Use them or target them? Seminars Cell Developmental Biology, 2017. DOI: 10.1016/j.semcdb.2017.08.00

Book chapter

1. **Bastos N**, Melo SA. Quantitative Analysis of Precursors MicroRNAs and Their Respective Mature MicroRNAs in Cancer Exosomes Overtime. Methods in Molecular Biology, 2018. DOI: 10.1007/978-1-4939-7601-0_11.

SUMMARY

Patients diagnosed with inoperable pancreatic ductal adenocarcinoma (PDAC) typically face an average survival of 11 months. The standard of care falls short in extending patient lifespans, underscoring the need for novel therapeutic approaches. While immunotherapy holds transformative promise in cancer care, its impact on PDAC is limited. New tactics that sensitize PDAC to immunomodulation are crucial for improving patient outcomes.

Extracellular vesicles (EVs) secreted by cancer cells (cEVs) carry molecular cargo capable of modulating the immune response. This thesis focuses on exploring intercellular communication facilitated by EVs as a mean to uncover strategies for bolstering the immune reaction against PDAC. We have studied whether inhibition of communication mediated by cEVs could sensitize PDAC tumors to immunomodulatory therapies.

We developed a genetically engineered mouse model (GEMM) that allows cEVs secretion impairment via Rab27a knockout (KO) in a PDAC context. Rab27a is essential for cEVs exocytosis. Interestingly, Rab27a KO mice displayed earlier disease onset and reduced overall survival, particularly in an immunocompetent background, indicating immune system involvement in the observed phenotype. Detailed tumor analysis revealed dysregulated immune responses and inflammation, driven by MRP8⁺ myeloid cells and Th17 T cells. Mechanistically, we found that MRP8 prompts cancer associated fibroblasts to secrete pro-inflammatory cytokines, driving CD4⁺ T cells toward a pro-tumorigenic Th17 phenotype. We also noted that loss of TSP1⁺ cEVs is critical to these changes by mediating CD11b⁺MRP8⁺ myeloid cells recruitment. Anti-inflammatory therapy and CD4⁺ T cell depletion mitigated aggressive Rab27a KO tumor traits, with no impact on Rab27a wild-type tumors. In human PDAC, Rab27a expression loss was associated with disease progression, and 24% of PDAC patients lacked Rab27a expression. Notably, patients with low Rab27a and high MRP8⁺ cell infiltration faced poorer prognosis. Molecularly, we identified an epigenetic mechanism mediated by SMC3, a cohesin complex component, that regulates Rab27a expression in PDAC. SMC3 methylation disrupted chromatin folding, impairing Rab27a cis-regulation. This prevented the interaction of an enhancer within the Rab27a gene with its promoter, inhibiting gene expression.

The work presented in this thesis represents a leap forward by unveiling the tumor suppressive role of cEVs in PDAC and deciphering the underlying mechanism. Importantly, we identified a potential therapeutic avenue for a subset of PDAC patients, involving immune response modulation. This discovery paves the way for the clinical testing of our findings and their potential to enhance patient outcomes.

SUMMARY (PORTUGUESE)

Pacientes diagnosticados com adenocarcinoma ductal do pâncreas (ADP) irressuscitável apresentam uma sobrevida média de apenas 11 meses. As terapias usadas na clínica falham na capacidade de estender a sobrevida destes pacientes, reforçando a necessidade de identificar novas terapêuticas. Recentemente, a imunoterapia emergiu como uma das mais promissoras e eficazes terapêuticas no tratamento do cancro, mas a sua eficácia no contexto do ADP ainda é bastante limitada. O desenvolvimento de estratégias que sensibilizem o ADP a terapêuticas como a imunoterapia tem o potencial de melhorar consideravelmente a sobrevida destes pacientes. Vesículas extracelulares secretadas por células cancerígenas (cVEs) são portadoras de conteúdo como ácidos desoxirribonucleicos, ácidos ribonucleicos, proteínas e metabolitos com a capacidade de modular a resposta imune. Esta dissertação foca-se em explorar a comunicação intercelular mediada por cVEs como um mecanismo de potenciar a resposta do sistema imune no contexto do ADP. Mais especificamente, investigar se a inibição da comunicação intercelular mediada por cVEs potencia a resposta a terapias imuno-modulatórias.

No âmbito desta tese desenvolvemos um modelo animal geneticamente modificado que nos permite inibir a secreção de cVEs, via o knockout (KO) do gene Rab27a, no contexto do ADP. Previamente, a proteína Rab27a foi identificada como fundamental na exocitose de cVEs. Na análise deste novo modelo verificamos que murganhos Rab27a KO apresentam uma progressão mais célere da doença bem como uma redução na sobrevida, especificamente num contexto imunocompetente. Estas alterações são potenciadas por uma desregulação na resposta imune bem como num processo inflamatório, mediado por células mielóides MRP8⁺ e células T Th17. Do ponto de vista mecanístico, verificamos que a proteína MRP8 libertada por células mielóides promove a secreção de citocinas pro-inflamatórias por parte de fibroblastos associados ao cancro, que por sua vez medeiam a diferenciação de células T CD4⁺ para o subtipo Th17, caracterizado como pro-tumorigénico no ADP. Adicionalmente, identificamos a proteína TSP1 presente em cVEs como mediadora destes processos, nomeadamente o recrutamento das células mielóides MRP8⁺. O uso de anti-inflamatórios e a depleção de células T CD4⁺ inibe a progressão tumoral em murganhos Rab27a KO mas não apresenta qualquer efeito em murganhos wild-type, demonstrando a especificidade do mecanismo.

Em amostras humanas de ADP verificamos uma perda de expressão de Rab27a ao longo da progressão tumoral, culminando em 24% dos pacientes não apresentarem expressão de Rab27a no tecido tumoral. Em adição, verificamos que pacientes com baixa expressão

de Rab27a e elevada infiltração de células MRP8⁺ no tumor apresentam um pior prognóstico.

Do ponto de vista molecular, identificamos um mecanismo epigenético mediado por SMC3, um componente do complexo coesina, que regula a expressão de Rab27a em ADP. Metilação do gene SMC3 promove uma alteração na estrutura da cromatina que interfere com a regulação em cis do gene Rab27a. Especificamente, esta alteração interfere com a interação entre a região promotora e o acentuassomo presente no gene Rab27a, inibindo a sua expressão.

O trabalho contemplado nesta tese representa um avanço significativo na área visto que se trata da primeira descrição do papel supressor tumoral de cVEs em ADP. Adicionalmente, a descoberta de um subgrupo de pacientes com ADP que podem beneficiar de terapêuticas dirigidas à resposta imune abre a porta ao teste na clínica das nossas observações.

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LIST OF ABBREVIATIONS

α -SMA	Alpha-smooth muscle actin
AAV	Adeno-associated virus
ADM	Acinar-to-ductal metaplasias
AKT	Protein kinase B
APC	Antigen-presenting cells
apCAFs	Antigen-presenting cancer-associated fibroblasts
ARF6	ADP-ribosylation factor 6
ATG	Autophagy-related
BRCA	Breast cancer gene
CAFs	Cancer-associated fibroblasts
CAR T-cell	Chimeric antigen receptor T-cell
CDNK2A	Cyclin Dependent Kinase Inhibitor 2A
cEVs	Cancer extracellular vesicles
ChIP	Chromatin immunoprecipitation
CLEC7A	C-type lectin domain family 7 member A
CT	Computerized tomography
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
DCK	Deoxycytidine Kinase
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DNMIVD	DNA methylation interactive visualization database
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition

ERK	Extracellular signal-regulated kinase
ERT2	Estrogen receptor
ESCRT	Endosomal sorting complexes required for transport
EVs	Extracellular vesicles
FACS	Fluorescent activated cell sorting
FAP	Fibroblast activation protein alpha
FDR	False discovery rate
FGFR	Fibroblast growth factor receptor
FLP	Flippase
FSC	Forward scatter
GEF	Guanine nucleotide exchange factor
GEMM	Genetically engineered mouse model
GEPIA	Gene expression profiling interactive analysis
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSEA	Gene set enrichment analysis
HE	Hematoxylin and eosin
HEP	Humane endpoint
iCAFs	Inflammatory cancer-associated fibroblasts
ICAM-1	Intercellular adhesion molecule 1
IF	Immunofluorescence
IHC	Immunohistochemistry
IL	Interleukin
ILVs	Intraluminal vesicles
INK4A	Inhibitors of CDK4 A
IVIS	In vivo imaging system

IPMN	Intraductal papillary mucinous neoplasm
KO	Knockout
KPC	LSL-Kras ^{G12D/+} ; LSL-Tp53 ^{R172H/+} ; Pdx1-Cre
KPC iRab27a	LSL-Kras ^{G12D/+} ; LSL-Tp53 ^{R172H/+} ; Pdx1-Cre; R26 ^{LSL-FLPoERT2/+} ; Rab27a ^{Frt/Frt}
KPC Rab27a	LSL-Kras ^{G12D/+} ; LSL-Tp53 ^{R172H/+} ; Pdx1-Cre; Rab27a ^{Frt/Frt}
KPF CD63-mCherry	FSF-Kras ^{G12D/+} ; Tp53 ^{Frt/+} ; Pdx1-Flp; R26 ^{CD63-XFP/+}
KRAS	Kirsten rat sarcoma virus gene
LC/ESI–MS/MS	Liquid chromatography-electrospray ionization tandem mass spectrometry
LFQ	Label-free quantification
LKB1	Liver kinase B1
LSL	Lox-stop-lox
LMP1	Latent membrane protein 1
MAPK	Mitogen-activated protein kinase
MCN	Mucinous cystic neoplasm
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MiRNAS	MicroRNAs
MLH1	Human mutL homolog 1
MMP	Matrix metalloproteinase
mPFS	Median progression-free survival
MPO	Myeloperoxidase
mOS	Median overall survival
MRI	Magnetic resonance imaging
MRP8	Migration inhibitory factor- (MIF)-related protein-8
MSI-H	Microsatellite instability-high

MUC-1	Mucin 1
MVBs	Multiple vesicular bodies
myCAFs	Myofibroblastic cancer-associated fibroblasts
NTA	Nanoparticle tracking analysis
NTE	Next to enhancer
nV	Non-vesicular
PanIN	Pancreatic intraepithelial neoplasia
PARP	Poly adenosine diphosphate (ADP)-ribose polymerase
PC	Pancreatic cancer
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PD-L2	Programmed cell death ligand 2
PDAC	Pancreatic ductal adenocarcinoma
PDX	Patient derived xenograft
PDX-1	Pancreatic and duodenal homeobox 1
PI3K	Phosphatidylinositol 3-kinase
PKT	Ptf1a-Cre; LSL-Kras ^{G12D/+} ; Tgfbr2 ^{loxP/loxP}
PKT iRab27a	Ptf1a-Cre; LSL-Kras ^{G12D/+} ; Tgfbr2 ^{loxP/loxP} ; R26 ^{LSL-FLPoERT2/+} ; Rab27a ^{Frt/Frt}
PKT Rab27a	Ptf1a-Cre; LSL-Kras ^{G12D/+} ; Tgfbr2 ^{loxP/loxP} ; Rab27a ^{Frt/Frt}
Pdx1 Rab27aKO	Pdx1-Flp; Rab27a ^{Frt/Frt}
PRSS1	Protease serine 1
PS	Ponceau S
Ptf1a iRab27a	Ptf1a-Cre; R26 ^{LSL-FLPoERT2/+} ; Rab27a ^{Frt/Frt}
RAF	Rapidly accelerated fibrosarcoma
RNA	Ribonucleic acid
ROCK1	Rho-associated protein kinase 1

ROS	Reactive oxygen species
SCF	Stem cell factor
SCR	Scramble
SEC	Size exclusion chromatography
SEM	Standard error of the mean
Sg	Small guided
Sh	Short hairpin
SHH	Sonic hedgehog
SMAD4	SMAD Family Member 4
SMC3	Structural maintenance of chromosomes 3
SRC	Proto-oncogene c-Src
SSC	Side scatter
STAT	Signal Transducer And Activator Of Transcription
TAM	Tamoxifen
TAMs	Tumor-associated macrophages
TCGA	Cancer Genome Atlas Program
TCR	T-cell receptor complex
TGF- β	Transforming growth factor beta
TIL	Tumor-infiltrating lymphocytes
TIMP	Tissue inhibitor of metalloproteinase
TLR4	Toll-like receptor 4
TNF- α	Tumor necrosis factor alpha
TP53	Tumor protein p53
TSAP6	Tumor suppressor-activated pathway 6
TSG101	Tumor susceptibility 101
TSP1	Thrombospondin-1

VEGF	Vascular endothelial growth factor
Zeb1	Zinc finger e-box binding homeobox 1
WT	Wild type
WT-1	Wilms' tumor gene 1
YAP	Yes-associated protein

PART I - INTRODUCTION

1. Pancreatic Cancer

Pancreatic cancer is a malignant tumor that originates in the cells of the pancreas, a glandular organ located in the abdomen behind the stomach. It is a highly aggressive and lethal form of cancer, often diagnosed at advanced stages when treatment options are limited. Understanding the risk factors, clinical presentation, diagnosis, pathophysiology and treatment are crucial to guide future research in pancreatic cancer, ultimately to advance the basic understanding of this deadly disease and improve patient care.

1.1 Epidemiology

Pancreatic cancer represents the 14th leading cause of new cancer cases in the world and is more incident in men (Sung, Ferlay et al. 2021). Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer (90%) (Klein 2021). Despite its low incidence, PDAC is one of the leading causes of cancer-related deaths, with almost as many deaths as new diagnosis per year (Sung, Ferlay et al. 2021). On average, PDAC patients at diagnosis present a median survival time of 10-12 months (Principe, Underwood et al. 2021).

Incidence and mortality of PDAC is mostly associated with the increasing age in world's population (Rawla, Sunkara et al. 2019). PDAC is a disease that is rarely diagnosed before the age of 40 and its risk increases with age (Klein 2021). In addition, the risk of death also increases dramatically with age being below 2 deaths per 100,000 person-years in PDAC patients aged between 35–39 years, to over 90 deaths per 100,000 person-years for patients aged over 80 years (Klein 2021).

Despite PDAC tumorigenesis being a highly complex process and dependent on several factors, key risk factors associated with this disease have been identified. Cigarette smoking, obesity, alcohol consumption, metabolic syndrome, diabetes and pancreatitis (both acute and chronic inflammation of the pancreas) are some of the risk factors already described (Becker, Hernandez et al. 2014, Rawla, Sunkara et al. 2019, Alhobayb, Peravali et al. 2021, Klein 2021, Gandhi, de la Fuente et al. 2022, Zhong, Liu et al. 2023). Inherited DNA variants can also constitute an increased risk to develop PDAC, representing

approximately 10% of all cases (Wood, Canto et al. 2022). Behavioral changes and cancer screening programs in high-risk groups could be key to improve PDAC patients' outcome.

1.2 Clinical presentation and diagnosis

PDAC is well-known for its silent progression, with symptoms often appearing at advanced stages. This leads to 90% of PDAC patients being diagnosed at a late stage in which over 50% present metastases (Kommalapati, Tella et al. 2018, Siegel, Miller et al. 2021).

Thus far, pancreatic lesions are detected by imaging techniques such as endoscopic ultrasound, CT scan or MRI. Diagnosis of PDAC relies on tumor biopsies to confirm and stage the cancer lesion accurately. Efforts have been made to develop new diagnostic tools that would allow early detection of PDAC. CA 19-9 blood levels has been the most commonly used non-invasive biomarker to signal the presence of a pancreatic cancer lesion and to manage the progression of the disease during treatment (Poruk, Gay et al. 2013). However, CA 19-9 does not present the sensitivity and specificity necessary to screen healthy individuals (Homma and Tsuchiya 1991, Kim, Lee et al. 2004). Therefore, the identification of better biomarkers has been the focus of extensive research in recent years (Sturm, Ettrich et al. 2022). Despite exciting results in the fields of circulating microRNAs, DNA and exosomes, these biomarkers still lack validation in larger cohorts of PDAC patients to represent valid options in the clinic (Melo, Luecke et al. 2015, Moutinho-Ribeiro, Adem et al. 2022, Moutinho-Ribeiro, Batista et al. 2022, Sturm, Ettrich et al. 2022). A better understanding of PDAC initiation and development could allow the identification of novel molecular markers that specifically diagnose patients at earlier stages of cancer development.

1.3 Pathophysiology

PDAC arises from the exocrine compartment of the pancreas, which is composed of acinar and ductal cells (Rawla, Sunkara et al. 2019). Despite its ductal morphology, there is no clear evidence of what is the cell of origin of PDAC (Malinova, Veghini et al. 2021).

Pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN), and mucinous cystic neoplasm (MCN) are the three different precursor lesions from which PDAC develops (Yonezawa, Higashi et al. 2008). The most prevalent type of PDAC

precursor lesions are PanINs (Singh and Maitra 2007). These lesions have morphologically altered ductal cells with escalating degrees of dysplastic growth which ranges from 1-3 (Singh and Maitra 2007). PanINs are non-invasive and do not penetrate the basement membrane (Singh and Maitra 2007). In a similar fashion, IPMN grow in the main or accessory pancreatic ducts and produce mucin (Machado, al Qadhi et al. 2015). Lastly, MCN are epithelial neoplasms that produce mucin and are usually composed of cystic masses rich in mucus and/or hemorrhagic material (Singh and Maitra 2007). Opposite to PanIN and IPMN, MCN are hardly ever related to pancreatic ducts (Singh and Maitra 2007).

Genetic alterations are key to the development of PDAC precursor lesions and, ultimately, progression to a malignant tumor. Activating *KRAS* mutations are the most prevalent alteration in PDAC, accounting for 92% of cases (Bailey, Chang et al. 2016). These mutations are the driving force of PDAC precursor lesions development (Grant, Hua et al. 2016). Oncogenic *KRAS* presents a constantly active form driving cancer initiation through activation of several cancer-related pathways such as PI3K/AKT, Ral-GEF and RAF/ERK (Eser, Schnieke et al. 2014). In addition, mutations that lead to inactivation of the tumor suppressor genes *CDKN2A*, *TP53*, and *SMAD4* are also commonly present in PDAC (Hezel, Kimmelman et al. 2006). These mutations succeed the appearance of *KRAS* mutations and are crucial for the progression of precursor lesions and, ultimately, PDAC development since *KRAS* mutations on their own are not sufficient to form a tumor (Aguirre, Bardeesy et al. 2003, Hingorani, Wang et al. 2005, Bardeesy, Cheng et al. 2006). As mentioned before, germline mutations can also confer an increased predisposition for PDAC development including those present in the tumor suppressor genes *BRCA1/2*, *LKB1*, *MLH1*, *PRSS1* and *INK4A* (Hezel, Kimmelman et al. 2006). However, these alterations are not sufficient to induce cancer initiation on its own but rather promote the progression of precursor lesions.

Several other mutations are also described to be present in PDAC which contribute to inter-tumor heterogeneity and reinforces the complexity of its biology (Hezel, Kimmelman et al. 2006, Grant, Hua et al. 2016). With this in mind, recent efforts have been made to stratify PDAC tumors based on their transcriptomic profiles to further understand the molecular mechanisms in play during PDAC progression and to, potentially, identify novel therapeutic avenues. Thus far, three main PDAC subtypes have been identified: the squamous/basal-like tumors (also described as quasimesenchymal), exocrine-like tumors and classical tumors (Collisson, Bailey et al. 2019). Basal-like tumors surge with the most aggressive phenotype, characterized by expression of genes associated with a mesenchymal phenotype, leading to a worst prognosis (Collisson, Sadanandam et al. 2011, Moffitt, Marayati et al. 2015, Bailey, Chang et al. 2016). Classical tumors are associated with the

expression of epithelial and adhesion genes (Collisson, Bailey et al. 2019). Exocrine-like tumors are mostly associated with the expression of digestive enzymes (Collisson, Bailey et al. 2019). Interestingly, some reports also suggest a fourth subtype described as immunogenic, due to an enrichment in immune-related pathways (Bailey, Chang et al. 2016). This indicates that components of the tumor microenvironment can be crucial to stratify PDAC tumors and, potentially, determine patients' outcome.

1.4 Tumor microenvironment

Pancreatic ductal adenocarcinoma presents a highly desmoplastic microenvironment enriched in cancer-associated fibroblasts and low immune cells infiltration (Dougan 2017) (Figure 1). Histologically, PDAC is characterized by the presence of a minority of cancer cells surrounded by a dense stromal compartment (Ullman, Burchard et al. 2022).

In fact, desmoplasia represents a hallmark of PDAC. Deposition of extracellular matrix (ECM), which consists of collagen, glycosaminoglycan, and others, has been demonstrated to inhibit vascularization, hinder immune cells infiltration, and impair therapeutic drugs perfusion into the tumor milieu (Murakami, Hiroshima et al. 2019). In addition, the presence of ECM is described to promote cancer cell survival, growth and invasiveness through interactions with receptors present in cancer cells' membrane (Murakami, Hiroshima et al. 2019). Nonetheless, it is important to mention that not all ECM components present a tumor-promoting function. Recent findings have demonstrated that stromal components can act to restrain, rather than support, PDAC (Özdemir, Pentcheva-Hoang et al. 2014, Rhim, Oberstein et al. 2014, Chen, Kim et al. 2021). Depletion of collagen I, one of major components of PDAC stroma, leads to a remodeling of the immune cell response and drives PDAC progression in preclinical models (Chen, Kim et al. 2021).

Cancer-associated fibroblasts (CAFs) are critical for ECM deposition (Chen, McAndrews et al. 2021). In a similar fashion to ECM components, CAFs also have a contradictory role in PDAC (Chen, McAndrews et al. 2021). Early research demonstrated that CAFs present a tumor-promoting function supporting cancer cells proliferation and invasion as well as modulating an immunosuppressive microenvironment (Kalluri 2016, Vennin, Méléneć et al. 2019, Francescone, Barbosa Vendramini-Costa et al. 2021, Hutton, Heider et al. 2021). However, CAFs have been recently described as a highly heterogenous population, which ultimately dictates their function (Chen, McAndrews et al. 2021). In part, this is believed to be due to the multitude of progenitor cells that can give rise to this population (Chen, McAndrews et al. 2021, Masugi 2022). Three major CAFs subpopulations have been

described in PDAC: myfibroblastic CAFs (myCAFs), inflammatory CAFs (iCAFs) and antigen-presenting CAFs (apCAFs). The myCAFs subpopulation is mainly associated with production of ECM and is usually located in proximity to cancer cells (Öhlund, Handly-Santana et al. 2017). On the other hand, iCAFs are usually present in the periphery of the tumor and are characterized by the expression of inflammatory cytokines (Öhlund, Handly-Santana et al. 2017). The apCAFs subpopulation is a less prevalent population that expresses MHC class II and CD74 which are molecules characteristic of antigen-presenting cells (APC) (Elyada, Bolisetty et al. 2019). Recent studies have demonstrated that myCAFs present a tumor-restraining role whereas iCAFs are associated with a tumor-promoting function mostly through a differential polarization of the immune response (Özdemir, Pentcheva-Hoang et al. 2014, Biffi, Oni et al. 2019, McAndrews, Chen et al. 2022). Interestingly, the ability of CAFs to differentiate from one subtype to another was already described, which increases the degree of complexity of studying the function of CAFs *in vivo* (Öhlund, Handly-Santana et al. 2017). Further studies will be needed to identify/validate the role of the subpopulations of CAFs described thus far, as well as how CAFs subpopulations plasticity occurs in PDAC.

PDAC is mainly regarded as a “non-immunogenic” cancer type. The presence of an extensive ECM and CAFs surrounding cancer lesions creates a “shield-like” structure that inhibits overall infiltration by immune cells (Murakami, Hiroshima et al. 2019). In addition, PDAC cells have the ability to suppress the immune response through secretion of chemokines (such as stromal cell-derived factor 1) and cytokines (such as IL-1, IL-6, TGF- β and granulocyte–macrophage colony-stimulating factor, GM-CSF) (Ullman, Burchard et al. 2022).

Within the PDAC microenvironment, the presence of immunosuppressive cells is prevalent from early stages of disease development (Clark, Hingorani et al. 2007). One of the major populations of immune cells present are the myeloid-derived suppressor cells (MDSC). KRAS activation in PDAC cells leads to the production of GM-CSF, which promotes recruitment and expansion of MDSC and disease progression (Bayne, Beatty et al. 2012, Pylayeva-Gupta, Lee et al. 2012). MDSC in PDAC are associated with inhibition of an anti-tumor T cell response (Clark, Hingorani et al. 2007, Bayne, Beatty et al. 2012). MDSC are able to downregulate the T-cell receptor complex (TCR) through arginase-1–dependent consumption and L-cysteine sequestration, and impair TCR function through the production of reactive oxygen and nitrogen species (Ullman, Burchard et al. 2022). Additionally, MDSC are able to inhibit T cells proliferative capacity (Clark, Hingorani et al. 2007).

Tumor-associated macrophages (TAMs) also represent a prevalent population in the PDAC microenvironment (Clark, Hingorani et al. 2007, Zhu, Herndon et al. 2017). TAMs are commonly divided into two main classes: M1 and M2 (Poh and Ernst 2021). M1 macrophages are associated with an anti-tumoral response through secretion of pro-inflammatory cytokines (such as TNF- α , IL-1 β and IL-12) as well as effector molecules (such as reactive nitrogen intermediates) (Poh and Ernst 2021). On the other hand, M2 macrophages present an anti-inflammatory phenotype through secretion of different cytokines, such as TGF- β and IL-10, which supports disease progression (Poh and Ernst 2021). This subtype of TAMs also has the ability to hinder T cells activity in an arginase-1 dependent mechanism in a similar fashion to MDSC, and promote T cells apoptosis through expression of PD-L1 and Dectin-1/galectin-9 (Ullman, Burchard et al. 2022). In its majority, PDAC is infiltrated by M2 macrophages which further supports an immunosuppressive microenvironment depleted of effector T cells (Kurahara, Shintchi et al. 2011, Muller, Haghnejad et al. 2022).

Despite being a scarcer population, T cells are also able to infiltrate the PDAC microenvironment. However, the subsets of T cell present (Th2, Tregs and Th17) are mostly associated with tumor-promoting functions (Wachsmann, Pop et al. 2012, Ullman, Burchard et al. 2022). Th2 T cells are described as having an anti-inflammatory function through secretion of IL-13 and IL-4. This T cell subset induces polarization and proliferation of M2 macrophages further supporting an anti-inflammatory microenvironment (Foucher, Ghigo et al. 2018). In addition, a Th2 response inhibits a Th1 cell polarization, which has a defined anti-tumoral function (Protti and De Monte 2013). On the other hand, Tregs function is mainly associated with impairment of an effective anti-tumoral dendritic cells (DCs) and CD8⁺ T cells response (Wachsmann, Pop et al. 2012). In PDAC, Tregs infiltrate increases with disease progression and is associated with worst prognosis. Regarding Th17 T cells, its role in PDAC is not so well defined. However, recent research has demonstrated the important role of Th17 and IL-17 in the development and progression of PanINs as well as in CD8⁺ T cells exclusion from the tumor microenvironment (McAllister, Bailey et al. 2014, Zhang, Chandra et al. 2020).

Finally, CD8⁺ T cells, or effector cells, comprise only a small percentage of the overall immune cell infiltrate (below 7%) in the PDAC microenvironment and usually present deficient function due to the expression of co-inhibitory molecules (Stromnes, Hulbert et al. 2017). Nonetheless, the presence of CD8⁺ T cells can have good prognostic value in specific PDAC contexts (Balachandran, Łuksza et al. 2017).

Altogether, this indicates that tumor microenvironment components present specific roles that can be context dependent. Further research is critical to better understand the dynamics of the PDAC microenvironment throughout disease progression. Dissecting the dynamic behavior of PDAC TME could allow the development of therapeutic strategies specific for certain tumor landscapes and disease contexts.

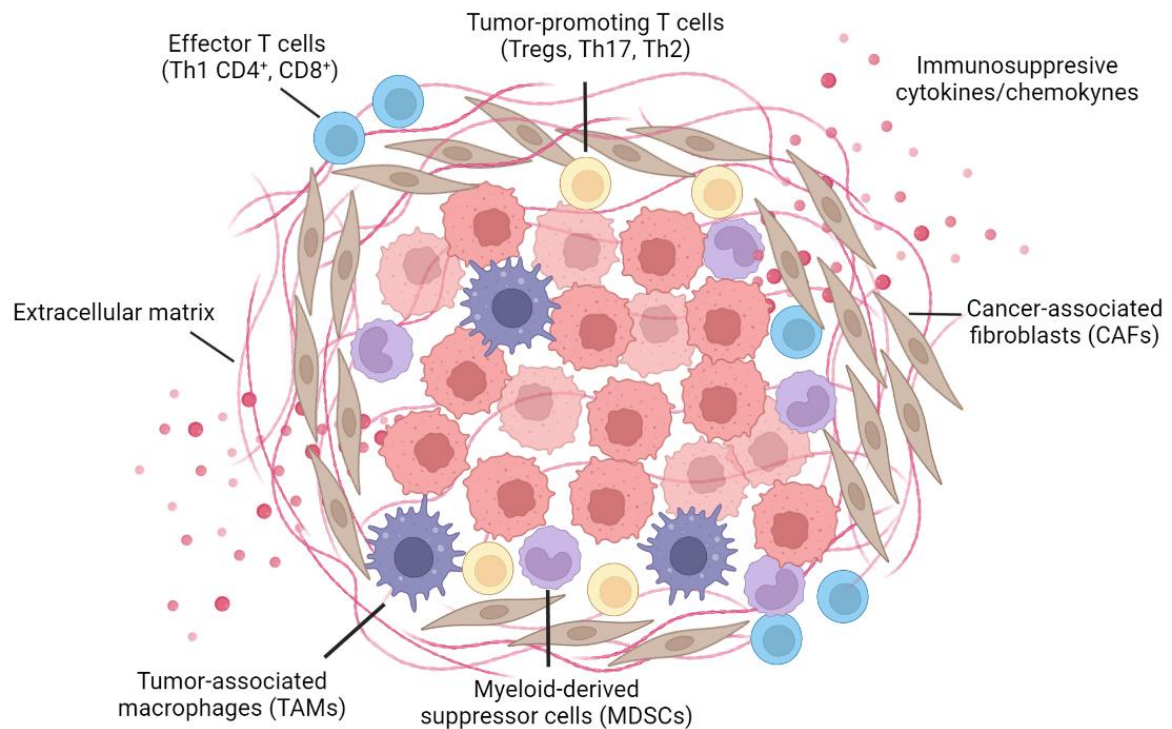


Figure 1 – PDAC microenvironment. The tumor microenvironment of PDAC is mainly characterized by the presence of a dense extracellular matrix and cancer-associated fibroblasts (CAFs). Effector T cells are excluded from the tumor milieu and their activity is compromised by the presence of immunosuppressive cytokines and chemokines released by cancer cells, myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) and tumor-promoting T cells (Tregs, Th17 and Th2). Adapted from (Timmer, Geboers et al. 2021).

1.5 Treatment

Despite major breakthroughs in cancer treatment, treatment of PDAC has not improved significantly. Surgery remains the only curative option but since the majority of PDAC patients are diagnosed at advanced stages of the disease, a great percentage is not suited for this approach (around 85%) (Ansari, Gustafsson et al. 2015). Within eligible patients, the 5-year survival rate after surgical intervention is also only 10% (Neoptolemos, Stocken et al. 2004, Oettle, Neuhaus et al. 2013). The main therapeutic options still rely on chemotherapeutic agents, namely gemcitabine and folfirinox, with median overall survivals of 6.8 and 11.1 months, respectively, in the metastatic disease (Conroy, Desseigne et al. 2011). In familial cases of PDAC, namely the ones that present mutations in the *BRCA* genes, the use of platinum-based chemotherapies presents better results (Principe, Underwood et al. 2021). In addition, due to the defects in DNA damage repair machinery present due to *BRCA* mutations, the use of poly adenosine diphosphate (ADP)-ribose polymerase (PARP) inhibitors has been approved as a maintenance therapy in cases that did not progress on platinum-based chemotherapy (Chi, Chung et al. 2021). PARP is a common pathway to repair DNA alterations which in BRCA-deficient cancer cells represents a highly effective target to promote cell death. However, these cases represent only a small fraction of the total number of PDAC patients, urging the necessity to identify novel therapeutic strategies.

Unfortunately, several targeted approaches in large-scale clinical trials have been mostly unsuccessful (Hosein, Dougan et al. 2022). These efforts have focused on targeting key oncogenic pathways in PDAC and different components of the tumor microenvironment (Ho, Jaffee et al. 2020, Hosein, Dougan et al. 2022). Nonetheless, clinical trials have demonstrated that the use of a personalized and targeted approach is possible and can improve response rates. For instance, in a retrospective analysis of the “Know Your Tumor” registry trial, PDAC patients that received matched therapies, meaning a targeted therapeutic approach based on genetic alterations present, survived longer than the ones that received unmatched therapy (approximately 2.6 years versus 1.3 years) (Pishvaian, Blais et al. 2020). In another trial, the PancSeq program, they demonstrated that the use of whole-exome sequencing of tumor biopsies allowed to guide therapeutic decisions in 24% of the patients (Aguirre, Nowak et al. 2018). However, the small percentage of patients that present mutations for which there are developed targeted therapies hinders the applicability of these approaches in the clinic.

KRAS is the clear driver of about 92% of PDAC tumors. Therefore, it is the most promising therapeutic target. The development of KRAS inhibitors has been demanding due to the KRAS protein structure, which presents no druggable pockets on its surface. However, several small-molecule inhibitors (MRTX849, MRTX1133, RMC-6236, and others) and novel therapeutic approaches (such as the iExosomes) have been developed in recent times with promising results in preclinical models and phase I clinical trials (Kamerkar, LeBleu et al. 2017, Bekaii-Saab, Spira et al. 2022, Gustafson, Wildes et al. 2022, Wang, Allen et al. 2022). Follow-up on the results of these clinical trials will demonstrate if using any of these KRAS-targeted therapies in PDAC is beneficial for patient care.

Due to the high prevalence of stroma in PDAC tissue and its role in tumorigenesis, several attempts have been made to target specific features of the tumor microenvironment (Ho, Jaffee et al. 2020). One of the main focuses has been the ECM. Initial efforts were made in targeting proteins that are able to remodel the ECM, such as matrix metalloproteinases (MMPs). MMPs are a family of proteolytic enzymes that possess the ability to degrade various proteins of the ECM, such as collagen and gelatin, ultimately leading to its remodeling (Quintero-Fabián, Arreola et al. 2019). In PDAC, MMPs are aberrantly expressed and are associated with metastatic disease and worst prognosis (Bramhall, Neoptolemos et al. 1997, Matsuyama, Takao et al. 2002, Jones, Humphreys et al. 2004). However, the use of multi-MMP inhibitors such as marimastat and tanomastat did not improve PDAC patients' outcome (Bramhall, Rosemurgy et al. 2001, Bramhall, Schulz et al. 2002, Moore, Hamm et al. 2003). In a similar fashion, targeting of the sonic hedgehog (SHH) signaling was performed. Studies have demonstrated that SHH signaling promotes desmoplasia through mediation of pancreatic stellate cells differentiation and CAFs invasion (Bailey, Swanson et al. 2008). Additionally, SHH inhibition leads to an increase in neoangiogenesis which ultimately determines drug perfusion and effectiveness (Olive, Jacobetz et al. 2009). However, the use of SHH inhibitors in combination with chemotherapy showed no benefit in clinical trials (De Jesus-Acosta, O'Dwyer et al. 2014, Catenacci, Junttila et al. 2015).

In another type of approach, hyaluronan, which represents one of the main components of the PDAC microenvironment, was targeted. Despite promising results in preclinical models, the use of hyaluronan in combination with different chemotherapeutic drugs presented conflicting results in clinical trials (Jacobetz, Chan et al. 2013, Hingorani, Zheng et al. 2018, Ramanathan, McDonough et al. 2019). Ultimately, indicating that remodeling of the ECM alone is not sufficient to improve chemotherapy efficacy.

Finally, targeting of cells of the tumor microenvironment responsible for ECM deposition, such as CAFs, has also been tested. FAP⁺ fibroblasts were described as possessing a tumor-promoting role in PDAC, however, targeting of FAP presented no benefit in a phase II clinical trial when used in combination with gemcitabine (Nugent, Cunningham et al. 2007, Lo, Wang et al. 2015, McAndrews, Chen et al. 2022).

The complexity and multitude of roles of the different components of the tumor microenvironment could have compromised the success of the clinical trials performed thus far. Strategies that aim to potentiate an anti-tumor immune response have also been the focus of research in recent years despite the prevalent immunosuppressive microenvironment present in PDAC.

2. Immunotherapy

Immunotherapy is a revolutionary approach to cancer treatment that promotes the use of the immune system to recognize, target, and eliminate cancer cells. It represents a paradigm shift in cancer therapy, focusing on enhancing the body's own immune response to fight cancer. Despite its great promise and application in several cancer types, some remain elusive to this therapeutic strategy highlighting the necessity of future research to unlock immunotherapy full potential and application across all types of cancer.

2.1 Types of immunotherapy

The concept of using the immune system to treat cancer dates back to the nineteenth century. William Coley first reported that treating cancer patients with inactivated *S. pyogenes* and *Serratia marcescens* led to a strong immune reaction, ultimately, achieving favorable responses in different cancer types (Decker and Safdar 2009). This finding encouraged extensive research in the fields of immunology and cancer which culminated in the development of several approaches that are able to potentiate an anti-tumor response and improve massively cancer patients' prognosis (Esfahani, Roudaia et al. 2020). Thus far, the most extensive researched and validated categories of immunotherapeutic approaches are: checkpoint inhibitors, which aim to unleash an intra-tumor T cell response; adoptive cell therapies, which are based on the development and systemic delivery of tumor-specific immune cells; and therapeutic vaccines, which aim to potentiate an anti-tumor response (Waldman, Fritz et al. 2020) (Figure 2).

Checkpoint inhibitors

Immune checkpoints are molecules that regulate the immune response (Waldman, Fritz et al. 2020). These checkpoints play a critical role in maintaining immune homeostasis and preventing immune hyperactivation (He and Xu 2020). In the cancer context, cancer cells exploit these checkpoints to evade immune recognition and elimination, ultimately, supporting disease progression (Waldman, Fritz et al. 2020). The use of inhibitors of these checkpoints aims to block these inhibitory signals, thus reactivating the anti-tumoral immune response.

Cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) are the most studied immune checkpoint molecules, namely of T cell response. CTLA-4 regulates T cell activation by inhibiting CD28 receptor function, which is a co-stimulatory molecule, through direct contact, competition for co-stimulatory ligands and recruitment of inhibitory molecules (Intlekofer and Thompson 2013). With this in mind, the use of inhibitory molecules against CTLA-4 was explored to potentiate T cell response in cancer. In preclinical models, the use of neutralizing anti-CTLA-4 antibodies promoted anti-tumoral immunity and hampered disease progression in several cancer types (Leach, Krummel et al. 1996, Yang, Zou et al. 1997, Hurwitz, Yu et al. 1998, Fecci, Ochiai et al. 2007). However, this response is dependent on cancer cells immunogenicity and tumor burden (Yang, Zou et al. 1997, Waldman, Fritz et al. 2020). The use of CTLA-4 inhibitors, such as ipilimumab, led to great improvements in patients' prognosis mainly in melanoma and lung cancer, but also in renal cell carcinoma and prostate cancer (Yang, Hughes et al. 2007, Lynch, Bondarenko et al. 2012, Kwon, Drake et al. 2014, Schadendorf, Hodi et al. 2015).

PD-1 is an immune checkpoint receptor expressed on activated T cells (Waldman, Fritz et al. 2020). Upon interaction with its ligands, programmed cell death ligand 1 and 2 (PD-L1 and PD-L2), a signaling cascade is activated suppressing T cell response (Waldman, Fritz et al. 2020). In physiological conditions, PD-L1 and PD-L2 are commonly expressed by APCs (Waldman, Fritz et al. 2020). However, reports have shown that PD-1 ligands are commonly overexpressed in different cancer types (Ohaegbulam, Assal et al. 2015). In fact, PD-1 ligands overexpression in cancer cell lines constrains CD8⁺ T cell cytotoxic activity leading to an increase in tumor growth and invasiveness (Iwai, Ishida et al. 2002). These findings supported the development of blocking antibodies targeting the PD-1/PD-L axis. Strikingly, this approach has demonstrated to induce durable anti-tumor responses in clinical trials performed in different types of cancer (Okazaki, Chikuma et al. 2013, Zou, Wolchok et al. 2016). This culminated on the approval of three different antibodies (pembrolizumab, nivolumab and atezolimumab) targeting the PD-1/PD-L inhibitory axis for the treatment of advanced melanoma, non-small-cell lung cancer, renal cell carcinoma and others (Sharma, Hu-Lieskovan et al. 2017).

Checkpoint inhibitors have shown remarkable efficacy in various cancer types; however, response rates can vary among patients, and the ones that respond can ultimately develop resistance (Sharma, Hu-Lieskovan et al. 2017). Ongoing research focuses on identifying predictive biomarkers to select patients who are more likely to benefit from checkpoint inhibitor therapy and identifying key molecules that mediate resistance mechanisms (Sharma, Hu-Lieskovan et al. 2017, Vredevoogd, Kuilman et al. 2019, Krijgsman, Kemper et al. 2021).

Adoptive cell therapies

Adoptive cell therapies involve the infusion of autologous or allogenic T cells into cancer patients to elicit an anti-tumor response (Rosenberg and Restifo 2015). In this field, the first approach that was developed was based on the use of tumor-infiltrating lymphocytes (TIL). In this case, lymphocytes are isolated from a tumor and expanded *ex-vivo*, ultimately, to be reinfused intravenously into the same patient. The first successful adoptive transfer was performed in melanoma patients leading to an objective response of 34%. However, the duration of response was short (median time of 4 months) (Rosenberg, Yannelli et al. 1994). Trials using lymphodepletion prior to adoptive T-cell transfer have demonstrated a clear improvement in clinical response, leading to complete disease regression in 22% of melanoma patients (Rosenberg, Yang et al. 2011). The main issue regarding this approach is the necessity of T cells with tumor reactivity being present in the tumor milieu, which does not occur in all cancer types (Waldman, Fritz et al. 2020).

To surpass this hurdle, studies have explored the use of TCR-engineered lymphocytes which led to the development of chimeric antigen receptor T cell (CAR T-cell) therapy. CARs are designed to recognize specific tumor-associated antigens and can bypass antigen presentation by MHC molecules and directly recognize a target molecule in the cell membrane of cancer cells (Waldman, Fritz et al. 2020). This leads to great improvements in therapy efficacy since MHC downregulation is a common feature of cancer cells (Dhatchinamoorthy, Colbert et al. 2021). In CAR T-cell therapy, T cells derived from the patient, or an allogeneic donor can be used. This type of therapy has achieved remarkable success mainly in hematologic malignancies, particularly in the treatment of acute lymphoblastic leukemia, and only moderate success in solid tumors (Kershaw, Westwood et al. 2006, Beatty, Haas et al. 2014, Fry, Shah et al. 2018).

A drawback associated with this approach is the necessity of a tumor-restricted antigen present at the cancer cells' membrane in order to develop an effective CAR T-cell therapy. In addition, loss of antigen expression by cancer cells could lead to therapy resistance (Lemoine, Ruella et al. 2021). Nonetheless, the use of a combination of CAR T-cell therapies targeting several tumor antigens and the development of CAR T-cells proficient in immune stimulatory cytokines secretion to recruit and enhance anti-tumoral T-cells function have been already employed and present promising results (Lemoine, Ruella et al. 2021).

Therapeutic vaccines

Therapeutic vaccines are designed to stimulate or enhance the immune response against cancer-specific antigens (Waldman, Fritz et al. 2020). The development of techniques such as next generation sequencing has allowed to determine the mutational load present in a tumor's DNA and, consequently, identify neoantigens. In theory, a higher mutational burden would lead to a higher number of neoantigens which, in turn, could trigger an effective anti-tumor immune response and determine the response to immune checkpoint inhibitors (Saxena, van der Burg et al. 2021). However, this is not always the case (Maleki Vareki 2018). This highlights that not all neoantigens are immunogenic and represent suitable targets in the development of cancer vaccines. Recently, the use of *in silico* approaches has allowed to predict the immunogenic neoantigens/peptides based on the patient's MHC molecules and, ultimately, develop personalized and effective vaccines (Waldman, Fritz et al. 2020). Different approaches can be used to formulate and deliver cancer vaccines such as synthetic peptides, mRNA and DNA plasmids as well as neoantigen-loaded DCs. Thus far, the optimal method to elicit a strong and durable immune response is not clear (Sahin and Türeci 2018).

The use of this therapeutic approach has had outstanding results in melanoma patients leading to complete responses in the majority of them (Ott, Hu et al. 2017, Sahin, Derhovanessian et al. 2017). However, the development of therapeutic vaccines presents some challenges such as the identification of optimal tumor antigens. Despite major improvements in bioinformatic approaches, identification of immunogenic neoantigens associated with a response mediated by MHC class II molecules is far from optimal (Nielsen, Lund et al. 2010). Cost and time to produce personalized vaccines also represents a major hurdle that needs to be surpassed (Waldman, Fritz et al. 2020). Ongoing research focuses on enhancing the efficacy and broadening the applicability of therapeutic vaccines in cancer treatment.

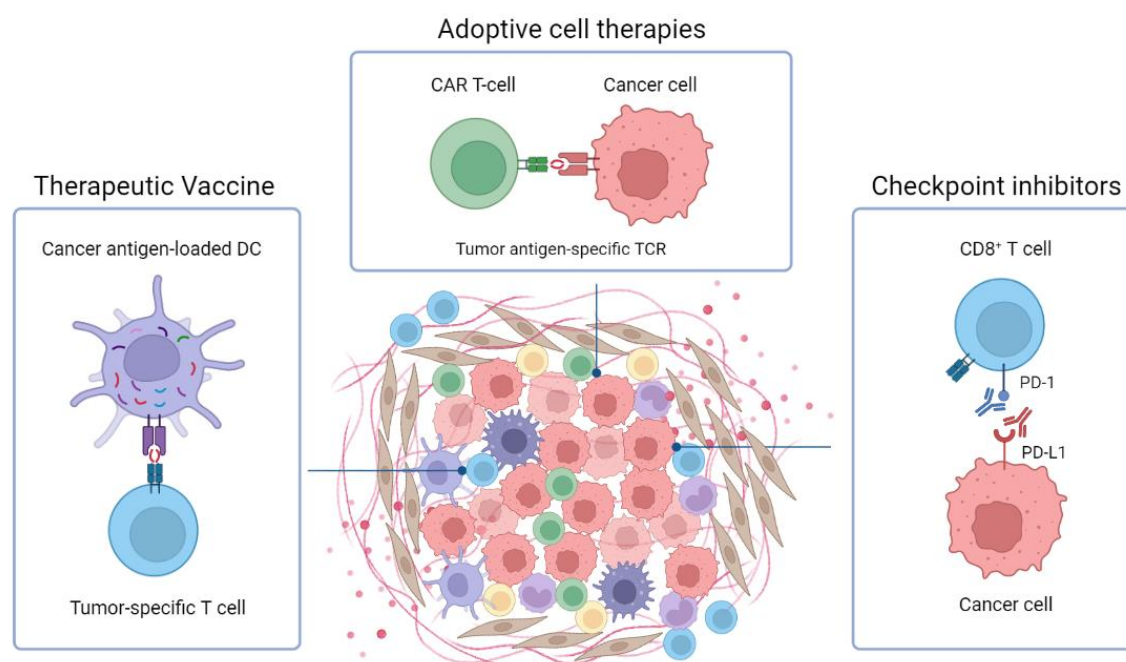


Figure 2 – Types of immunotherapy. The most well validated immunotherapeutic approaches focus on checkpoint inhibitors, which through the use of blocking antibodies against inhibitory molecules of immune response, such as PD-1 and PD-L1, aim to potentiate an intra-tumor T cell response; adoptive cell therapies, which are based on the development and systemic delivery of T cells with a TCR specific to a tumor antigen (CAR T-cell); and therapeutic vaccines, which aim to trigger an anti-tumor immune response through the delivery of immunogenic cancer antigens. Adapted from (Li, Miao et al. 2021).

2.2 Immunotherapy in pancreatic cancer

Despite immunotherapy representing a breakthrough in the treatment of various solid cancers, PDAC remains refractory to this therapeutic approach (Royal, Levy et al. 2010, Mohindra, Kircher et al. 2015, Wainberg, Hochster et al. 2020). Only a small percentage (1-2%) of PDAC patients are eligible for immunotherapy because of their MSI-H status (Marcus, Lemery et al. 2019, Claudio, Lodewijk et al. 2021).

Early efforts focused on the use of checkpoint inhibitors alone or in combination with chemotherapeutic agents for the treatment of PDAC. The potential benefit of using such combination strategy lays on the ability of cytotoxic drugs to induce immunogenic cancer cell death and reduce immune suppressive mechanisms (Galluzzi, Humeau et al. 2020). From the clinical trials performed thus far, the one that presented better results was based on a combination strategy of anti-PD-1 and a gemcitabine/nab-paclitaxel regimen. The use

of this therapeutic approach led to a median progression-free survival (mPFS) of 9.1 months and a median overall survival (mOS) of 15 months in metastatic PDAC patients which represents an improvement in comparison with the mOS outcomes achieved with gemcitabine/nab-paclitaxel (8.5 months) and FOLFIRINOX (11.1 months) (Conroy, Desseigne et al. 2011, Von Hoff, Ervin et al. 2013, Weiss, Blaydorn et al. 2018). However, the use of this type of immunotherapeutic approach does not show consistent outcomes between different cohorts of patients and the use of other types of checkpoint inhibitors, such as anti CTLA-4, does not present as promising results (Aglietta, Barone et al. 2014, Mohindra, Kircher et al. 2015, Weiss, Waypa et al. 2017, Bockorny, Semenisty et al. 2020). Ultimately, this indicates that the use of this therapeutic approach is far from being a reality in the treatment of PDAC patients.

Due to the lack of a clinical response when using checkpoint inhibitors, other types of immunotherapy have been explored in the treatment of PDAC, namely the use of CAR T-cell therapy. Unfortunately, this approach has presented conflicting results (Timmer, Geboers et al. 2021). The use of $\alpha\beta$ T cell therapy in combination with chemotherapy resulted in an mOS of 18.7 months from diagnosis, indicating a survival benefit in comparison to the abovementioned mOS of standard chemotherapeutic options (Kumai, Mizukoshi et al. 2021). Whereas the use of CAR T cells specific for mesothelin, a commonly overexpressed protein in PDAC, and V γ 9V δ 2 T cells, a subset of T cells with a potent anti-tumor response through recognition of phosphoantigens, were unable to improve patients' outcome (Aoki, Matsushita et al. 2017, Beatty, O'Hara et al. 2018). Potentially, this variability in response could be associated with CAR T cells specificity and the ability of these cells to infiltrate and persist in the tumor milieu.

Several clinical trials performed in PDAC have also explored the use of cancer vaccines. The developed vaccines are either focused on a specific neoantigen or a multitude of cancer antigens (Timmer, Geboers et al. 2021). One of the main targets explored has been the mutated KRAS, since it is present in more than 90% of PDAC patients. Studies have demonstrated the feasibility of this approach and presented encouraging results (Wedén, Klemp et al. 2011, Palmer, Valle et al. 2020). The combination of several peptide vaccines targeting the most common KRAS mutations, recombinant GM-CSF and gemcitabine led to an increase of around 10 months of the mOS of PDAC patients with resected primary tumors in comparison with gemcitabine alone in a phase 1/2 clinical trial (Palmer, Valle et al. 2020). GM-CSF is commonly used in combination with peptide vaccines since it promotes DCs activation and antigen presentation (Ushach and Zlotnik 2016). Another major target of cancer vaccines for the treatment of PDAC has been WT-1. WT-1 seems crucial for pancreatic cancer cells growth and invasion, with its overexpression being

present in around 75% of PDAC cases (Oji, Nakamori et al. 2004, Koido, Okamoto et al. 2017). Unfortunately, data from a clinical trial including 85 PDAC patients demonstrated that the use of a WT-1 peptide vaccine in combination with gemcitabine did not improve patients' outcome in comparison with gemcitabine alone (Nishida, Ishikawa et al. 2018). However, the use of a DC-based vaccine pulsed with WT-1/MUC-1 peptides in combination with gemcitabine presented a favorable outcome (mPFS and mOS of 17.7 and 46.4 months, respectively) (Nagai, Adachi et al. 2020). This indicates that a strategy targeting multiple cancer antigens rather than a single one could present a benefit in the immune response generated.

Following this, treatment regimens using GVAX, an approach that uses irradiated allogeneic cancer cells with no growth capacity as a mean to present a multitude of PDAC antigens, have also been explored. Thus far, the use of GVAX alone or in combination with different therapeutic strategies failed to improve prognosis in comparison with gemcitabine (Le, Picozzi et al. 2019, Tsujikawa, Crocenzi et al. 2020). Nonetheless, patients with increased CD8⁺ T cells and reduced TAMs and MDSCs in the tumor microenvironment presented a better outcome upon treatment with GVAX-based therapy (Tsujikawa, Crocenzi et al. 2020). This result points to a potential benefit of using this type of immunotherapy in a specific subset of patients rather than in all PDAC cases.

Finally, personalized cancer vaccines have also been tested in different PDAC clinical trials (Yanagimoto, Shiomi et al. 2010, Qiu, Yun et al. 2013, Mehrotra, Britten et al. 2017). Within these studies, the approach that presented better results was based on alpha-galactosyl (α -Gal) expressing DCs pulsed with tumor lysates in combination with cytokine-induced killer cells (Qiu, Yun et al. 2013). Pre-treated PDAC patients presented a major improvement in mOS from diagnosis (24.7 months) with an effective anti-tumor immune response taking place (Qiu, Yun et al. 2013). However, other personalized approaches failed to produce similar results (Yanagimoto, Shiomi et al. 2010, Mehrotra, Britten et al. 2017).

Besides the frequent late diagnosis of PDAC patients, the lack of effective therapeutic options contributes immensely to the dismal prognosis of this disease. Therefore, the identification and development of novel therapeutic strategies is still a major focus in the pancreatic cancer field. Unfortunately, the success of recent clinical trials, including the ones based on immunotherapy, has been underwhelming. Identification of sensitizing strategies or of subsets of patients eligible for approved effective therapies, could be key to improve therapy efficacy. In addition, the field has been moving towards personalized medicine as an effort to improve patients' outcome.

3. Extracellular Vesicles

Extracellular vesicles (EVs) are small, membrane-bound vesicles that are released by cells into the extracellular space. EVs mediate communication between cells through interaction and transfer of bioactive molecules, including proteins, lipids, and nucleic acids (Bastos, Ruivo et al. 2018). The three types of classical EVs are exosomes, microvesicles and apoptotic bodies (Sheta, Taha et al. 2023). Exosomes are nano-size vesicles of endosomal origin, ranging from 40 to 150 nanometers; microvesicles are larger vesicles released directly from the plasma membrane, typically ranging from 150 to 1000 nanometers in size; and apoptotic bodies present a size from 100 to 5000 nanometers and are generated during apoptosis (Sheta, Taha et al. 2023). These different types of EVs mostly differ in their biogenesis pathways (Figure 3).

3.1 Biogenesis of extracellular vesicles

The endocytic pathway was initially characterized as a cellular mechanism involved in the recycling or degradation of various cellular components. However, more recent studies have revealed its crucial role in exosomes biogenesis. The process begins with endocytosis, where an endocytic vesicle derived from the plasma membrane is formed and subsequently fuses with the early endosome. Through a series of maturation steps, the early endosomes progress into late endosomes or multivesicular bodies (MVBs). Within MVBs, intraluminal vesicles (ILVs) are generated through inward budding of the endosomal membrane. Finally, the late endosomes can either merge with lysosomes for degradation, recycle to the trans-Golgi network, or fuse with the plasma membrane, leading to the release of ILVs as exosomes (Colombo, Raposo et al. 2014, Sheta, Taha et al. 2023).

During the endocytic pathway, the endosomal sorting complex required for transport (ESCRT) machinery plays a crucial role in vesicle budding and protein sorting (Sheta, Taha et al. 2023). The ESCRT complex consists of five main components: ESCRT-0, -I, -II, -III, and Vps4. Initially, the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a key component of the ESCRT-0 complex, recognizes and sorts ubiquitinated proteins into early endosomes. ESCRT-I and ESCRT-II then facilitate the inward budding of the endosomal membrane. ESCRT-III and Vps4 subsequently mediate the formation of ILVs (Sheta, Taha et al. 2023). However, it is worth noting that exosomes biogenesis can also occur independently of ESCRT complexes. Studies have demonstrated that ceramide can form raft-like structures and mediate the inward budding necessary for ILVs formation

(Trajkovic, Hsu et al. 2008). In fact, the loss of sphingomyelinase, an enzyme involved in sphingolipid breakdown into ceramide, impairs exosomes secretion in various models (Chairoungdua, Smith et al. 2010, Kosaka, Iguchi et al. 2010, Yuyama, Sun et al. 2012). Additionally, tetraspanins such as CD63, CD9 and CD81, commonly identified as exosomal markers, play a role in protein sorting into exosomes through mechanisms independent of ESCRT. CD63 regulates the shuttling of latent membrane protein 1 (LMP1) into exosomes, preventing its lysosomal degradation (Verweij, van Eijndhoven et al. 2011). CD9 facilitates the loading of CD10 into exosomes, while CD81 exhibits microdomains crucial for cargo sorting (Mazurov, Barbashova et al. 2013, Perez-Hernandez, Gutiérrez-Vázquez et al. 2013). On the other hand, tetraspanin-6 inhibits the interaction between syntenin and its partners ALIX and syndecan, leading to the degradation of MVB cargo through the lysosomal pathway (Ghossoub, Chéry et al. 2020).

The involvement of Rab proteins is essential throughout the different steps of the endosomal pathway. The Rab family consists of 60 different small GTPases that play a regulatory role in vesicle budding, endosomes mobility, endosomes docking and fusion with the plasma membrane, as well as exosomes release (Colombo, Raposo et al. 2014). For example, Rab4 and Rab5 mediate the recycling and trafficking of endosomes, and the fusion of endosomes, respectively, in the initial stages of the endocytic pathway (Gorvel, Chavrier et al. 1991, McCaffrey, Bielli et al. 2001). Rab11 and Rab35 are primarily associated with early stages of the endocytic pathway, regulating recycling and sorting processes (Stenmark 2009). However, these Rabs have also been implicated in exosomes release (Savina, Vidal et al. 2002, Hsu, Morohashi et al. 2010).

In addition, Rab27a and Rab27b are involved in the regulation of docking and fusion of MVBs with the plasma membrane, and the mobility of MVBs towards the plasma membrane, respectively (Ostrowski, Carmo et al. 2010). Furthermore, Rab7 plays a role in later stages of the endocytic pathway, controlling the secretion of exosomes (Baietti, Zhang et al. 2012). However, other studies associate Rab7 as a key component in triggering the fusion of MVBs with lysosomes (Vanlandingham and Ceresa 2009). This dual role of Rab7 appears to be dependent on its ubiquitylation status (Rocha, Kuijl et al. 2009).

Microvesicles, in contrast to exosomes, are formed through a simpler process involving outward blebbing and shedding directly from the cell membrane. The contraction of the cytoskeleton plays a crucial role in facilitating this process (Bebelman, Smit et al. 2018). Interestingly, some of the machinery involved in exosomes biogenesis can also contribute to microvesicles formation. For example, the ESCRT complex has been implicated in the formation of microvesicles that are enriched in cell surface proteins (Nabhan, Hu et al.

2012). Additionally, sphingomyelinase appears to be important for mediating microvesicle production in a ceramide-dependent manner (Bianco, Perrotta et al. 2009).

Apoptotic EVs are generated during the process of apoptosis. In the initial stages of apoptosis, membrane blebbing on the cell surface is crucial in the formation of apoptotic EVs, and this process is regulated by Rho-associated protein kinase (ROCK1) (Coleman, Sahai et al. 2001). Similar to the formation of microvesicles, the cytoskeleton, particularly actomyosin, plays an important role in the formation of membrane blebs (Orlando, Stone et al. 2006). Subsequently, apoptotic membrane protrusions, known as apoptopodia, are formed, which can ultimately lead to the formation of apoptotic EVs (Li, Liao et al. 2020).

Various cellular processes, such as intracellular signaling and stress, can trigger the release of EVs. One example is the process of autophagy, which primarily involves organelles and proteins degradation, but studies have shown that autophagy-related proteins, such as ATG5 and ATG16L1, can also promote the fusion of MVBs with the plasma membrane, leading to the release of exosomes (Guo, Chitiprolu et al. 2017). Additionally, the ATG12–ATG3 complex can interact with an ESCRT-associated protein to regulate exosomes biogenesis (Murrow, Malhotra et al. 2015). Another cellular pathway that can impact EVs release is the DNA damage response (DDR), which frequently is activated in cancer. For example, TP53, a key regulator of DDR, modulates exosomes secretion through the activation of the tumor suppressor-activated pathway 6 (TSAP6) (Lespagnol, Duflaut et al. 2008). Alterations in TP53 expression and function are commonly observed in cancer, including PDAC, which may explain the changes in EVs communication in this context. Interestingly, these cellular responses can also influence the cargo (Wu, Zhang et al. 2021).

Proteins, RNA, DNA, and lipids constitute the contents of EVs. Regarding the lipid composition, EVs membranes are primarily composed of sphingomyelin, ceramide and cholesterol (Sheta, Taha et al. 2023). The content of EVs varies depending on their type, which in addition to size, allows to differentiate the various EVs subclasses. Exosomes, for instance, are characterized by the presence of the tetraspanin family members (CD63, CD81 and CD9), proteins belonging to the ESCRT machinery (ALIX, TSG101) and various Rab proteins (Sheta, Taha et al. 2023). On the other hand, microvesicles are characterized by the presence of Annexin A1 and ARF6, while apoptotic EVs exhibit Annexin V, a common marker of apoptosis (Sheta, Taha et al. 2023). However, the cargo of EVs is not solely determined by their subclass, but also by the cell from which they originate. For example, EVs released by pancreatic cancer cells contain mutated KRAS and TP53 DNA, which are common genetic alterations in this type of cancer (Kahlert, Melo et al. 2014). Furthermore, exosomes derived from cancer cells carry microRNAs and protein signatures that are

specific to particular cancer types (Schwarzenbach and Gahan 2019, Hoshino, Kim et al. 2020). Interestingly, the cargo present in EVs does not fully mirror the entire content of the originating cell. This suggests that the cargo carried by EVs is actively selected and implies a biological function for these vesicles. The discovery by Valadi and colleagues that mRNA contained within EVs can be translated into protein in recipient cells sparked significant interest in the field of EVs biology and the investigation of how EVs modulate recipient cells (Valadi, Ekström et al. 2007).

Recent research has shed light on the mechanisms through which EVs engage with recipient cells, primarily involving two main models of interaction: direct contact with receptors on the cell membrane and delivery of their cargo (Gurung, Perocheau et al. 2021). The content of EVs has the ability to interact with cell surface receptors, initiating signalling cascades within the recipient cell. This process is particularly significant in immunomodulatory responses. For instance, exosomes derived from DCs can stimulate T cells through MHC-peptide complexes or by presenting Toll-like receptor-ligands on their surface, which in turn leads to the bystander activation of DCs (Sobo-Vujanovic, Munich et al. 2014, Tkach, Kowal et al. 2017).

Furthermore, EVs can be internalized and, subsequently, deliver their cargo. This is an active and dynamic process primarily mediated by endocytosis (Bastos, Ruivo et al. 2018). Various mechanisms of uptake have been identified, including classical pathways dependent on clathrin or caveolae, as well as macropinocytosis and phagocytosis (Mulcahy, Pink et al. 2014). Remarkably, these mechanisms are not mutually exclusive, indicating that a single cell can employ multiple endocytic pathways to internalize EVs. Alternatively, fusion events can also occur and mediate EVs internalization into cells (Bastos, Ruivo et al. 2018).

The profound impact of EVs on recipient cells has sparked significant interest in unravelling their roles in both physiological processes and disease conditions. In the context of cancer, extensive research has been dedicated to comprehending the intricate functions of EVs in orchestrating interactions within the tumor microenvironment and governing various cancer-related processes.

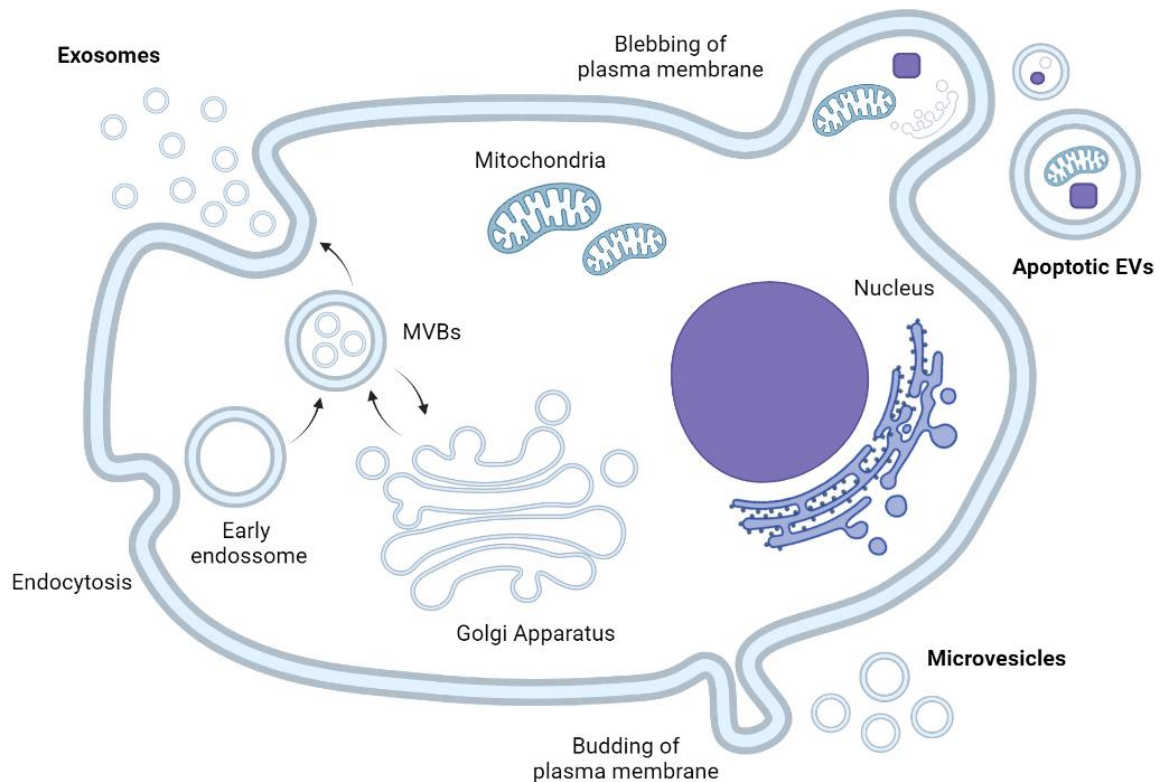


Figure 3 – Extracellular vesicles biogenesis. The three main types of EVs present different biogenesis pathways. Exosomes are derived from the endosomal pathway and are released to the extracellular space through the fusion of multivesicular bodies (MVBs) with the plasma membrane. Microvesicles are formed through direct budding of the plasma membrane. Apoptotic vesicles are a subproduct of apoptosis and are generated from membrane protrusions, called apoptopodia. Adapted from (Yates, Anthony et al. 2019).

3.2 Extracellular vesicles in cancer

Extensive evidence suggests that EVs play a significant role in all aspects of cancer, actively participating in every hallmark of the disease. These versatile vesicles have been shown to promote cancer cell survival, growth, and invasion, while also exerting influence on various components of the tumor microenvironment (Ruivo, Adem et al. 2017, Adem, Vieira et al. 2020). Notably, EVs derived from cancer cells possess the ability to transfer oncogenic cargo to recipient cells, thereby triggering phenotypic alterations and significantly contributing to the progression of the disease.

EVs play a noteworthy role in cancer even during its early stages, as evidenced by several studies. Transformed cells have been shown to transfer their oncogenic material to

unrelated cell populations, endowing them with oncogenic potential (Bergsmedh, Szeles et al. 2001, Antonyak, Li et al. 2011, Melo, Sugimoto et al. 2014). For instance, Bergsmedh and colleagues demonstrated that tumor DNA can be horizontally transferred through apoptotic bodies, resulting in the loss of contact inhibition and tumorigenicity in SCID mice of TP53 knockout cells (Bergsmedh, Szeles et al. 2001). Another study revealed that cancer microvesicles carrying the protein cross-linking enzyme tissue transglutaminase and fibronectin from different cancer cell lines can activate mitogenic signaling in fibroblasts, inducing their transformation (Antonyak, Li et al. 2011). Additionally, exosomal microRNAs derived from a breast cancer cell line have the ability to convert a non-tumorigenic breast cell line into a tumor-forming one (Melo, Sugimoto et al. 2014). However, it should be noted that transient rather than permanent effects were observed when cancer EVs containing oncogenic *H-ras* activated a cellular response resembling malignant transformation in non-tumorigenic cells, as reported by (Lee, Chennakrishnaiah et al. 2016).

Cancer EVs have been implicated in promoting the growth of cancer cells. A significant breakthrough in this regard was made by Al-Nedawi and colleagues who demonstrated the transfer of oncogenic EGFR mRNA between glioblastoma cells via EVs. This transfer lead to the activation of AKT and MAPK signaling pathways, ultimately promoting cell proliferation (Al-Nedawi, Meehan et al. 2008). Subsequent studies have further elucidated the role of EVs content in activating various cancer-related signaling pathways that are closely involved in cellular proliferation. For instance, colon cancer exosomes were found to shorten mitosis duration and activate STAT3 signaling, particularly under hypoxic conditions (Ren, Sun et al. 2019). More recently, our research has shown that EVs facilitate an intra-tumor communication network among different subpopulations of cancer cells, thereby supporting the progression of PDAC. Specifically, we demonstrated that cancer stem cells preferentially communicate with non-stem cancer cells through Agrin⁺ EVs, leading to the activation of YAP signaling (Ruivo, Bastos et al. 2022). YAP, a major component of the Hippo pathway, plays a critical role in promoting cell proliferation and regulating apoptosis (Han 2019).

Cancer EVs have been found to possess the ability to inhibit apoptosis in cancer cells and confer resistance to therapy-induced cell death. Numerous studies have provided evidence that microRNAs and proteins contained within exosomes promote chemoresistance in various cancer types (Lan, Zeng et al. 2019). For instance, exosomes derived from pancreatic cancer cells and enriched in microRNA-155 have been shown to reduce the expression of DCK, an enzyme involved in the metabolism of the chemotherapeutic drug gemcitabine. Furthermore, these cancer exosomes enhance the expression of superoxide dismutase and catalase, which suppresses basal and gemcitabine-induced reactive oxygen

species (ROS) formation, thereby contributing to chemoresistance (Patel, Khan et al. 2017). Additionally, the release of EVs by cancer cells can serve as a strategy to eliminate chemotherapeutic drugs, further promoting drug resistance (Safaei, Larson et al. 2005).

Cancer EVs play a significant role in promoting cancer cell invasion and metastasis. Once released into the extracellular space, these EVs can modulate cells within the tumor microenvironment or travel through the bloodstream and lymphatic system to regulate cells at distant sites, particularly sites of metastasis. Locally, cancer EVs have been found to promote cancer cell invasion by inducing epithelial-mesenchymal transition (EMT) or by facilitating degradation of the ECM. For instance, a study demonstrated that cancer exosomes enriched with TGF- β can trigger the expression of Snail and Zeb1, which are genes commonly associated with the EMT program, in pancreatic cancer cells (Nakayama, Miyoshi et al. 2022). Interestingly, not only cancer exosomes but also exosomes derived from stromal components can induce an EMT phenotype in cancer cells. Exosomes containing WNT, derived from cancer-associated fibroblasts, promote motility and metastasis of breast cancer cells by modulating cell polarity (Luga, Zhang et al. 2012). Moreover, studies have shown that cancer exosomes carry enzymes involved in extracellular matrix remodeling, such as MMP2, MMP9, and urokinase plasminogen activator, thereby facilitating remodeling of the ECM and favoring the dissemination of cancer cells (Dolo, D'Ascenzo et al. 1999).

Cancer EVs also play a crucial role in the formation of the pre-metastatic niche, prepping future sites of metastasis for cancer cell seeding and expansion. The involvement of EVs in this process was first described in melanoma, where cancer cell-derived exosomes “educate” bone marrow progenitor cells toward a pro-vasculogenic phenotype by activating the receptor tyrosine kinase MET. This leads to increased vascular leakiness in future metastatic sites, facilitating cancer cell seeding (Peinado, Alečković et al. 2012). Similarly, exosomes derived from pancreatic cancer cells contribute to the formation of a pre-metastatic niche in the liver. These cancer exosomes promote fibronectin production by hepatic stellate cells through an indirect mechanism mediated by TGF- β expressing Kupfer cells, thereby supporting pancreatic cancer cells metastasis (Costa-Silva, Aiello et al. 2015). Interestingly, integrins present on the surface of exosomes are crucial in determining their organotropism and, consequently, the sites of metastasis. In fact, altering the integrin profile of cancer exosomes can redirect the metastatic site of cancer cells in preclinical models (Hoshino, Costa-Silva et al. 2015).

In addition to their role in regulating cancer cell phenotype, cancer EVs are exploited by cancer cells to modulate other cells within the tumor microenvironment, thereby supporting

disease progression. One example is the ability of cancer cells to induce the differentiation of fibroblasts into myofibroblasts. This process is mediated by the activation of SMAD signaling through TGF- β and the transmembrane proteoglycan betaglycan present on the membrane of cancer exosomes (Webber, Steadman et al. 2010). Cancer EVs also play a role in modulating endothelial cells and promoting angiogenesis. Skog and colleagues demonstrated that microvesicles derived from glioblastoma cells are enriched in angiogenic proteins such as angiogenin, IL-6, IL-8, TIMP-1, VEGF and TIMP-2, and can stimulate endothelial cell proliferation (Skog, Würdinger et al. 2008). Other studies have shown that microRNAs present in cancer exosomes can induce a similar pro-angiogenic phenotype (Zeng, Li et al. 2018). Moreover, under hypoxic conditions, cancer cells alter the cargo of their EVs and secrete EVs enriched in pro-angiogenic molecules to promote neovascularization (Park, Tan et al. 2010, Svensson, Kucharczywska et al. 2011).

Cancer-derived EVs also play a crucial role in regulating the immune response in cancer. Numerous studies have demonstrated that cancer cells secrete EVs to hinder the anti-tumor response and create an immunosuppressive microenvironment by influencing various immune cells (Liu, Gu et al. 2015). For instance, early reports indicated that melanoma exosomes containing FasL induce apoptosis in activated T cells (Andreola, Rivoltini et al. 2002). Additionally, studies have shown that TGF- β ⁺ cancer exosomes can inhibit the function of natural killer cells and cytotoxic CD8⁺ T cells by suppressing the NKG2D receptor, a crucial molecule involved in cytokines and granules release (Clayton, Mitchell et al. 2008). Cancer exosomes have also been observed to promote the differentiation and expansion of immune regulatory cells. In ovarian cancer, exosomes derived from cancer cells facilitate the conversion of CD4⁺ T cells into Treg cells, enhancing their suppressor activity and resistance to apoptosis (Szajnik, Czystowska et al. 2010). Furthermore, exosomes containing prostaglandin E2 and TGF- β can stimulate the accumulation and activation of MDSCs, leading to elevated levels of inflammatory cytokines in the tumor microenvironment and accelerated disease progression (Xiang, Poliakov et al. 2009). Tumor exosomes can also mediate the conversion of TAMs cytokine profile, promoting tumor invasion and metastasis (Marton, Vizler et al. 2012). Recent findings have highlighted the packaging of PD-L1 in exosomes derived from glioblastoma, melanoma, and breast cancer, contributing to an immunosuppressive phenotype and predicting the response to anti-PD-1 treatment (Chen, Huang et al. 2018, Ricklefs, Alayo et al. 2018, Yang, Li et al. 2018).

However, it has also been observed that cancer exosomes can elicit an immune response against tumor cells. Treatment of bone-marrow derived DCs with tumor exosomes *ex vivo* results in a robust induction of an anti-tumor response mediated by CD8⁺ T cells (Wolfers,

Lozier et al. 2001). This approach has shown to be effective in expanding tumor-specific cytotoxic T cells in clinical settings. Exosomes derived from the ascites of cancer patients contain various tumor-specific antigens, which can be utilized to cross-present peptides to autologous T cells via DCs (Andre, Scharzt et al. 2002).

These findings underscore the notion that the function of EVs in cancer is heavily influenced by the context and stage of disease progression. There seems to be a delicate balance between activating and inhibiting an anti-tumor response mediated by EVs, contributing to the intricate biology of these vesicles. The role of EVs in modulating not only cancer immunity but other cancer-related processes throughout different stages of disease progression could be crucial in understanding cancer dynamics. Manipulating EVs holds immense potential in influencing various components of the tumor microenvironment and determining therapy response. To fully comprehend the role of these vesicles, further research employing appropriate preclinical models is imperative.

PART II - OBJECTIVES

Pancreatic ductal adenocarcinoma (PDAC) is associated with a dismal prognosis (Siegel, Miller et al. 2019, Sung, Ferlay et al. 2021). Late diagnosis and limited effective treatment options contribute to the high lethality of PDAC (Sultana, Smith et al. 2007, Becker, Hernandez et al. 2014). While immunotherapy has revolutionized cancer treatment (Gettinger, Horn et al. 2015, Hodi, Chesney et al. 2016), PDAC has shown to be resistant to this approach (Amin, Baine et al. 2020, Rahma, Katz et al. 2021). Therefore, it is crucial to identify strategies that sensitize PDAC patients to immunotherapy and identify patient subsets that are suitable for this therapeutic approach. Cancer extracellular vesicles (cEVs) play a significant role in regulating various cancer-related processes, including the immune response (Liu, Gu et al. 2015, Kurywchak, Tavormina et al. 2018). Thus, targeting cEVs represents an intriguing avenue for modulating cancer immunity and enhancing the response to immunotherapy.

The primary objective of this thesis is to investigate the impact of cEVs-mediated communication on the initiation and progression of pancreatic cancer within the natural context of disease development. Furthermore, we aimed to explore how impairing this communication could influence the efficacy of immunomodulatory therapies. We hypothesized that in a scenario of impaired cEVs communication, there would be a more responsive intratumoral immune cell repertoire, ultimately leading to an improved response to immunotherapeutic approaches. The identification of a subset of PDAC patients who could benefit from these therapeutic options holds great potential for enhancing patient care.

The main objectives of this thesis are:

1. To investigate the impact of Rab27a-dependent cEVs communication on the initiation and progression of PDAC.
2. To elucidate the molecular cargo carried by cEVs that plays a pivotal role in modulating PDAC progression.
3. To determine how is the tumor microenvironment of PDAC tumors reprogrammed by Rab27a-dependent cEVs communication.
4. To evaluate whether Rab27a-dependent cEVs communication impacts the efficacy of therapies that modulate the immune response.
5. Unravel the underlying mechanisms regulating Rab27a expression and Rab27a-dependent cEVs communication throughout the progression of PDAC.

The findings of objective number 5 have been published in one original article present for the first time in a thesis:

1. **Bastos N***, Castaldo SA, Adem B, Machado JC, Melo CA, Melo SA

SMC3 epigenetic silencing regulates Rab27a expression and drives pancreatic cancer progression. Journal of Translational Medicine, 2023 DOI: 10.1186/s12967-023-04448-1

The findings of objectives number 1-4 are contemplated in a manuscript currently in preparation.

The experimental work, results and discussion of this thesis are presented in **PART III** and in **PART IV**. The main conclusions of this work constitute **PART V**. The methodology used to address the specific objectives of this thesis is described in **PART VI**. Finally, the complete version of the article is reproduced in **Appendix I**.

PART III – RESULTS

1. Evaluation of the impact of Rab27a-dependent cEVs communication in PDAC progression

Cancer extracellular vesicles (cEVs) have been described to promote cancer progression through the modulation of several cancer-related processes. However, the role of cEVs in cancer, namely PDAC, in a natural setting of disease development has never been assessed before. Here, we describe that Rab27a represents the best target to modulate cEVs communication *in vivo*. Then, using PDAC genetically engineered mouse models (GEMMs), we demonstrate that Rab27a and Rab27a-dependent cEVs communication have a tumor suppressive role.

1.1 Rab27a is the RabGTPase that best correlates with cEVs release in PDAC

In order to determine the best Rab GTPase as a surrogate marker for secretion of EVs by cancer cells and as target to impair cEVs communication in PDAC, we screened for four Rab GTPases (Rab5, Rab7, Rab27a and Rab27b) in three human PDAC cell lines (PANC-1, BxPC-3 and MIA PaCa-2). Each Rab GTPase corresponds to a specific step in EVs biogenesis of endosomal origin, from early endosomes to exocytosis. We demonstrated that Rab27a and 27b are not expressed equally in the panel of cell lines, whereas Rab5 and 7 are (Figure 1A). MIA PaCa-2 is the human PDAC cell line that expresses the higher amounts of Rab27a and 27b proteins (Figure 1A). As expected, MIA PaCa-2 is also the cell line that secretes the highest number of vesicles in comparison with PANC-1 and BxPC-3 (Figure 1B). Most importantly, amongst the four Rab GTPases analyzed Rab27a protein levels is the one that correlates the best with the number of secreted vesicles by cancer cells (Figure 1C). In addition, previous work also demonstrates that Rab27a expression is crucial for EVs secretion (Ostrowski, Carmo et al. 2010, Peinado, Alečković et al. 2012, Ruivo, Bastos et al. 2022). Based on our results and previously published data, we have focused our research on Rab27a and Rab27a-dependent EVs communication in PDAC.

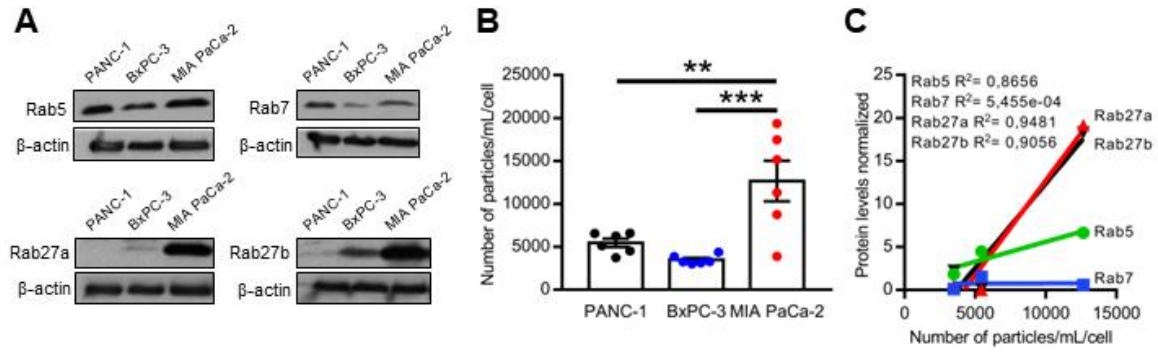


Figure 1. Rab27a protein levels correlate the best with the number of extracellular vesicles released in PDAC. (A) Representative Rab5, Rab7, Rab27a and Rab27b western blot in PANC-1, BxPC-3 and MIA PaCa-2 cells. β-actin was used as loading control. **(B)** Number of particles per cell and per mL released by PANC-1, BxPC-3 and MIA PaCa-2 cells (n=6). One-way anova **p<0.01, ***p<0.001. **(C)** Correlation between the normalized Rab5 (green), Rab7 (blue), Rab27a (red) and Rab27b (black) protein levels and the number of particles secreted per cell and per mL in PANC-1, BxPC-3 and MIA PaCa-2 cells. Linear regression. Data are Mean±SEM.

1.2 Impaired cEVs secretion through loss of Rab27a accelerates disease onset and reduces survival in an immunocompetent background

To impair the secretion of cEVs in PDAC we developed a GEMM to conditionally knockout (KO) Rab27a in cancer cells. FRT sites were introduced into a region flanking exon 4 of the Rab27a gene (Figure 2A). Upon the action of the recombinase flippase, exon 4 is excised, resulting in loss of Rab27a expression. The *Rab27a^{Frt/Frt}* allele was then crossed to the PDAC GEMM PKT (*Ptf1a-Cre; LSL-Kras^{G12D/+}; Tgfbr2^{loxP/loxP}*). PKT is a Cre-driven model that spontaneously develops PDAC and recapitulates key aspects of the human disease (Ijichi, Chytil et al. 2006). To establish an inducible and conditional model to cancer cells, we used the *R26^{LSL-FLPOERT2/+}* allele to obtain the PKT iRab27a (*Ptf1a-Cre; LSL-Kras^{G12D/+}; Tgfbr2^{loxP/loxP}; R26^{LSL-FLPOERT2/+}; Rab27a^{Frt/Frt}*) (Goodrich, Talhouk et al. 2018). This mouse spontaneously develops PDAC, and Rab27a recombination occurs specifically in cancer cells upon tamoxifen treatment (Figure 2B). As a control, we used the same model without the *R26^{LSL-FLPOERT2/+}* allele (PKT Rab27a; Figure 2B). Tamoxifen treatments were performed in both groups from birth. Surprisingly, KO of Rab27a in cancer cells resulted in earlier disease onset and a significant decrease in overall survival (Figure 2C and 2D). Histopathological analysis of primary tumors of mice euthanized at 35 days of age confirmed a significant increase in surface area of PDAC in the pancreas of Rab27a KO tumors (Figure 2E). We confirmed that recombination of the Rab27a allele occurs in the pancreas of tamoxifen-treated PKT iRab27a mice (Figure 2F). We isolated cancer cells

(EPCAM⁺) and established cancer cell lines from a second PDAC model, the KPC iRab27a (*LSL-Kras*^{G12D/+}; *LSL-Tp53*^{R172H/+}; *Pdx1-Cre*; *R26*^{LSL-FLPoERT2/+}; *Rab27a*^{Frt/Frt}) and demonstrated a significant reduction in Rab27a expression in tamoxifen-treated mice (Figure 2G) (Hingorani, Wang et al. 2005). Importantly, we confirmed that Rab27a KO resulted in impaired secretion of EVs by cancer cells (Figure 2H). We showed a significant reduction in CD63⁺ and Alix⁺ EVs isolated from Rab27a KO cancer cells, confirming that Rab27a KO negatively impacts the exocytosis of cEVs (Figure 2H).

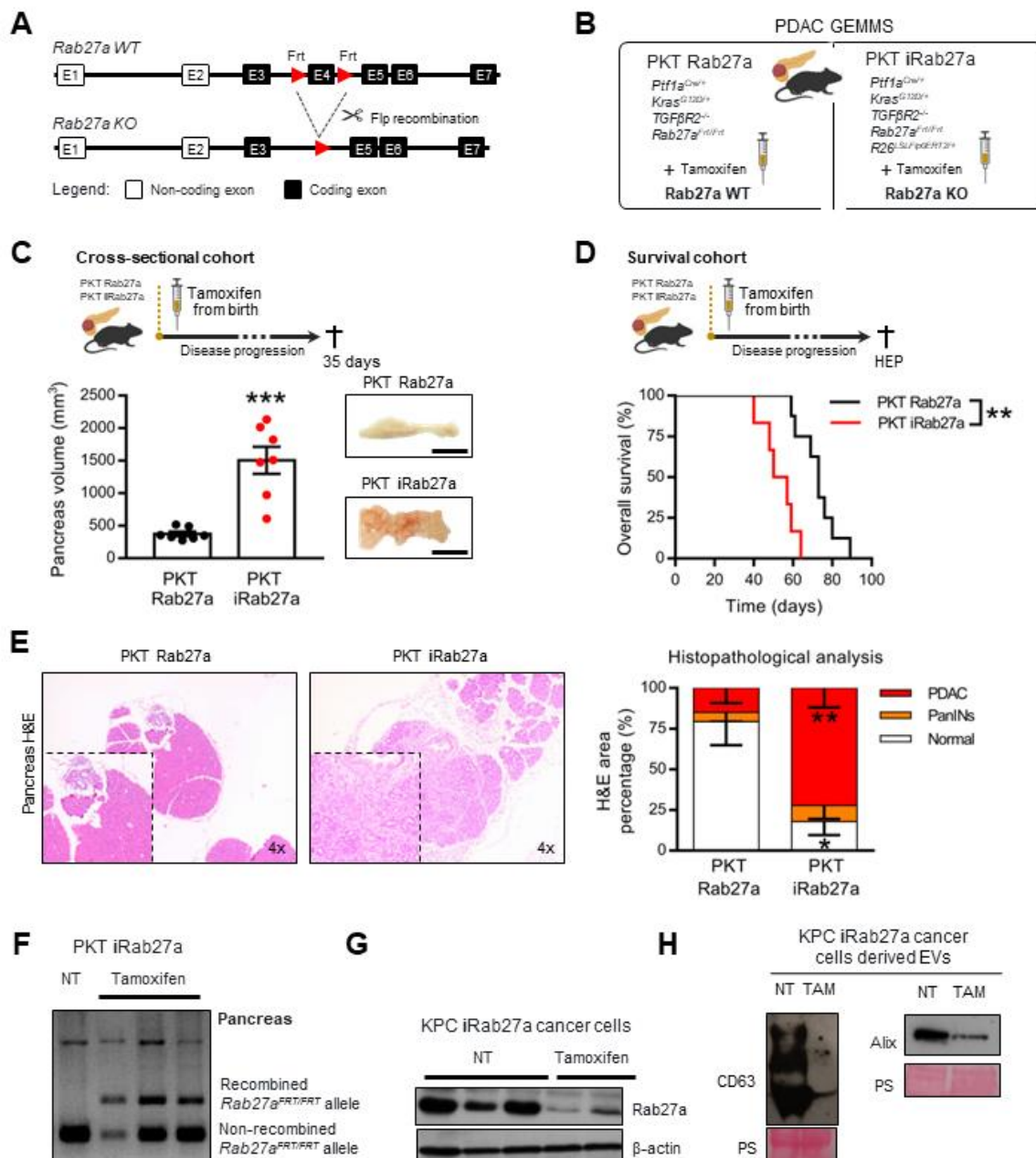


Figure 2. Rab27a knockout in cancer cells impairs cEVs release and promotes PDAC progression. (A) Schematic representation of the Rab27a^{FRT/FRT} allele. FRT sites were placed flanking the exon 4 of the Rab27a gene. Upon flippase action, exon 4 is excised leading to the Rab27a KO allele. (B) PKT iRab27a, an inducible and conditional Rab27a knockout GEMM that spontaneously develops PDAC. Rab27a knockout is achieved specifically in cancer cells upon tamoxifen treatment. The PKT Rab27a model represents the control group treated with tamoxifen that, due to the lack of the LSL-FlpO-ERT2, expresses Rab27a. (C) Representative pancreas images (right) and pancreas volume (left) of PKT Rab27a (n=7) and PKT iRab27a (n=7) treated with tamoxifen from birth and euthanized at 35 days (Unpaired t-test *** $p < 0.001$). Scale bar represents 10mm. (D) Kaplan-Meier survival curve of PKT Rab27a (n=8) and PKT iRab27a (n=6) mice treated with tamoxifen from birth (Log-rank Mantel-Cox test ** $p < 0.01$). (E) Representative photos of H&E staining of PKT Rab27a and PKT iRab27a pancreas (4x, with zoom inset) and histopathological analysis of the percentage of normal, PanIns and PDAC area (Unpaired t-test * $p < 0.05$, ** $p < 0.01$). (F) Representative PCR of Rab27a allele recombination in tumors of PKT iRab27a mice non-treated (NT, n=1) or tamoxifen treated (n=3). (G) Representative Rab27a western blot in cancer cells isolated from tumors of KPC iRab27a non-treated (n=3) or tamoxifen treated (n=2) mice. (H) Representative CD63 and Alix western blot in EVs derived from cancer cells isolated from tumors of KPC iRab27a non-treated (NT) or tamoxifen (TAM) treated mice. Ponceau S (PS) was used as a loading control. Data are mean $\pm$ SEM.

Considering the potential of cEVs to reprogram the immune response, we asked the question if the immune system could mediate the phenotype induced by Rab27a KO (Liu, Gu et al. 2015, Kurywchak, Tavormina et al. 2018, Liu, Wu et al. 2020). We crossed PKT iRab27a mice with the *Rag2*^{-/-} allele (PKT iRab27a *Rag2*^{-/-} mice have no mature B or T cells) and monitored the disease progression of Rab27a KO tumors in an immunodeficient background (Hao and Rajewsky 2001). Interestingly, the lack of an adaptive immune system rescues the phenotype of Rab27a KO mice resulting in a significant increase in overall survival of immunodeficient (PKT iRab27a *Rag2*^{-/-}) in comparison to immunocompetent mice (PKT iRab27a, Figure 3A). Moreover, PKT mice with Rab27a WT expression in cancer cells in an immunodeficient background (PKT *Rag2*^{-/-}) did not present changes in overall survival, indicating that the adaptive immune system plays a role in disease progression specifically in a Rab27a KO context (Figure 3B). To further confirm the role of the immune system in the phenotype observed, we orthotopically injected KPC iRab27a EPCAM⁺ cells derived from a non-treated mouse (Rab27a WT) in immunodeficient mice (NOD scid). One group of the immunodeficient mice was treated with tamoxifen resulting in Rab27a KO in cancer cells (Figure 3C). Interestingly, there were no differences in tumor weight between groups at 5 weeks post-inoculation (Figure 3C). This data shows that differences in tumor growth

are not intrinsic to cancer cells Rab27a KO phenotype but rather are dependent on the microenvironment, more specifically, the immune response.

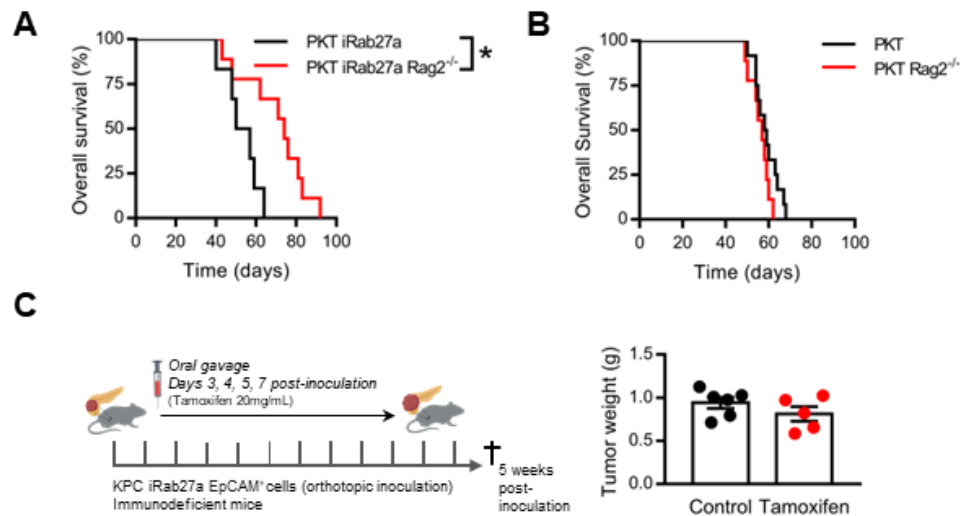


Figure 3. Lack of a proficient immune system hinders disease progression specifically in a Rab27a knockout context in PDAC. (A) Kaplan-Meier survival curve of PKT iRab27a (n=6) and PKT iRab27a Rag2^{-/-} (n=9) mice treated with tamoxifen from birth (Log-rank Mantel-Cox test * $p < 0.05$). Survival curve of PKT iRab27a mice is the same as in Figure 2D. **(B)** Kaplan-Meier survival curve of PKT (n=12) and PKT Rag2^{-/-} (n=9) mice (Log-rank Mantel-Cox test). **(C)** Experimental layout (left) and tumor weight (right) of immunodeficient mice orthotopically injected with EpCAM⁺ cells isolated from a tumor of a KPC iRab27a non-treated mouse. Tamoxifen treatment was performed in a subset of mice at days 3, 4, 5 and 7 post-inoculation (Control n=6, Tamoxifen n=5, Unpaired t-test). Data are mean $\pm$ SEM.

In summary, our data demonstrate that inhibition of cEVs secretion through KO of Rab27a in cancer cells promotes disease progression in PDAC GEMMs. The phenotype observed is dependent on the presence of an intact immune system.

2. Identification of the molecular cargo in cEVs that modulates PDAC progression

We demonstrated that impairment of Rab27a-dependent cEVs communication leads to an earlier disease onset and faster progression in PDAC which is dependent on a proficient immune system. Here, we aimed to identify the main routes of intratumoral cEVs communication and what are the molecular effectors modulating these recipient cells.

2.1 PDAC cEVs communicate with endothelial cells and carry proteins associated with angiogenesis and immune response

The GTPase Rab27a regulates the late stages of the endocytic pathway, mediating the exocytosis of EVs into the extracellular space (Ostrowski, Carmo et al. 2010, Bastos, Ruivo et al. 2018). We demonstrated that KO of Rab27a in cancer cells resulted in a significant reduction in the number of secreted EVs (Figure 2H). Thus, we asked the question which cells of the tumor microenvironment cancer cells communicate with through cEVs, ultimately, impacting disease progression. To address this question, we used a unique GEMM to identify the biodistribution of PDAC cEVs, the KPF CD63-mCherry model (*FSF-Kras^{G12D/+}; Tp53^{Frt/+}; Pdx1-Flp; R26^{CD63-XFP/+}*, Figure 4A) (Adem, Bastos et al. 2021). KPF CD63-mCherry mice spontaneously develop PDAC, and cancer cells secrete CD63-mCherry positive cEVs (Figure 4A) (Adem, Bastos et al. 2021). Interestingly, when analysing the natural endogenous distribution of cEVs in PDAC tumors we discovered that CD31⁺ endothelial cells are one of the major recipient cells of PDAC cEVs in the tumor microenvironment (Figure 4B). These data pointed to a potential role of cEVs in regulating angiogenesis in PDAC.

Thus, we examined the protein content of cEVs. We started by isolating cEVs from 4 different human PDAC cell lines (BxPC-3, PANC-1, T3M4, and MIA PaCa-2) and performed liquid chromatography-electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). We previously performed a biophysical characterization of the purified vesicles (Ruivo, Bastos et al. 2022). We identified 185 proteins in human PDAC EVs (Spreadsheet S1). Interestingly, gene ontology analysis identified pathways associated with angiogenesis, immunity and inflammation (Figure 4C and Spreadsheet S2). This data highlights that cEVs can modulate these cancer-related processes. In fact, we confirmed that Rab27a KO tumors have a significant increase in angiogenesis as reflected by the number of vessels quantified by the presence of CD31⁺ endothelial cells (Figure 4D). Therefore, considering the cargo of cEVs, the nature of one of their major recipient cells and how crucial the presence of a proficient immune response is, in the phenotype observed, in a context of impaired cEVs communication, we hypothesized that angiogenesis and recruitment/activation of immune cells could be important in disease progression of Rab27a KO tumors.

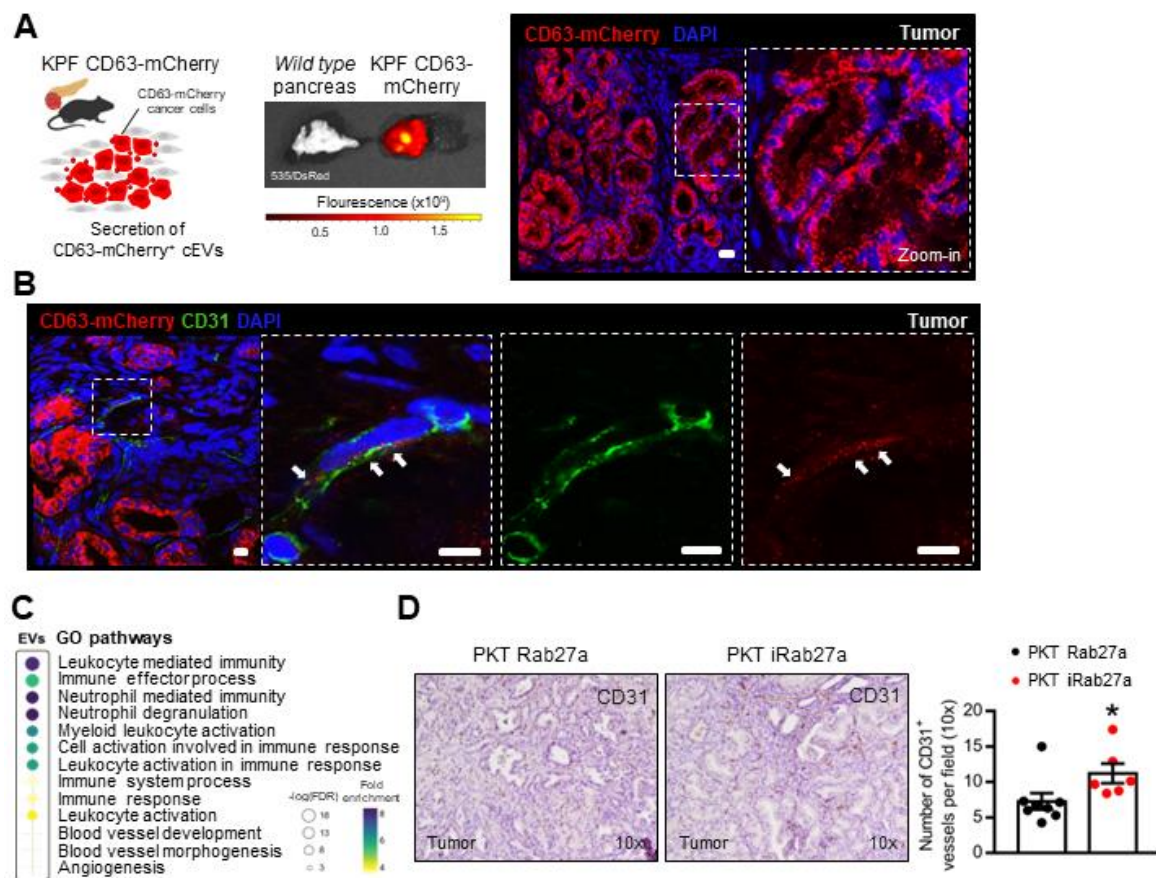


Figure 4. PDAC cEVs modulate endothelial cells through their cargo. (A) Schematic of the KPF CD63-mCherry mouse model (*FSF-Kras*^{G12D/+}; *Tp53*^{Frt/+}; *Pdx1-Flp*; *R26*^{CD63-XFP/+}) in which cancer cells express CD63-mCherry and secrete CD63-mCherry⁺ EVs (left). Representative IVIS image of a wild type pancreas and a KPF CD63-mCherry tumor at the timepoint of euthanasia using the 535 laser and DsRed emission filter (middle). Fluorescence intensity ranges from red (least intense) to yellow (most intense). Representative immunofluorescence of CD63-mCherry (red) in a KPF CD63-mCherry tumor (right). DAPI was used to stain nuclei. Scale bar 20μm. **(B)** Representative immunofluorescence of CD63-mCherry (red) and CD31 (green) in tumors of KPF CD63-mCherry. DAPI was used to stain nuclei. Arrows indicate CD63⁺ EVs taken up by CD31⁺ cells. Scale bar 10μm. **(C)** GO pathways enriched in total proteins identified in cancer EVs related with immunity and angiogenesis. **(D)** Representative CD31 IHC images (10x, top) and respective quantification (bottom) in PKT Rab27a (n=8) and PKT iRab27a (n=6) tumors (Unpaired t-test *p<0.05). Data are mean±SEM.

2.2 Secretion of TSP1⁺ cEVs is dependent on Rab27a expression and targeting TSP1 in PDAC results in worse prognosis and neoangiogenesis

Thrombospondin-1 (TSP1) is a protein associated with angiogenesis and immunity and is enriched in cEVs (Spreadsheet S2) (Bornstein 2009, Lopez-Dee, Pidcock et al. 2011). The

role of TSP1 in mediating an anti-inflammatory response and impairing angiogenesis by inhibiting endothelial cell migration and proliferation is well established (Bornstein 2009, Lopez-Dee, Pidcock et al. 2011). Given the described functions of TSP1, we hypothesized that loss of TSP1⁺ cEVs in Rab27a KO tumors triggers neoangiogenesis, which could at least partially explain disease acceleration of these tumors.

First, we validated the presence of TSP1 in cEVs isolated from the MIA PaCa-2 cell line by size-exclusion chromatography (SEC; Figure 5A-C). TSP1 was also identified in non-vesicular fractions (nV), albeit at much lower levels (Figure 5A-C). TSP1 was also present in cEVs isolated from a murine pancreatic cancer cell line derived from a KPC tumor (Figure 5D). To demonstrate that secretion of TSP1⁺ cEVs is dependent on Rab27a expression, we established a stable clone using the MIA PaCa-2 cell line expressing a doxycycline-inducible (Tet-On) short hairpin that targets Rab27a. Size-exclusion chromatography analysis of cEVs and nV fractions demonstrated that upon doxycycline treatment (Rab27a downregulation) TSP1 levels were significantly decreased (Figure 5E), indicating that Rab27a regulates the secretion of TSP1⁺ cEVs.

To validate our hypothesis, we orthotopically injected adeno-associated virus (AAV) carrying a GFP-tagged short hairpin that targets TSP1 (AAV GFP-shTSP1) into the pancreas of PKT mice and evaluated disease progression (Figure 5F). Notably, in line with impairment of secretion of cEVs by KO of Rab27a in PDAC cells, TSP1 knockdown resulted in worse overall survival in comparison to PKT mice injected with AAV GFP-tagged scramble short hairpin (AAV GFP-shSCR, Figure 5F). Expression of the shRNA in PKT tumors at the timepoint of euthanasia was confirmed by IVIS[®] Spectrum imaging (Figure 5G). Most importantly, TSP1 downregulation led to a significant increase in the number of vessels (CD31⁺ endothelial cells) in PKT tumors (Figure 5H). In summary, we conclude that secretion of TSP1⁺ cEVs is dependent on Rab27a expression, and that downregulation of TSP1 in PDAC phenocopies the worse prognosis and neoangiogenesis observed in Rab27a KO tumors.

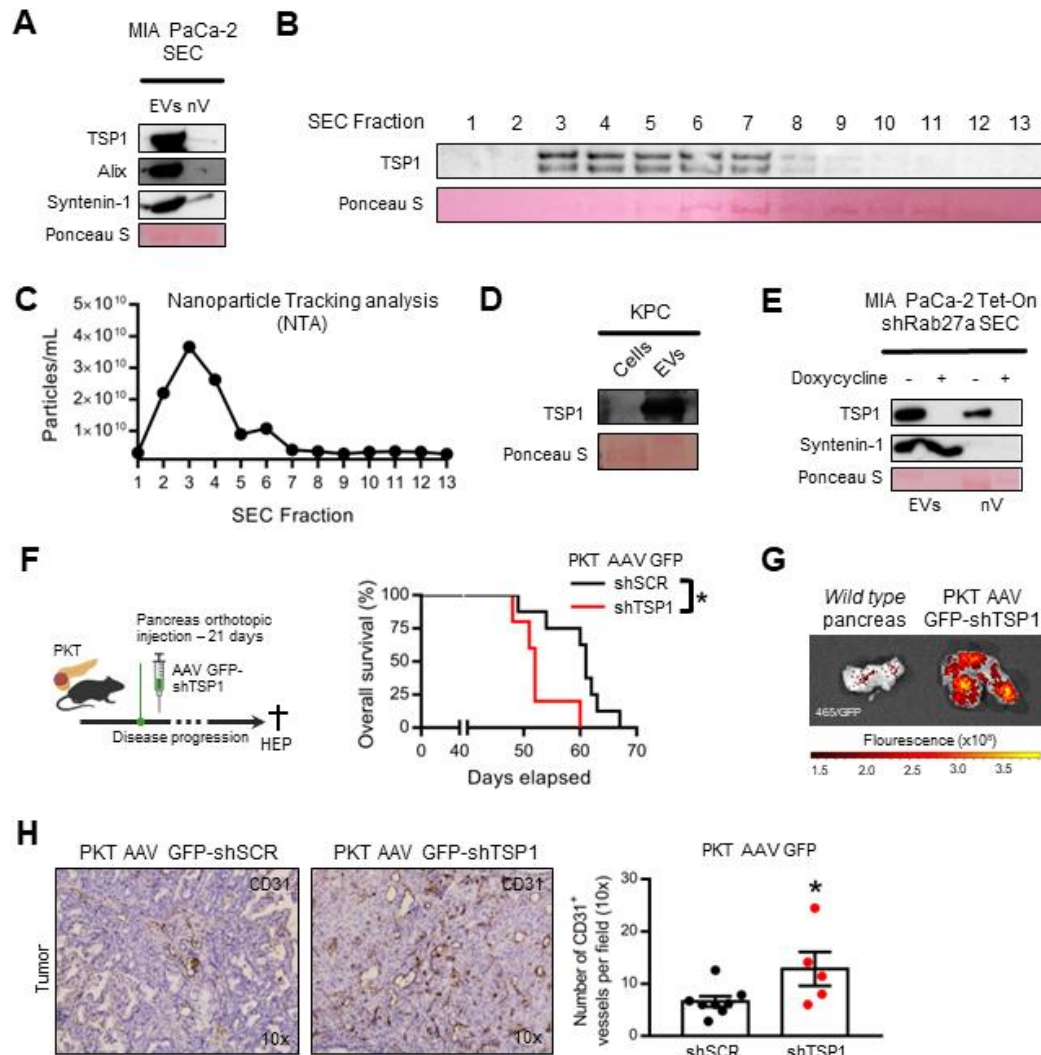


Figure 5. Rab27a regulates TSP1⁺ EVs release and loss of TSP1 leads to neo-angiogenesis in PDAC GEMMs. (A) Representative western blot of TSP1 in EVs and non-vesicular (nV) fractions isolated by SEC from MIA PaCa-2. Alix and Syntenin-1 were used as EVs markers. Ponceau S was used as a loading control. (B) Representative TSP1 western blot in the 13 fractions obtained by SEC from MIA PaCa-2. Ponceau S was used as loading control. (C) Nanoparticle tracking analysis (NTA) of the 13 fractions obtained by SEC. (D) Representative TSP1 western blot in cells and EVs derived from a KPC cell line. Ponceau S was used as loading control. (E) Representative western blot of TSP1 in EVs and non-vesicular (nV) fractions isolated by SEC from MIA PaCa-2 Tet-On shRab27a cultured in the presence (Rab27a knockdown) or absence (Rab27a WT) of doxycycline. Syntenin-1 was used as an EVs marker. Ponceau S was used as loading control. (F) Experiment schematic (left) and Kaplan-Meier survival curve (right) of PKT mice orthotopically injected with AAV-shSCR (n=8) or AAV-shTSP1 (n=5) at 21 days of age (Log-rank Mantel-Cox test *p<0.05). (G) Representative IVIS image of a wild type pancreas and a PKT AAV GFP-shTSP1 tumor at the timepoint of euthanasia using the 465 laser and GFP emission filter. Fluorescence intensity ranges from red (least intense) to yellow (most intense). (H) Representative CD31 IHC images (10x, left) and respective quantification (right) in PKT AAV-shSCR (n=8) and PKT AAV-shTSP1 (n=5) tumors (Unpaired t-test *p<0.05). Data are mean±SEM.

3. Identification of the alterations present in the tumor microenvironment upon impairment of Rab27a-dependent cEVs communication in PDAC

We identified TSP1 as a major component of cEVs and a regulator of neoangiogenesis, ultimately, modulating PDAC progression. We next set to identify the molecular and cellular alterations in the tumor microenvironment that occur upon loss of Rab27a and TSP1⁺ cEVs.

3.1 Th17 immune response prevails in PDAC upon cEVs impairment

To identify the mechanisms that drive disease progression in Rab27a KO tumors mediated by loss of cEVs communication, we performed LC/ESI-MS/MS of whole tumors. Unsupervised analysis identified 328 differentially expressed proteins between Rab27a KO and WT tumors (Heatmap, Figure 6A, Spreadsheet S3). Proteins enriched in Rab27a KO versus WT tumors were mostly associated with immune response-related pathways, as shown by Reactome analysis (Figure 6B). Interestingly, among those proteins, we found the pattern recognition receptor CLEC7a that is mainly expressed by myeloid cells and is involved in promoting a Th17 response, which was previously described as tumor-promoting in PDAC (Figure 6C) (Huang, Wang et al. 2012, McAllister, Bailey et al. 2014). These data further support a role for the immune system in PDAC cEVs impaired tumorigenesis.

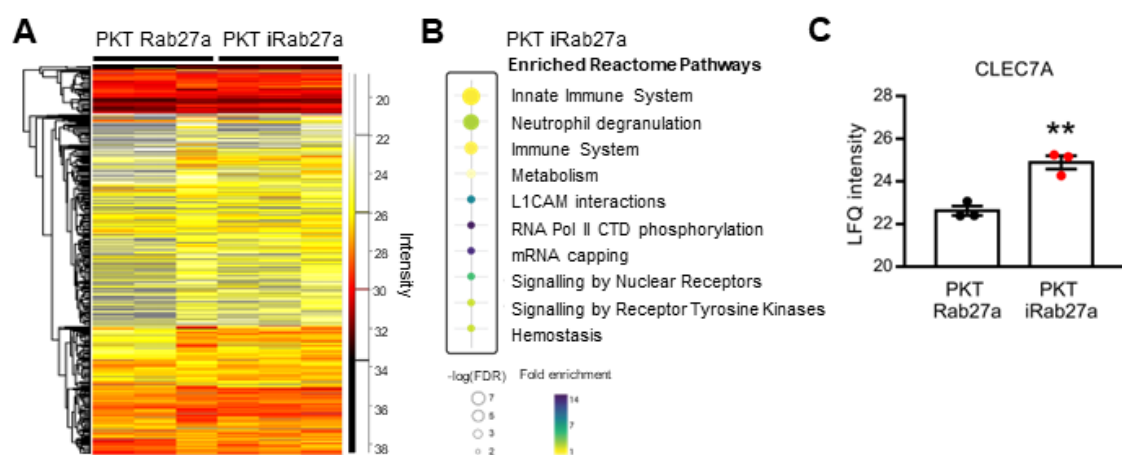


Figure 6. Rab27a knockout tumors present an enrichment in immune-related pathways. (A) Heatmap depicting protein clusters in PKT Rab27a (n=3) and PKT iRab27a (n=3) whole tumors. **(B)** Top 10 enriched reactome pathways in PKT iRab27a tumors. Ranking based on $-\log(\text{FDR})$. **(C)**

CLEC7A LFQ intensity in PKT Rab27a (n=3) and PKT iRab27a (n=3) tumors (Unpaired t-test $**p<0.01$). Data are mean $\pm$ SEM.

To identify the landscape of immune cell populations altered in Rab27a KO tumors, we screened for immune-related cytokines, which can dictate the nature of immune cell populations, using whole tumor extracts. Interestingly, we found upregulation of IL-6 and IL-23 (Figure 7A), two inflammatory cytokines associated with Th17 differentiation and immune response (Kimura and Kishimoto 2010, Toussiot 2012). We further validated the overexpression of IL-6 in PKT as well as KPC Rab27a KO tumors by western blot (Figure 7B and 7C). Thus, both mass spectrometry and cytokine array data pointed to a potential Th17 immune response in Rab27a KO tumors. We further showed a significant increase in the Th17/Th1 cell ratio in Rab27a KO tumors, demonstrating a switch in the immune cell repertoire and an increase in the Th17 response (Figure 7D). ROR γ T and T-bet were used as surrogate markers of Th17 and Th1 immune responses, respectively. Importantly, we demonstrated that ROR γ T Th17 T cells were also significantly increased in a second Rab27a KO PDAC mouse model (KPC iRab27a, Figure 7E). In addition, the presence of CD4 $^{+}$ T cells was also significantly increased in both models (Figure 7F). This could indicate that more CD4 $^{+}$ T cells are actively being recruited to Rab27a KO tumors with enhanced angiogenesis, and preferentially differentiate into a Th17 subset in the presence of inflammatory cytokines such as IL-6 and IL-23. In summary, our data demonstrate that a Th17 immune response prevails in Rab27a KO tumors.

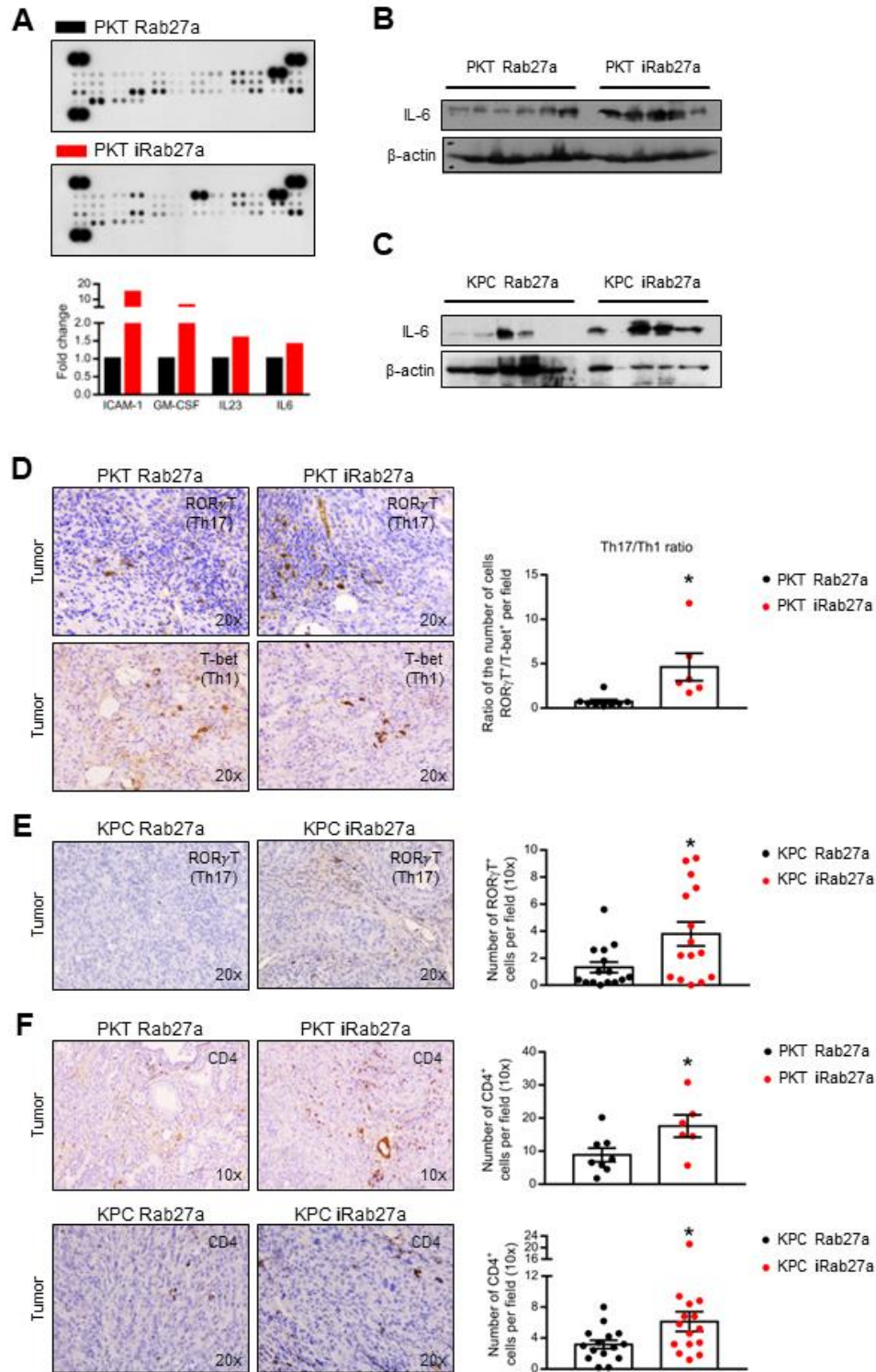


Figure 7. Rab27a knockout tumors present an enrichment in inflammatory cytokines driving a Th17 response. (A) Representative cytokine array (left) and respective quantification (right) of highlighted proteins (ICAM-1, GM-CSF, IL-23 and IL-6) of PKT Rab27a and PKT iRab27a whole tumors. (B) Representative IL-6 western-blot in PKT Rab27a (n=6) and PKT iRab27a (n=5) tumors (Unpaired t-test ** $p < 0.01$). β -actin was used as a loading control. (C) Representative IL-6 western-blot in KPC Rab27a (n=5) and KPC iRab27a (n=5) tumors. β -actin was used as a loading control.

(D) Representative ROR γ T and T-bet IHC images (20x, left) and respective quantification (right) in PKT Rab27a (n=8) and PKT iRab27a (n=6) tumors (Unpaired t-test $*p<0.05$). Ratio between the average number of ROR γ T⁺ cells and the average number of T-bet⁺ cells was performed. **(E)** Representative ROR γ T IHC images (20x) and respective quantification (right) in KPC Rab27a (n=15) and KPC iRab27a (n=15) tumors (Unpaired t-test $*p<0.05$). **(F)** Representative CD4 IHC images (10x, left) and respective quantification (right) in PKT Rab27a (n=8) and PKT iRab27a (n=6) tumors (top) and in KPC Rab27a (n=15) and KPC iRab27a (n=15) tumors (bottom, unpaired t-test $*p<0.05$). Data are mean $\pm$ SEM.

3.2 PDAC with impaired secretion of cEVs are infiltrated by CD11b⁺MRP8⁺ myeloid cells that polarize a Th17 response

We next investigated the alterations in Rab27a KO tumors that result in a pro-Th17 cytokine rich tumor microenvironment. Together with IL-6 and IL-23, GM-CSF was upregulated in Rab27a KO tumors (Figure 7A). This protein has a wide range of effects on myeloid populations, including survival, activation, differentiation and mobilization (Kumar, Taghi Khani et al. 2022). We found that Rab27a KO tumors were highly infiltrated by myeloid CD11b⁺ cells, although no alterations in intratumoral macrophages or neutrophils were observed (Figure 8A-C). CD11b⁺ myeloid cells are a major source of the proinflammatory protein MRP8 (also called S100A8) previously shown to promote Th17 differentiation via upregulation of IL-6 by fibroblast-like synoviocytes in rheumatoid arthritis (Bhardwaj, Zolt et al. 1992, Loser, Vogl et al. 2010, Lee, Woo et al. 2013). With this in mind, we demonstrated a significant increase in MRP8⁺ cells in Rab27a KO tumors (PKT iRab27a and KPC iRab27a) and confirmed that these are of myeloid origin by immunofluorescence (Figure 8D and 8E). In addition, we also confirmed that TSP1 knockdown PDAC tumors have a significant increase in infiltration by MRP8⁺ cells (Figure 8F). Most importantly, in a cohort of human PDAC patients, concomitant low Rab27a expression and a high number of intratumoral MRP8⁺ cells is associated with worse prognosis in comparison with high Rab27a expression and a low number of intratumoral MRP8⁺ cells (Figure 8G).

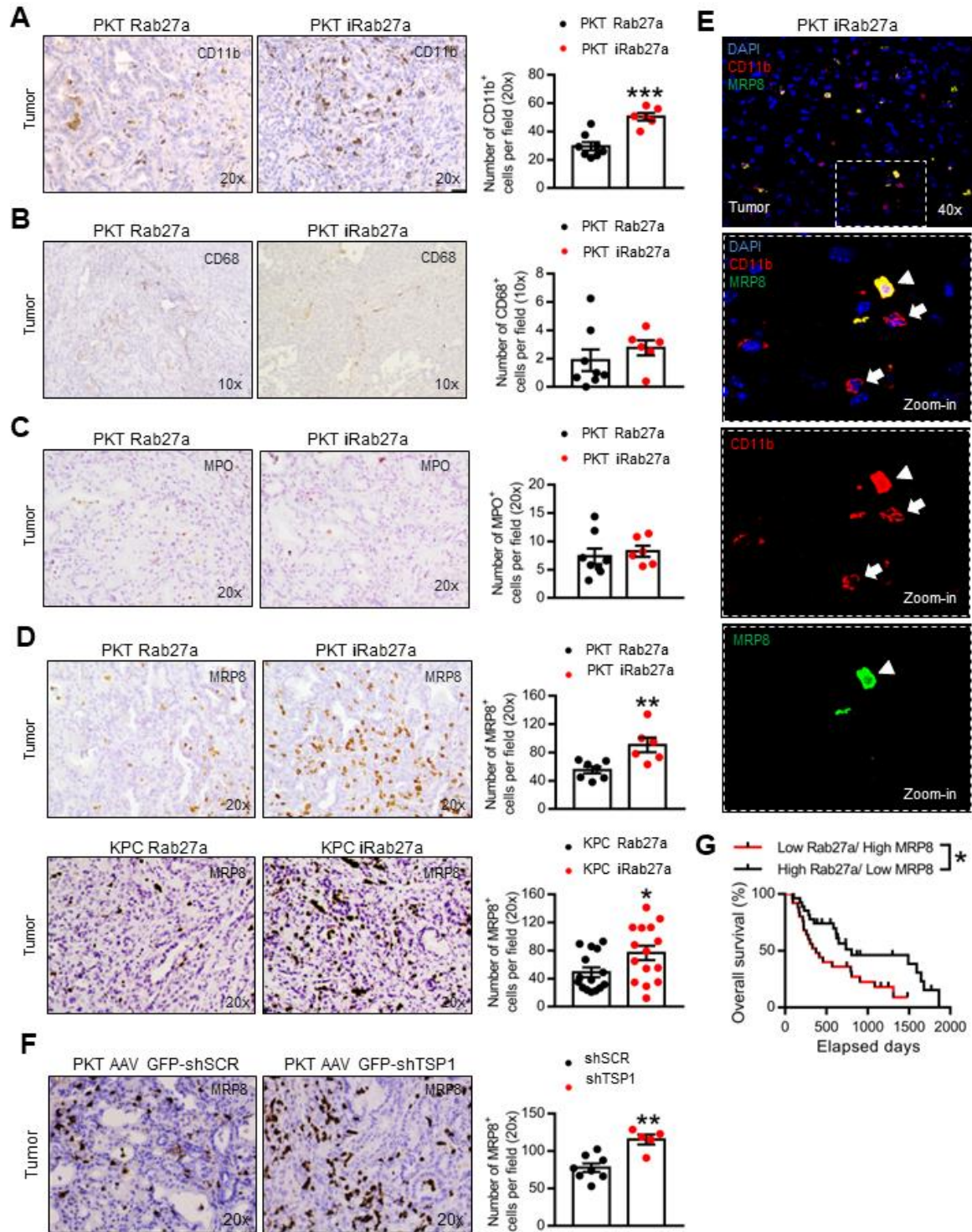


Figure 8. Rab27a knockout tumors present an enrichment in MRP8⁺ myeloid cells. (A) Representative CD11b IHC images (20x, left) and respective quantification (right) in PKT Rab27a (n=8) and PKT iRab27a (n=6) tumors (Unpaired t-test *** $p < 0.01$). (B, C) Representative CD68 (B) and MPO (C) IHC images (10x, left) and respective quantification (right) in PKT Rab27a (n=8) and PKT iRab27a (n=6) tumors (Unpaired t-test). (D) Representative MRP8 IHC images (20x, left) and respective quantification (right) in PKT Rab27a (n=7, one mouse was removed from the analysis since it was identified as an outlier) and PKT iRab27a (n=6) tumors (top) and in KPC Rab27a (n=15)

and KPC iRab27a (n=15) tumors (bottom, unpaired t-test $*p<0.05$, $**p<0.01$) **(E)** Representative IF images of PKT iRab27a tumors (40x and zoom-in). CD11b – red, MRP8 – green and DAPI – blue. Arrows indicate CD11b⁺ cells and triangles indicate CD11b⁺ MRP8⁺ cells. **(F)** Representative MRP8 IHC images (20x, left) and respective quantification (right) in PKT AAV-shSCR (n=8) and PKT AAV-shTSP1 (n=5) tumors (Unpaired t-test $**p<0.01$). **(G)** Kaplan-Meier survival curve of low Rab27a/High MRP8 (n=25) and High Rab27a/Low MRP8 (n=27) PDAC patients (right, Log-rank Mantel-Cox test $*p<0.05$). Data are mean $\pm$ SEM.

Thus, in parallel with the role of MRP8 in rheumatoid arthritis, we hypothesized that MRP8 could stimulate cancer-associated fibroblasts (CAFs) to produce IL-6 in Rab27a KO tumors (Lee, Woo et al. 2013). This inflammatory tumor microenvironment populated by MRP8⁺ myeloid cells and inflammatory CAFs (iCAFs – CAFs with increased expression of IL-6) could be the basis for the predominant Th17 immune response in Rab27a KO tumors (Lee, Woo et al. 2013, Öhlund, Handly-Santana et al. 2017). To test our hypothesis, we isolated CAFs from a PKT tumor and performed *ex vivo* treatments with recombinant MRP8 mouse protein. We showed that IL-6 expression by CAFs was significantly increased upon treatment with MRP8 protein (Figure 9A). Moreover, we showed that this activation is dependent on the expression of TLR4 in iCAFs (Figure 9B). TLR4 has been described as the major receptor for MRP proteins (Ehrchen, Sunderkötter et al. 2009, Loser, Vogl et al. 2010). We showed that CAFs isolated from a PKT tumor express TLR4 in the cytoplasm and at the cell membrane. Its downregulation by small interfering RNA reduced the expression of IL-6 by approximately 50%, in MRP8-stimulated CAFs (Figure 9B and 9C). Interestingly, the number of α SMA⁺ CAFs in Rab27a KO tumors was not altered (Figure 9D). Thus, we demonstrate that MRP8 expressed by CD11b⁺ myeloid cells stimulate CAFs to secrete IL-6.

To test whether the release of IL-6 by iCAFs is sufficient to promote the differentiation of CD4⁺ T cells into a Th17 phenotype in Rab27a KO tumors, we stimulated mouse PDAC CAFs with recombinant MRP8 mouse protein and cocultured these cells *ex vivo* with naïve CD4⁺ T cells isolated from the spleen of a WT mouse. We showed that IL-17 expression was significantly increased in CD4⁺ T cells cocultured with MRP8-stimulated CAFs compared to CD4⁺ T cells cocultured with nonstimulated CAFs (Figure 9E). To confirm that Th17 differentiation is dependent on IL-6 produced by iCAFs, the same experiment was performed in the presence of an IL-6 blocking antibody. We showed that IL-6 blockade reduced the expression of IL-17 in CD4⁺ T cells cocultured with MRP8-stimulated CAFs by half, validating its role in differentiating CD4⁺ T cells towards Th17 cells (Figure 9F).

In summary, at this point we provide evidence that loss of TSP1⁺ cEVs in Rab27a KO tumors results in neoangiogenesis and recruitment of MRP8⁺ myeloid cells that activate iCAFs in a TLR4-dependent manner to secrete IL-6, creating a Th17 prone tumor microenvironment.

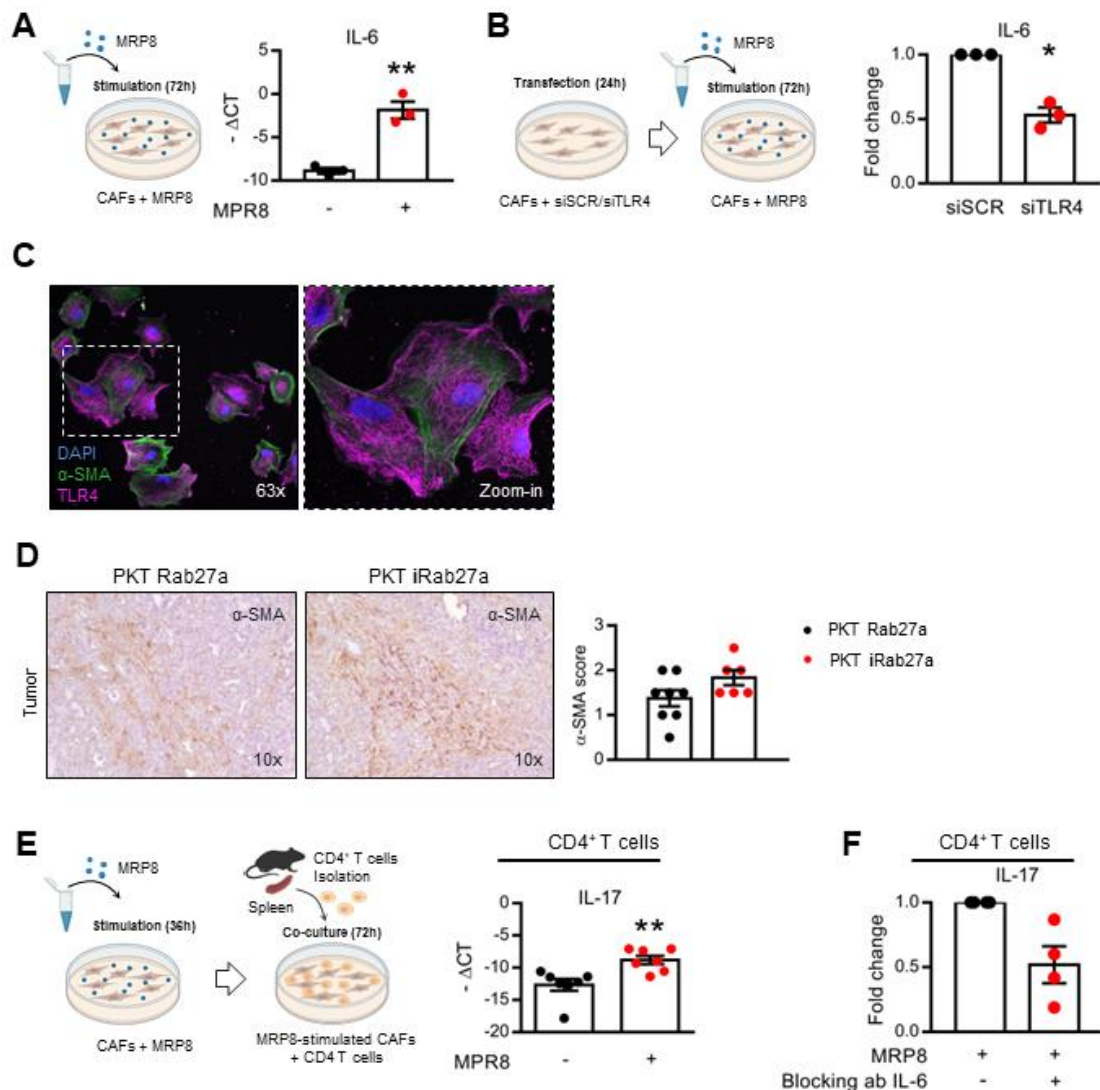


Figure 9. Myeloid-derived MRP8 promotes Th17 differentiation through CAFs-dependent IL-6 release. (A) Experimental layout (left) and IL-6 quantification by qPCR (right) in CAFs isolated from a PKT tumor stimulated with exogenous MRP8 (5µg/mL) (n=3, Unpaired t-test **p<0.01). (B) Experimental layout (left) and IL-6 quantification by qPCR (right) in CAFs isolated from a PKT tumor stimulated with exogenous MRP8 (5µg/mL) after transfection with a siSCR or siTLR4 (n=3, Unpaired t-test *p<0.05) (C) Representative IF images of CAFs isolated from a PKT tumor (63x and zoom-in). TLR4 – purple, α-SMA – green and DAPI – blue. (D) Representative α-SMA IHC images (10x, left) and respective quantification (right) in PKT Rab27a (n=8) and PKT iRab27a (n=6) tumors (Unpaired t-test). (E) Experimental layout (left) and IL-17 quantification by qPCR (right) in CD4⁺ T cells co-culture (n=3, Unpaired t-test **p<0.01). (F) Experimental layout (left) and IL-17 quantification by qPCR (right) in CD4⁺ T cells co-culture (n=3, Unpaired t-test *p<0.05).

cultured with CAFs isolated from a PKT tumor previously stimulated with exogenous MRP8 (5µg/mL) (n=7, Unpaired t-test ** $p < 0.01$). **(F)** IL-17 quantification by qPCR in CD4⁺ T cells co-cultured with CAFs isolated from a PKT tumor previously stimulated with exogenous MRP8 (5µg/mL) in the presence of a blocking antibody for IL-6 (2µg/mL) (n=4, Unpaired t-test). Data are mean±SEM.

3.3 Loss of Rab27a expression in healthy pancreas does not alter its histology or physiology

Chronic pancreatitis is one of the main risk factors associated with PDAC (Gandhi, de la Fuente et al. 2022). Since there are major alterations in Rab27a KO tumors that concern an inflammatory prone environment, we have set to determine if Rab27a KO in a healthy pancreas could foster an inflammatory environment simulating pancreatitis and driving PDAC initiation. To answer this, we developed the Pdx1 Rab27aKO model (*Pdx1-Flp; Rab27a^{Frt/Frt}*, Figure 10A). Loss of Rab27a expression in healthy pancreatic cells was confirmed by immunohistochemistry (Figure 10B). We observed that Rab27a KO mice euthanized at 8 weeks of age did not show alterations in body weight, pancreas volume and histology, blood glucose, amylase or insulin levels (Figure 10C-G). Additionally, to exclude any possible effects of the tamoxifen used in our cancer models, we developed the Ptf1a iRab27a model (*Ptf1a-Cre; R26^{LSL-FLPoERT2/+}; Rab27a^{Frt/Frt}*) in which Rab27a KO is induced by tamoxifen treatment (Figure 10H). Rab27a KO mice treated with tamoxifen from birth and sacrificed at 17 weeks presented no changes in body or pancreas weights in comparison to the controls (Figure 10I and 10J). Histological analysis confirmed no alterations in the pancreas (Figure 10K). Most importantly, cytokine profile of Rab27a KO healthy pancreas did not present major changes in comparison with Rab27a WT healthy pancreas, namely in IL-6, IL-23, GM-CSF or ICAM-1 (Figure 10L). Overall, our data shows that Rab27a KO does not alter healthy pancreas development and function, which indicates that our phenotype is cancer specific.

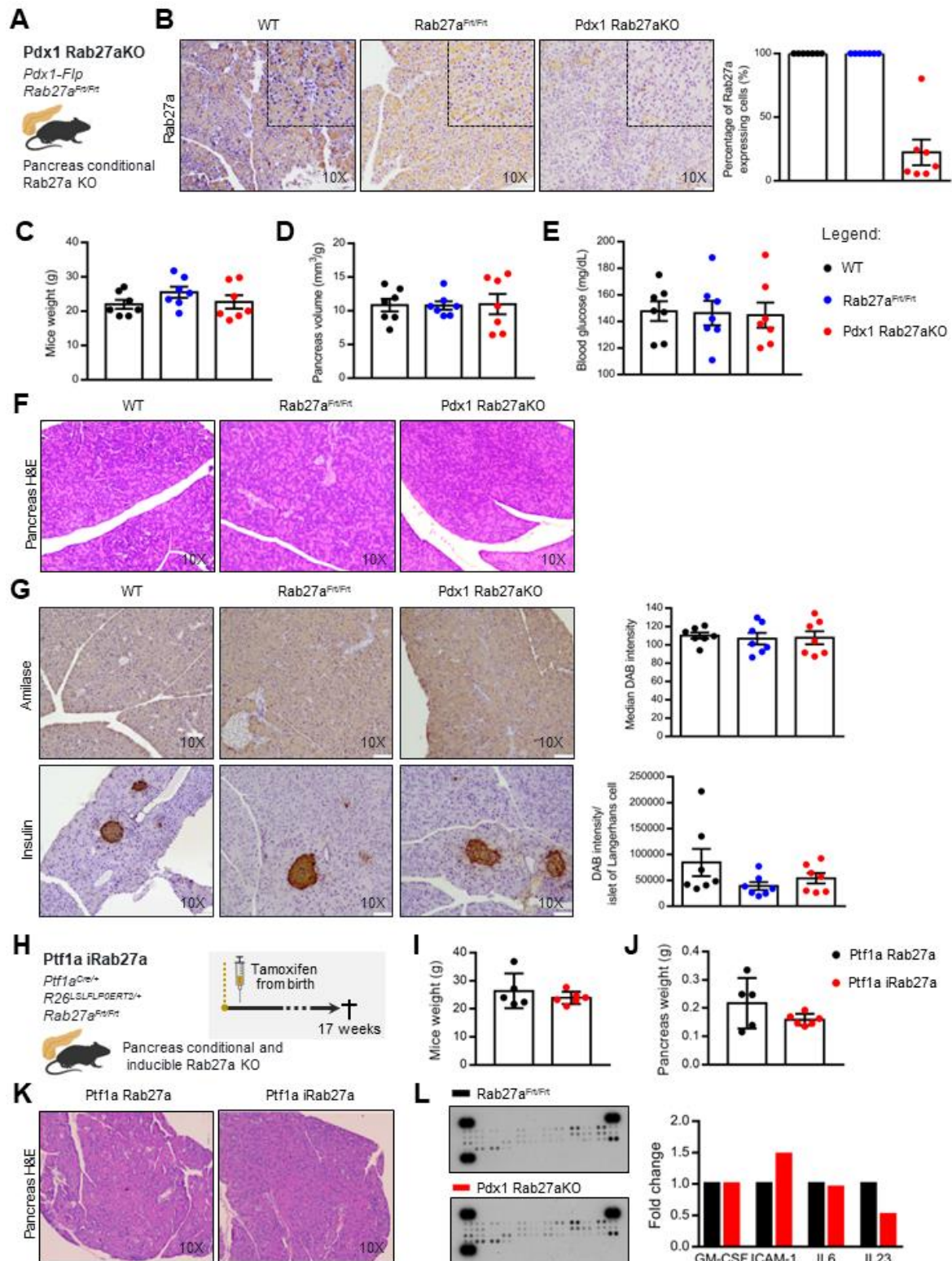


Figure 10. Rab27a knockout in pancreatic cells does not alter pancreas development and function. (A) Schematic representation of the Pdx1 Rab27aKO model. The expression of the Pdx1-Flp and Rab27a^{FRT/FRT} alleles allows the conditional Rab27a knockout in pancreas cells. (B) Representative Rab27a IHC images (10x and zoom-in, left) and respective quantification (right) in pancreas of WT (n=7), Rab27a^{FRT/FRT} (n=7) and Pdx1 Rab27aKO (n=7) mice (Unpaired t-test). (C, D, E) Mice weight (C), pancreas volume (D) and blood glucose (E) of WT (n=7), Rab27a^{FRT/FRT} (n=7)

and Pdx1 Rab27aKO (n=7) mice. **(F)** Representative photos of H&E staining of WT, Rab27a^{FRT/FRT} and Pdx1 Rab27aKO pancreas (10x). **(G)** Representative amilase and insulin IHC images (10x, left) and respective quantification (right) in pancreas of WT (n=7), Rab27a^{FRT/FRT} (n=7) and Pdx1 Rab27aKO (n=7) mice (Unpaired t-test). **(H)** Schematic representation of the Ptf1a iRab27a model. The expression of the Ptf1a-Cre, R26^{LSLFLPOERT2/+} and Rab27a^{FRT/FRT} alleles allows the conditional and inducible Rab27a knockout in pancreas cells. Tamoxifen treatments were performed from birth and mice were euthanized at 17 weeks. **(I, J)** Mice weight **(I)** and pancreas weight **(J)** of Ptf1a Rab27a (n=5) and Ptf1a iRab27a (n=6) mice. **(K)** Representative photos of H&E staining of Ptf1a Rab27a and Ptf1a iRab27a pancreas (10x). **(L)** Representative cytokine array (left) and respective quantification (right) of highlighted proteins (GM-CSF, ICAM-1, IL-6 and IL-23) of Rab27a^{FRT/FRT} and Pdx1 Rab27aKO pancreas. Data are mean±SEM.

3.4 Activation of SRC signaling in PDAC tumors with impaired secretion of cEVs fuels their proliferative capacity

We then asked how the tumor microenvironment of Rab27a KO tumors activates the proliferation of cancer cells, worsening the disease. Analysis of the mass spectrometry data of whole tumors identified overexpression of SRC protein (Figure 11A). STRING analysis demonstrated that SRC protein has the highest number of interacting proteins within overexpressed proteins in Rab27a KO tumors (Figure 11B). SRC promotes cell proliferation, survival and migration through activation of signaling in different cancer-related pathways (Jiao, Bi et al. 2018). Moreover, SRC was previously described to play a role in the interplay between the immune response and cancer (Liu, Pham et al. 2013). Gene set enrichment analysis (GSEA) identifies an enrichment for several signaling pathways activated by SRC, such as FGFR, MET, SCF-KIT and ERKs, in Rab27a KO tumors (Figure 11C) (Jin 2020, Pathania, Pentikäinen et al. 2021). Thus, we validated the activation of SRC signaling through pSRC staining in cancer cells in the two Rab27a KO PDAC models (PKT iRab27a and the KPC iRab27a, Figure 11D). Consequently, Rab27a KO tumors present enhanced proliferative activity, demonstrated by Ki67 (Figure 11E).

Most importantly, we demonstrate that Rab27a KO cells *in vitro*, in the absence of any component of the tumor microenvironment, do not show overactivation of SRC signaling (Figure 11F). Altogether, we provide evidence that the immune response is a key element in Rab27a KO tumors that feedbacks in the activation of SRC signaling in cancer cells, fuelling their proliferative capacity.

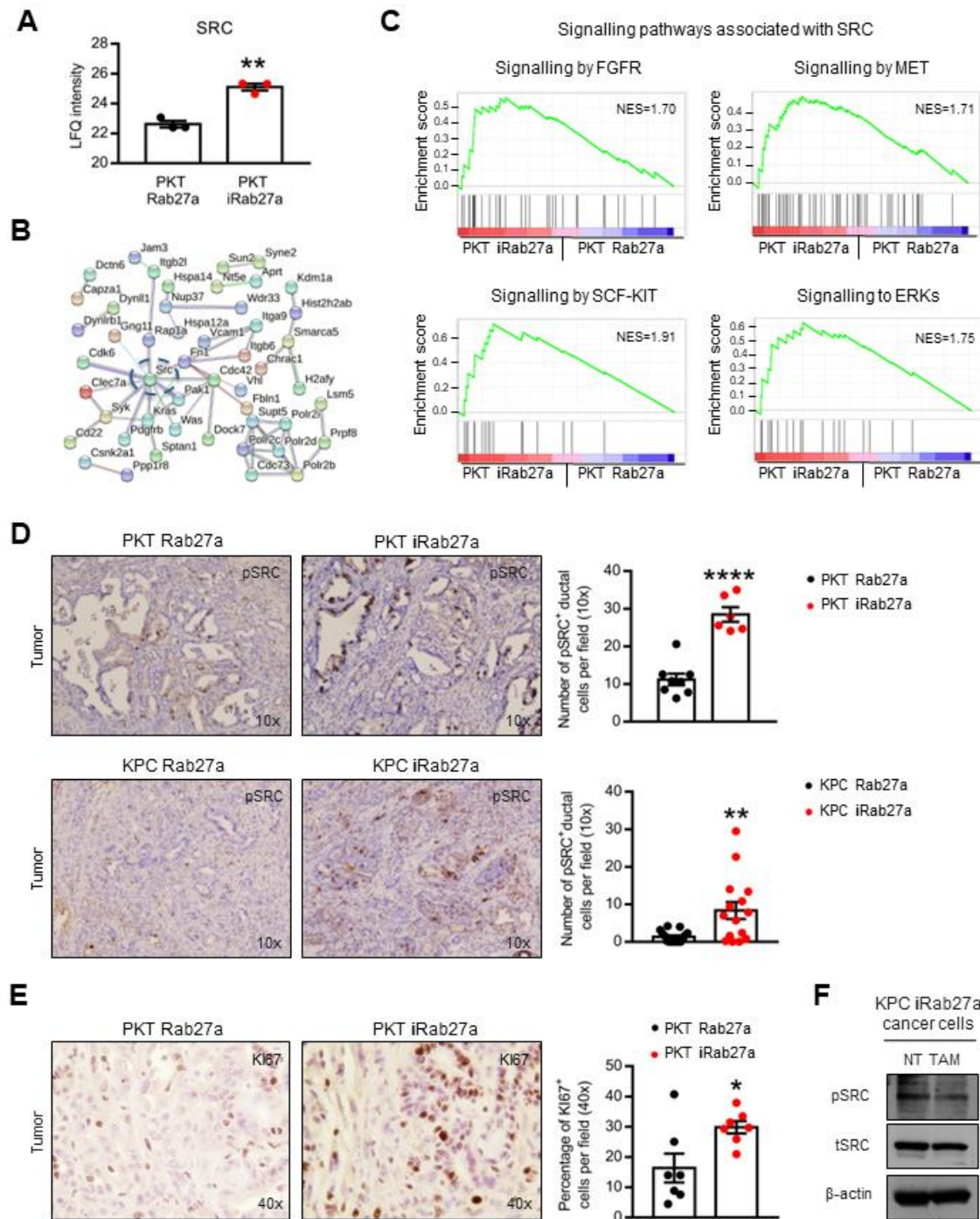


Figure 11. Rab27a knockout tumors present overactivation of SRC signaling. (A) SRC LFC intensity in PKT Rab27a (n=3) and PKT iRab27a (n=3) tumors (Unpaired t-test $**p < 0.01$). (B) String analysis of overexpressed proteins in PKT iRab27a tumors. Dashed circle depicts SRC. (C) Gene set enrichment analysis (GSEA) identifies proteins associated with signaling by FGFR, MET, SCF-KIT and to ERKs as overexpressed in PKT iRab27a tumors. (D) Representative pSRC IHC (10x, left) and respective quantification (right) in PKT Rab27a (n=8) and PKT iRab27a (n=6) mice (top) and KPC Rab27a (n=15) and KPC iRab27a (n=15) mice (bottom) treated with tamoxifen from birth (Unpaired t-test $**p < 0.01$, $****p < 0.0001$). (E) Representative KI67 IHC images (40x, left) and

respective quantification (right) in the pancreas of PKT Rab27a (n=7) and PKT iRab27a (n=7) mice euthanized at 35 days (Unpaired t-test * $p < 0.05$). **(F)** Representative total SRC (tSRC) and pSRC western blot in cancer cells isolated from tumors of KPC iRab27a non-treated (NT) or tamoxifen treated (TAM) mice. β -actin was used as a loading control. Data are mean $\pm$ SEM.

4. Evaluation of Rab27a-impaired PDAC tumors response to immunomodulatory therapies

We verified that the prevalence of MRP8⁺ myeloid cells is critical to promote a Th17 immune response in Rab27a-impaired tumors. Altogether, these alterations drive SRC signaling in cancer cells, fueling PDAC progression. With this in mind, we sought to explore the potential of using immunomodulatory therapies to target the prevailing inflammation and pro-tumorigenic immune response.

4.1 Loss of Rab27a expression in cancer cells sensitizes PDAC to immunomodulatory therapies

Given the inflammatory nature of CD11b⁺MRP8⁺ myeloid cells and the cytokines secreted by iCAFs, we treated Rab27a WT and KO mice (PKT and PKT iRab27a) with dexamethasone for 14 days starting at 21 days of age (Figure 12A). Dexamethasone is a glucocorticoid with anti-inflammatory and immune suppressant activities, and its role in suppressing IL-6 function was previously described (Levine, Larivée et al. 1993, Newton 2000). Interestingly, dexamethasone treatment impaired tumor growth in Rab27a KO tumors but not in Rab27a WT tumors (Figure 12B). Histopathological analysis of the pancreas of Rab27a KO mice treated with dexamethasone showed a significant increase in the normal area (Figure 12C). As expected, IL-6 was decreased in Rab27a KO mice treated with dexamethasone (Figure 12D). In addition, there was a significant decrease in the number of MRP8⁺ myeloid cells and CD4⁺ T cells, and decreased levels of pSRC in cancer cells of dexamethasone treated Rab27a KO tumors (Figure 12E and 12F). In summary, our data indicate that Rab27a impaired PDAC tumors rely on the presence of an inflammatory immune cell repertoire that drives disease progression.

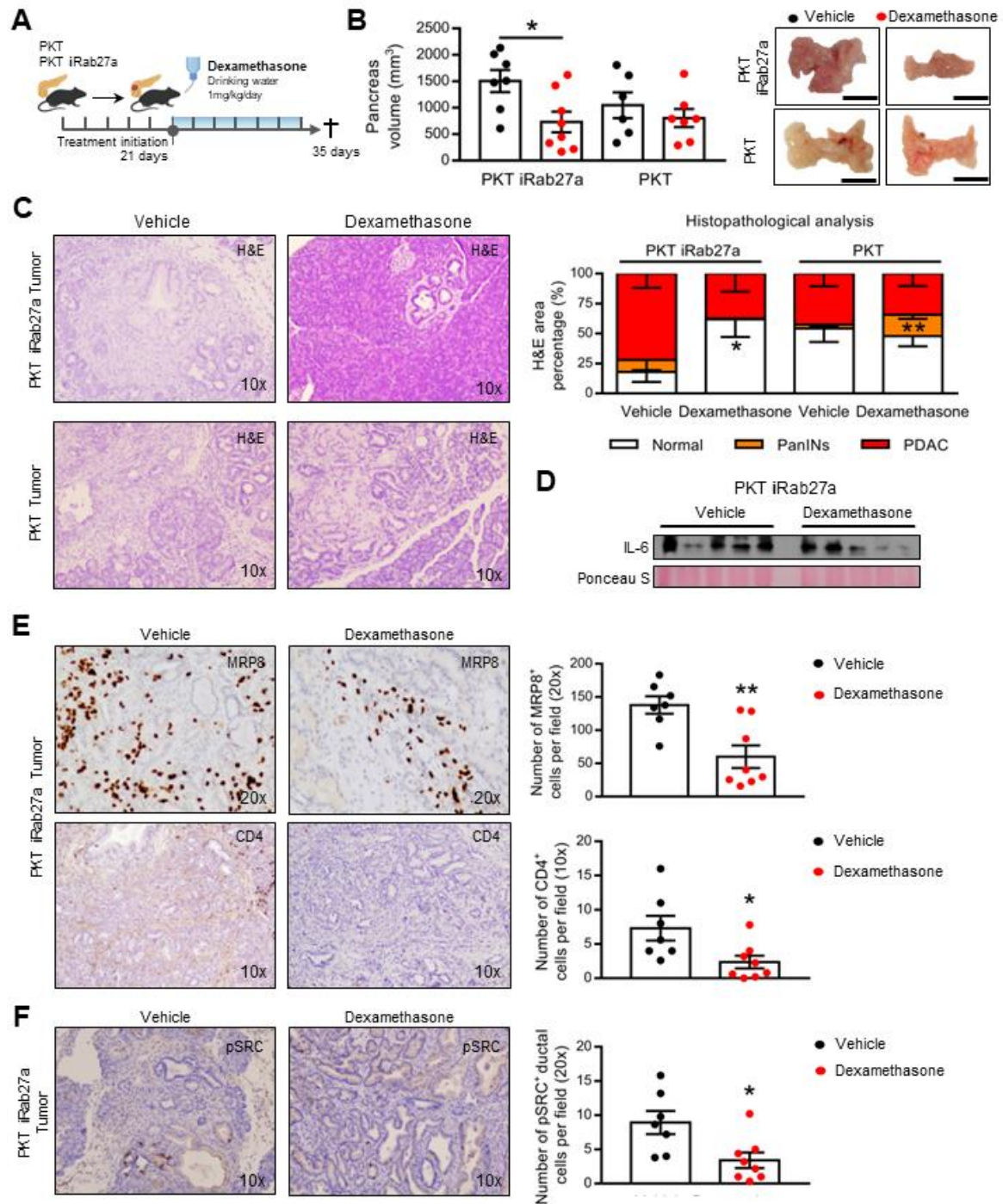


Figure 12. Dexamethasone treatment suppresses tumor growth specifically in a Rab27a knockout context in PDAC GEMMs. (A) Experimental layout of PKT and PKT iRab27a mice treated with dexamethasone in the drinking water (1mg/kg/day) starting at 21 days of age. **(B)** Representative pancreas images (right) and pancreas volume (left) of PKT and PKT iRab27a mice treated with vehicle (PKT – n=6, PKT iRab27a – n=7) or dexamethasone (PKT – n=7, PKT iRab27a – n=8) (Unpaired t-test $p < 0.05$). **(C)** Representative photos of H&E staining (left) of PKT and PKT iRab27a pancreas treated with vehicle or dexamethasone (10x) and histopathological analysis of the percentage of normal, PanINs and PDAC area (right, Unpaired t-test $p < 0.05$, $**p < 0.01$). **(D)**

Representative IL-6 western-blot in PKT iRab27a tumors treated with vehicle (n=5) or dexamethasone (n=5). Ponceau S was used as a loading control. **(E)** Representative MRP8 (top) and CD4 (bottom) IHC images (MRP8 - 20x and CD4 - 10x, left) and respective quantification (right) in PKT iRab27a tumors treated with vehicle (n=7) or dexamethasone (n=8) (Unpaired t-test * $p < 0.05$, ** $p < 0.01$). **(F)** Representative pSRC IHC images (10x, left) and respective quantification (right) in PKT iRab27a tumors treated with vehicle (n=7) or dexamethasone (n=8) (Unpaired t-test * $p < 0.05$). Data are mean $\pm$ SEM.

Finally, we treated Rab27a WT and KO mice (PKT and PKT iRab27a) weekly with an anti-CD4 depleting antibody, starting at 21 days of age (Figure 13A). A significant decrease in tumor growth was observed only in Rab27a KO and not in Rab27a WT tumors, both treated with anti-CD4 (Figure 13B). This indicates that CD4⁺ T cells, which present predominantly a Th17 phenotype in Rab27a KO tumors, have a tumor-promoting function in these tumors but not in Rab27a WT tumors. We also demonstrated a decrease in IL-17 levels in anti-CD4-treated Rab27a KO tumors, reflecting the Th17 immune response present in these tumors (Figure 13C). In addition, we showed a decrease in pSRC levels in cancer cells in Rab27a KO tumors treated with an anti-CD4 depleting antibody, further solidifying the role of the Th17 enriched tumor microenvironment in activating this pathway in Rab27a KO cancer cells (Figure 13D). We confirmed CD4⁺ T cell depletion in the tumor and spleen by flow cytometry (Figure 13E and 13F). In summary, we demonstrated the pro-tumorigenic Th17 nature of the CD4⁺ T cells specific to Rab27a KO tumors. Our data unravel the potential to target the Th17 immune response in low Rab27a expressing PDAC to impair tumor growth.

In contrast to the treatment of Rab27a KO tumors with dexamethasone or an anti-CD4 depleting antibody, gemcitabine treatment was ineffective to impair the disease progression of these tumors highlighting the benefit of using such immunotherapeutic approaches in comparison with the current PDAC standard of care (Figure 13G). Altogether, our data demonstrate the biological significance of the Th17 immune response and inflammation in the progression of Rab27a KO tumors.

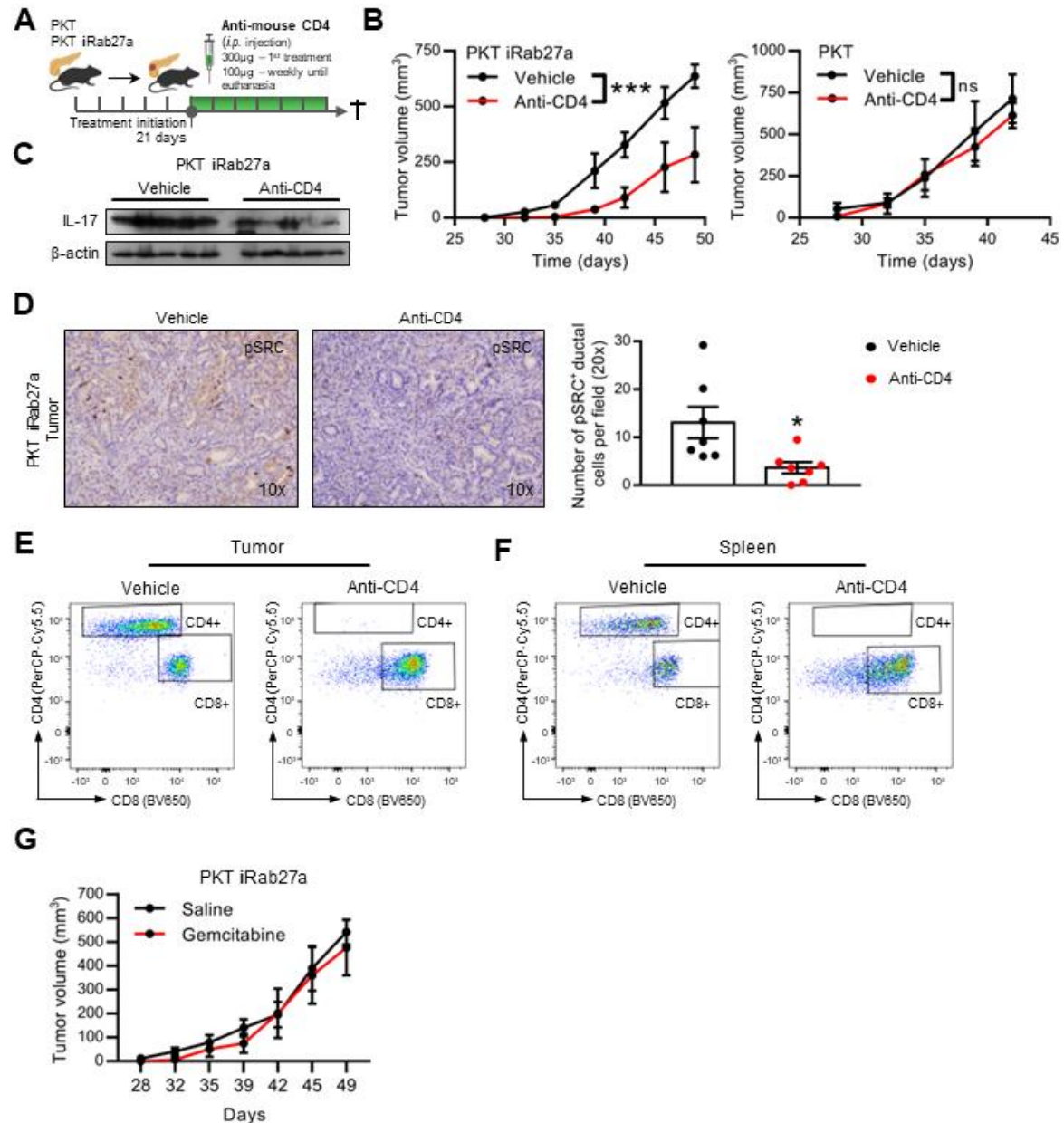


Figure 13. Anti-CD4 treatment suppresses tumor growth specifically in a Rab27a knockout context in PDAC GEMMs. (A) Experimental layout of PKT and PKT iRab27a mice treated with a depleting antibody targeting mouse CD4 starting at 21 days of age. The first treatment consists in 300µg of depleting antibody targeting mouse CD4 followed by 100µg of antibody treatment performed weekly until timepoint of euthanasia. **(B)** Tumor growth curve monitored by ultrasound of PKT and PKT iRab27a treated with vehicle (PKT – n=5, PKT iRab27a – n=7) or anti-CD4 (PKT – n=5, PKT iRab27a – n=7) (Two-way anova *** $p < 0.001$). **(C)** Representative IL-17 western-blot in PKT iRab27a tumors treated vehicle (n=5) or anti-CD4 (n=5). β -actin was used as a loading control. **(D)** Representative pSRC IHC images (10x, left) and respective quantification (right) in PKT iRab27a tumors treated with vehicle (n=7) or anti-CD4 (n=7) (Unpaired t-test * $p < 0.05$). **(E, F)** Representative flow cytometry plot of the tumor (**E**) and spleen (**F**) of PKT iRab27a mice treated with vehicle or anti-

CD4 stained for CD4 and CD8. TCR- β was used to identify T-cells. **(G)** Tumor growth curve monitored by ultrasound of PKT iRab27a treated with vehicle (n=6) or gemcitabine (n=5, 50mg/kg, 2 times per week) (Two-way anova). Data are mean $\pm$ SEM.

5. Identification of the mechanisms regulating Rab27a expression in PDAC

We verified using preclinical models that Rab27a and Rab27a-dependent cEVs communication have a tumor suppressive role through modulation of angiogenesis and the immune response in PDAC. Finally, we aimed to understand how Rab27a expression and function behaves throughout PDAC progression in the human disease and what are the regulatory mechanisms taking place in such process.

5.1 Rab27a expression decreases throughout PDAC progression in human samples

First, we analyzed the protein expression of Rab27a in a cohort of 179 human PDAC primary tumors, stages I through IV. We showed that around 24% of PDAC patients do not express Rab27a in the primary tumor (Figure 14A). We also observed that Rab27a expression decreased significantly in areas of the tumors that reflect the progression of the disease. First, we identified a significant decrease in Rab27a protein levels in cancer tissue in comparison with normal pancreas (Figure 14B). Second, the same decrease in Rab27a expression was observed in undifferentiated PDAC tissue areas in comparison to differentiated PDAC within the same tumor (Figure 14C).

Overall, our data indicate that, during disease progression, a decrease in Rab27a expression could be an advantage for tumor growth. Most importantly, we have identified a subset of PDAC patients who do not show expression of Rab27a in the primary tumor and could benefit from immunomodulatory therapies (Figure 15).

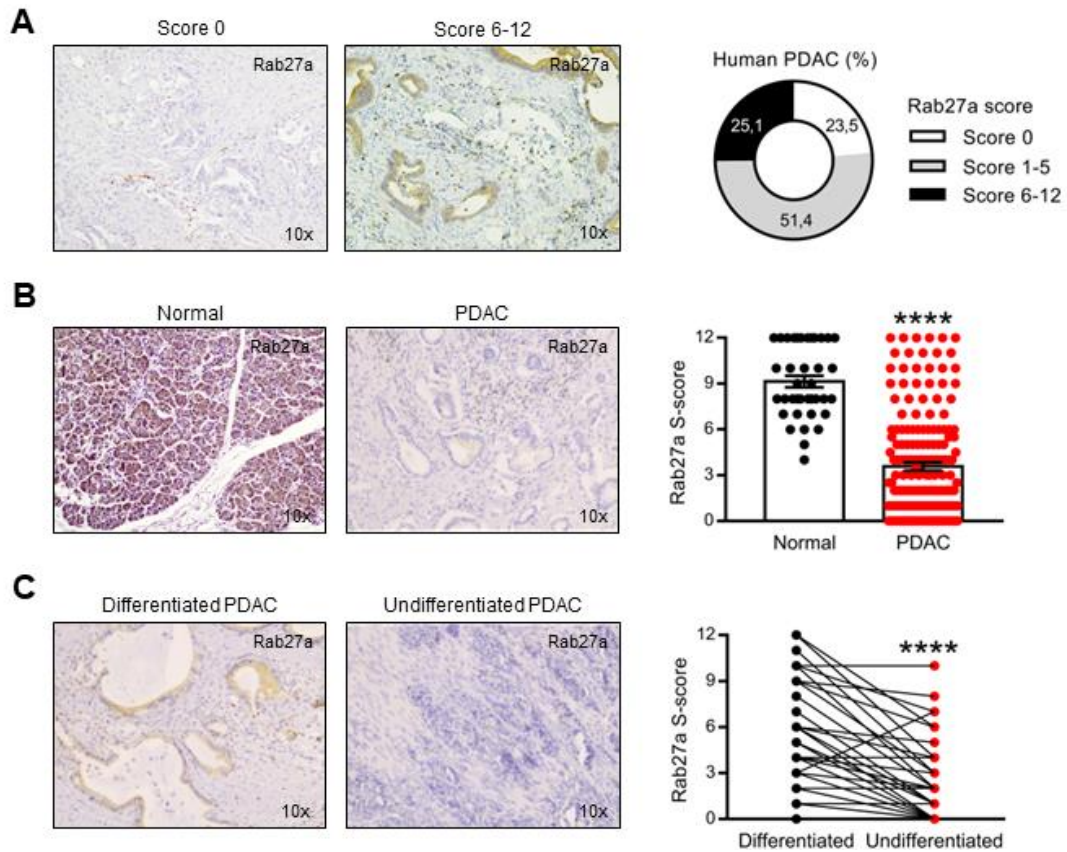


Figure 14. Rab27a is lost during PDAC progression. (A) Representative Rab27a IHC images in a human PDAC primary tumor with score 0 and score 6-12 (10x, left) and percentage of PDAC patients with score 0, score 1-5 and score 6-12 of Rab27a expression (n=179, right). (B) Representative Rab27a IHC images (10x, left) and respective quantification (right) in normal (n=38) and PDAC (n=179) issues from human specimens (Unpaired t-test **** $p<0.0001$). (C) Representative Rab27a IHC images (10x, left) and respective quantification (right) in differentiated and undifferentiated PDAC tissue from the same human specimen (n=52, Paired t-test **** $p<0.0001$). Data are mean $\pm$ SEM.

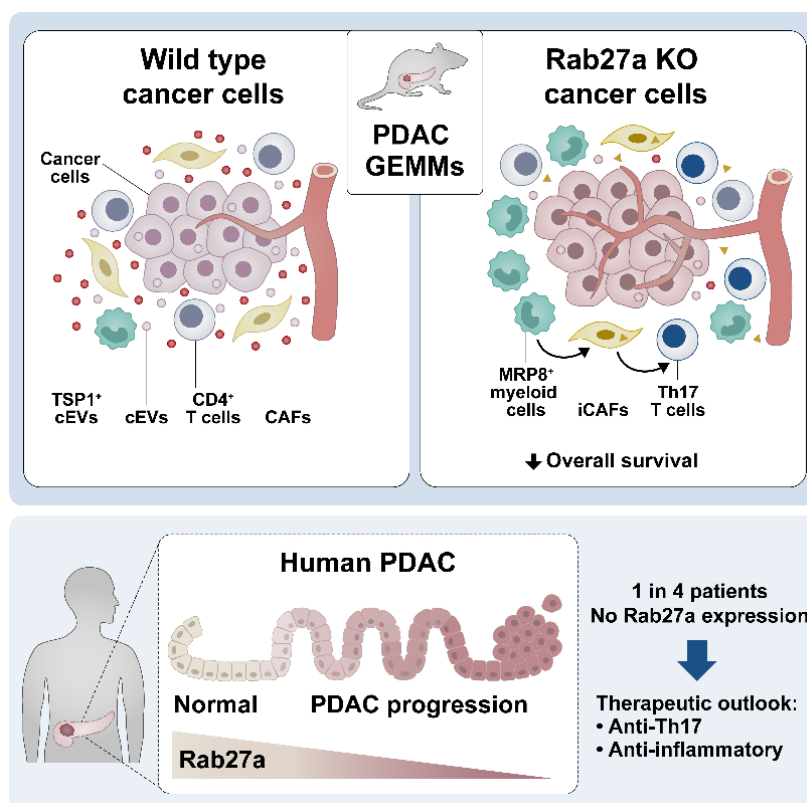


Figure 15. Schematic illustrating the pro-tumorigenic immune response present in a context of impaired Rab27a and cEVs communication in PDAC and the potential therapeutic outlook of PDAC patients based on Rab27a expression.

5.2 Epigenetic regulation of SMC3 modulates Rab27a expression in PDAC

Expression of Rab27a in human samples decreases during the course of the disease. In addition, we showed that loss of Rab27a expression accelerates disease progression in PDAC GEMMs. In sum, Rab27a gene behaves as a tumor suppressor in PDAC.

Therefore, we went on to look for the mechanism that regulates Rab27a expression in these tumors. Genetic and epigenetic mechanisms are the top candidates to regulate gene expression. We have queried the TCGA (Cancer Genome Atlas Program) for somatic mutations across the Rab27a gene in human PDAC samples and we have not identified any mutations that could cause the observed loss of expression of Rab27a in PDAC. Therefore, we went on and tested if epigenetic regulation could be the mechanism by which Rab27a expression is lost in PDAC. Methylation is frequently observed in human cancer and targets many genes involved in the progression of the disease. To test this hypothesis, we treated two human PDAC cell lines (PANC-1 and MIA PaCa-2) with the demethylating

agents, azacitidine and decitabine. Rab27a expression levels significantly increase in cancer cells treated with any of the two demethylating agents (Figure 16A and 16B). In stark contrast, treatments with the same demethylating agents did not alter Rab27a expression in a healthy pancreas cell line (hTERT-HPNE, Figure 16C). Hence, our data shows that an epigenetic regulatory mechanism of Rab27a expression occurs specifically in cancer. We went on and interrogated the DNMIIVD platform for Rab27a methylation levels in human PDAC tissue and healthy pancreas (Ding, Chen et al. 2020). Our data demonstrates that methylation levels were not different between healthy and human PDAC samples (Figure 16D). Therefore, we could conclude that Rab27a expression is indirectly regulated by an epigenetic-driven mechanism.

We next tested the hypothesis that indirect epigenetic regulation of transcription factors that bind Rab27a gene could control its expression and explain our observations. Thus, we screened the genome browser to identify transcription factors that bind the promoter region of Rab27a. In addition, we filtered for those transcription factors that, besides binding to Rab27a, are also methylated in human PDAC samples (Figure 16E). SMC3 emerged as one of the best candidates because it has two binding regions in the Rab27a gene and is methylated in PDAC (Figure 16F). SMC3 is part of the cohesin complex, which mediates sister chromatid cohesion, double-stranded DNA break repair and regulates gene expression (Sun, Nishino et al. 2013). We demonstrate that SMC3 methylation levels are increased in PDAC tissue in comparison to healthy pancreas using the DNMIIVD platform (Figure 16F) (Ding, Chen et al. 2020). Most importantly, high levels of SMC3 methylation are associated with worse prognosis in PDAC (Figure 16G) (Ding, Chen et al. 2020). Thus, we went on to validate that SMC3 is regulated by methylation and determine if the expression of Rab27a is dependent on the expression of SMC3. We treated PANC-1 and MIA PaCa-2 cell lines with demethylating agents, azacitidine and decitabine. In agreement with our previous observations, treatment with demethylating agents, led to a significant increase in SMC3 expression, suggesting that this gene is commonly methylated in PDAC (Figure 16H and 16I). Next, to understand if Rab27a expression is dependent on SMC3, we transfected MIA PaCa-2 cells with a short hairpin RNA (shRNA) targeting SMC3 and evaluated Rab27a protein levels. Upon SMC3 knockdown we observed a decrease in Rab27a protein levels suggesting that Rab27a could be regulated by SMC3 (Figure 16J). Notably, SMC3 expression levels positively correlate with Rab27a expression in human PDAC samples (analysis of TCGA data using the GEPIA platform; Figure 16K) (Tang, Li et al. 2017).

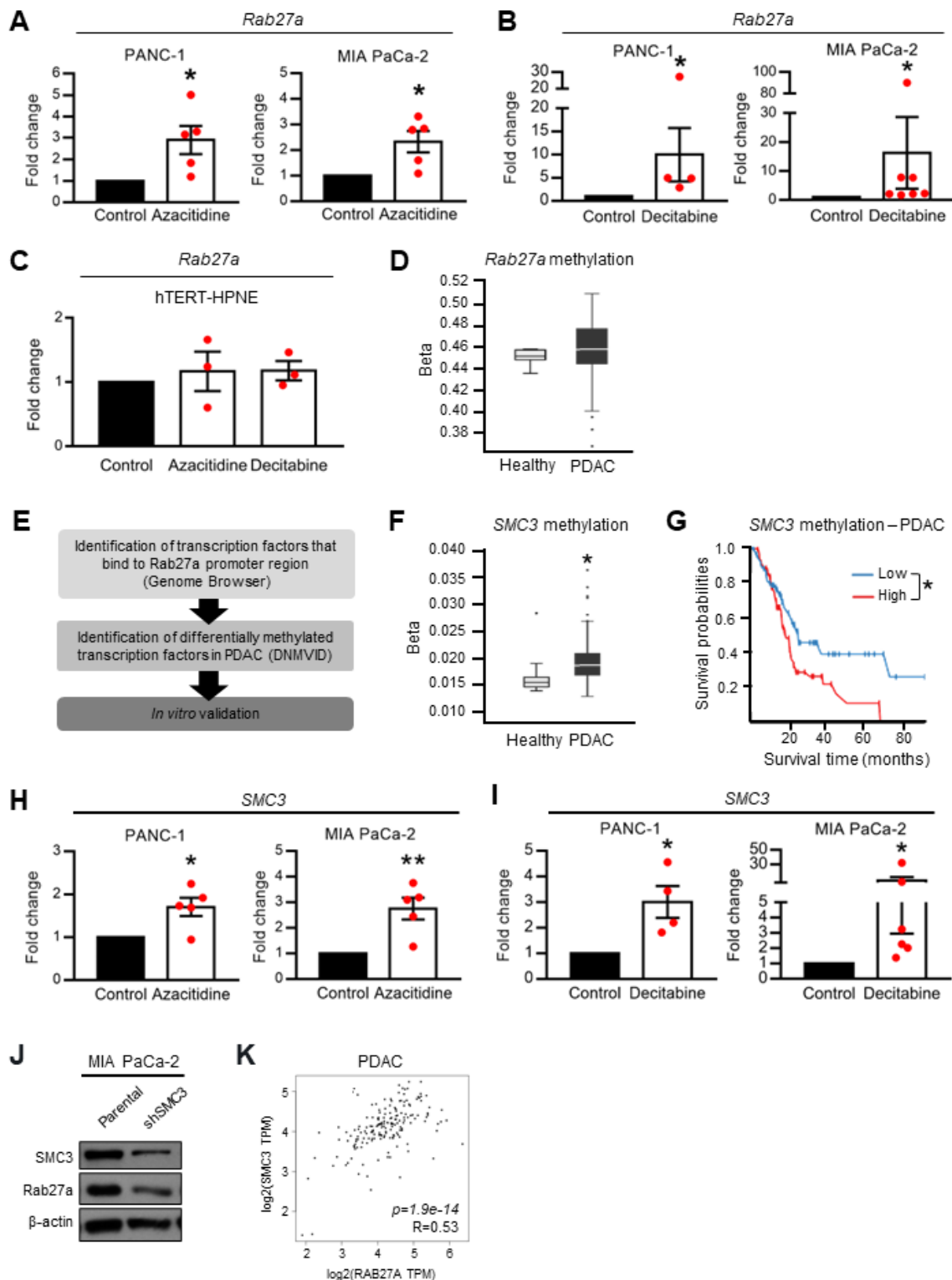


Figure 16. SMC3 epigenetic regulation modulates Rab27a expression.

(A) Fold change of Rab27a expression determined by qPCR in PANC-1 and MIA PaCa-2 treated with azacitidine (5 μ M – PANC-1, 1 μ M – MIA PaCa-2). N=5, unpaired t-test, * p <0.05. **(B)** Fold change of Rab27a expression determined by qPCR in PANC-1 and MIA PaCa-2 treated with decitabine (5 μ M – PANC-1, 1 μ M – MIA PaCa-2). N=4 in PANC-1, n=7 in MIA PaCa-2, unpaired t-test, * p <0.05. **(C)**

Fold change of Rab27a expression determined by qPCR in hPNE-hTERT treated with azacitidine (1 μ M) or decitabine (1 μ M) (n=3). **(D)** Rab27a methylation levels in healthy pancreas and PDAC in human samples. Data extracted from DNMIIVD platform. **(E)** Pipeline to determine potential targets that regulate Rab27a expression in PDAC. **(F)** SMC3 methylation levels in healthy pancreas and PDAC in human samples. Data extracted from DNMIIVD platform. Unpaired t-test, *p<0.05. **(G)** Kaplan-Meier curve of the overall survival of PDAC patients with low (blue) or high (red) levels of SMC3 methylation. Data extracted from DNMIIVD platform. Log-rank test, *p<0.05. **(H)** Fold change of SMC3 expression determined by qPCR in PANC-1 and MIA PaCa-2 treated with azacitidine (5 μ M – PANC-1, 1 μ M – MIA PaCa-2). N=5, unpaired t-test, *p<0.05, **p<0.01. **(I)** Fold change of SMC3 expression determined by qPCR in PANC-1 and MIA PaCa-2 treated with decitabine (5 μ M – PANC-1, 1 μ M – MIA PaCa-2). N=4 in PANC-1, n=6 in MIA PaCa-2, unpaired t-test, *p<0.05. **(J)** Representative western blot of SMC3 and Rab27a in MIA PaCa-2 parental cell line or transfected with a shSMC3. **(K)** RNA expression correlation of SMC3 and Rab27a in human PDAC samples. R=0.53, p=1.9x10⁻¹⁴. Data extracted from TCGA. Data are Mean $\pm$ SEM.

5.3 SMC3 mediates Rab27a expression by supporting promoter-enhancer interactions

SMC3 within the cohesin complex regulates gene expression through 3D genome organization facilitating promoter and enhancer contacts (Cuartero, Weiss et al. 2018). Using the UCSC genome browser chromatin-immunoprecipitation sequencing (ChIP-seq) tracks for SMC3, we verified that SMC3 is able to bind the promoter region of Rab27a gene as well as two other regions within close proximity (Figure 17A) (Kent, Sugnet et al. 2002). Based on the presence of high histone 3 lysine 4 mono-methylation and histone 3 lysine 27 acetylation marks present in one of the regions, we defined these locations as enhancer and next to enhancer (NTE) regions (Figure 17A). We postulated that NTE serves as an anchor region to bring Rab27a enhancer and promoter regions together. Indeed, we have used available databases to study in situ chromatin interactions by means of Hi-C chromatin structure analysis (Krietenstein, Abraham et al. 2020). We showed that NTE, where SMC3 binds with high affinity, contacts with the promoter region of Rab27a (Figure 17A). To support our hypothesis, we validated through ChIP-qPCR the binding of SMC3 to these three regions. In accordance with what is observed in SMC3 ChIP-seq datasets, we observed high SMC3 binding to the promoter and NTE regions in a human PDAC cell line (Figure 17B). In order to confirm that the region defined as an enhancer, and specifically the SMC3 binding site, has the ability to promote gene expression, we cloned this sequence into a pGL3-Promoter luciferase reporter plasmid. Strikingly, both the sense and anti-sense sequences of the SMC3 binding site in the enhancer region led to an increase in the

luminescence signal, indicating that these sequences have enhancer function (Figure 17C). Next, to determine the impact on Rab27a expression of the enhancer and NTE regions, we designed different CRISPR sgRNA sequences to target both regions (Figure 17D). Small-guided RNA sequences E1, E2 and E3 were designed to target the enhancer region of Rab27a (Figure 17D). Additionally, sgRNA sequence NTE1 was designed to target the SMC3 binding site in the NTE region (Figure 17D). We show that all sgRNA sequences led to a decrease in Rab27a protein levels (Figure 17E). The combination of three sgRNA sequences targeting the enhancer region and the single sgRNA targeting the NTE region were the conditions that most impacted Rab27a protein levels (Figure 17E). These results demonstrate that the enhancer and the NTE regions are important for Rab27a expression. In addition, the NTE region also appears to be a crucial anchor point, through an SMC3-dependent spatial gene organization mechanism (Figure 17F).

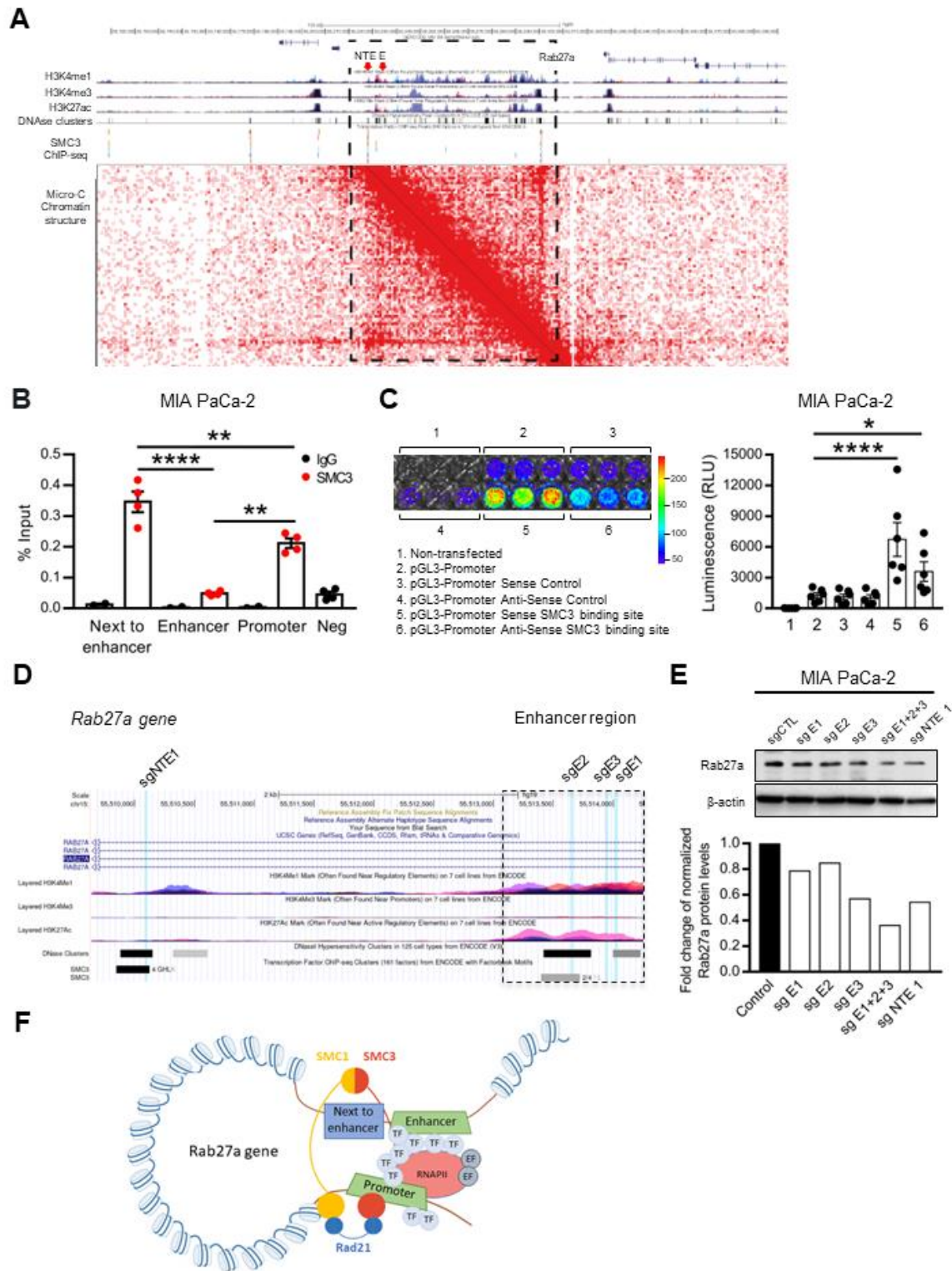


Figure 17. SMC3 binding to the *Rab27a* gene is crucial for gene expression. (A) Hi-C in the *Rab27a* gene in the H1-hESC cell line. Dashed lines highlight the *Rab27a* gene. (B) ChIP-qPCR of SMC3 for different binding sites in the *Rab27a* gene (Promoter, Enhancer and Next to enhancer regions) in MIA PaCa-2 cells (n=4). ChIP using IgG antibody was used to identify regions of specific binding. Negative region for SMC3 binding was used to confirm specificity of the enrichment. One-

way anova ** $p < 0.01$, **** $p < 0.0001$. **(C)** Representative luminescent imaging by IVIS (left) and quantification (right) of MIA PaCa-2 cells non-transfected (1), transfected with pGL3-Promoter empty vector (2), pGL3-Promoter vector with a control sequence (sense, 3), pGL3-Promoter vector with a control sequence (anti-sense, 4), pGL3-Promoter vector with SMC3 binding site in the enhancer region of Rab27a gene (sense, 5) or pGL3-Promoter vector with SMC3 binding site in the enhancer region of Rab27a gene (anti-sense, 6). $N=6$, two-way anova, * $p < 0.05$, **** $p < 0.0001$. **(D)** Genome browser panel highlighting regions in the Rab27a gene targeted by sgE1, sgE2, sgE3 and sg NTE1. Sg1 and 3 target regions of the Rab27a enhancer. SgE2 targets SMC3 binding site in the Rab27a enhancer region and sgNTE1 targets SMC3 binding site in a region next to the Rab27a enhancer. Dashed lines identify the enhancer region in the Rab27a gene. **(E)** Representative western blot for Rab27a (top) and respective quantification (bottom) in MIA PaCa-2 cells transduced with a control sg (intron region of Rab27a gene), a sg for Rab27a enhancer region 1, a sg for Rab27a enhancer region 2, a sg for Rab27a enhancer region 3, a combination of sgs for different regions of the Rab27a enhancer (1+2+3) or a sg for next to enhancer region 1. β -actin was used as loading control. **(F)** Schematic of the proposed mechanism of SMC3 binding to the Rab27a gene. Data are Mean $\pm$ SEM.

PART IV – DISCUSSION

Our work focused on elucidating the pivotal interplay between cancer cells and the immune response through cEVs. We demonstrate the release of immunomodulatory cargo within EVs by cancer cells. Disruption of EVs-mediated communication from PDAC cells promotes disease progression, specifically in the presence of the immune system. Remarkably, the presence of a pro-tumorigenic immune response is exclusive to tumors exhibiting impaired cEVs secretion (Rab27a KO), distinct from tumors proficient in cEVs secretion. The immunomodulatory effects of cEVs primarily stem from their ability to reprogram endothelial cells, influencing angiogenesis and fostering the recruitment/expansion of MRP8⁺myeloid cells. Myeloid-derived MRP8 prompts Th17 differentiation via interaction with iCAFs. Notably, we establish that the Rab27a-dependent cEVs communication network is lost as PDAC progresses in human disease, orchestrated by an epigenetic mechanism mediated by SMC3. The main findings of our work present a potential clinical application through the identification of a subset of PDAC tumors that respond to immunomodulatory therapies.

The role attributed to cEVs in this study likely pertains specifically to exosomes, rather than the diverse EVs subsets released by cancer cells. This specificity arises from Rab27a exclusive involvement in the later stages of the endocytic pathway and subsequent exosomes release. Further investigations employing alternative models are required to elucidate the functions of other subpopulations in cancer progression.

Earlier studies have demonstrated that Rab27a knockdown impairs tumor growth across various cancer types (Bobrie, Krumeich et al. 2012, Peinado, Alečković et al. 2012). In the context of PDAC, Rab27a knockdown did not yield discernible disparities in tumor growth (Kren, Michaud et al. 2020). However, these studies were either conducted in an immunodeficient background or in an immunocompetent setting where cancer cells were orthotopically injected into the pancreas. Whereas in our study we assess the role of cEVs in the natural setting of disease development. Within this framework, compromised cEVs secretion via Rab27 KO potentiated PDAC progression. This highlights the pivotal role that model systems play in studying the functions of cEVs in cancer biology. Although, it is relevant to state that these roles may diverge across different cancer types.

Communication between cancer and immune cells through EVs has been identified to exert inhibitory effects on antitumor responses and promoting an immunosuppressive microenvironment (Liu, Gu et al. 2015). In contrast to our findings, a recent study reported a decline in CD11b⁺Ly6C⁺Ly6G⁺ granulocytic cells without notable alterations in other CD11b⁺ populations within the pancreas of mice injected with a KPC shRab27a cell line

(Kren, Michaud et al. 2020). Our approach incorporated two distinct GEMMs that spontaneously develop PDAC. How immune cells recruitment and expansion are shaped in a model where natural progression of the disease occurs versus an orthotopic model, could be relevant to the differences observed. Importantly, despite the mouse origin of the KPC cell line, some degree of MHC incompatibility may persist upon injection into immunocompetent mice, potentially influencing the antitumor immune response.

Chronic pancreatitis is an established risk factor for PDAC (Gandhi, de la Fuente et al. 2022). Earlier studies unveiled that constitutional KO of both Rab27a and Rab27b genes in mice leads to a state of chronic inflammation characterized by an increase in inflammatory cytokine levels and the expansion of myeloid cells (Alexander, Ramstead et al. 2017). We have demonstrated that Rab27a deficiency specifically in healthy pancreatic cells fails to induce pancreatitis or any inflammatory condition. Given that constitutional KO model disrupts gene expression and function across all cells within the organism, the inflammatory state observed in the double Rab27a and Rab27b KO mice could possibly be attributed to gene expression impairment across diverse cell types rather than a particular cell type. In fact, the role of Rab27a in regulating granule exocytosis and motility in various immune cells, as well as dense granules in platelets, has been well-documented (Haddad, Wu et al. 2001, Bolasco, Tracey-White et al. 2011, Ramadass and Catz 2016). As a consequence, Rab27a impairment may compromise immune cells function, potentially accounting for alterations in the immune response present in the double KO mice.

TSP1, a matricellular glycoprotein, is secreted by diverse cell types (Lopez-Dee, Pidcock et al. 2011). Our findings demonstrated that TSP1 is mostly released within EVs rather than in free-form. While we cannot completely disregard the possibility of the secreted form of TSP1 influencing the observed phenotypes, the enrichment of TSP1 in cEVs renders this scenario unlikely. Notably, TSP1 alone was not capable of triggering a Th17 immune response. Therefore, it is evident that additional components within cEVs (protein or RNA) and/or modifications in the tumor microenvironment following Rab27a KO are key in modulating this multifaceted mechanism supporting disease progression.

Dexamethasone administration and CD4⁺ T cell depletion individually disrupted the protumorigenic microenvironment and suppressed tumor growth exclusively in Rab27a KO tumors. The contributory role of Th17 T cells and IL-17 in promoting disease progression during early stages of PDAC was previously described (McAllister, Bailey et al. 2014). IL-17RA and IL-17RC, primary receptors for IL-17, are frequently expressed in PDAC premalignant lesions (McAllister, Bailey et al. 2014, Zhang, Zoltan et al. 2018). Upon binding to its receptors, IL-17 induces signaling through various cancer-related pathways via SRC

activation (Zhao, Chen et al. 2019). Consequently, overactivation of SRC signaling observed in Rab27a KO tumors is likely mediated by an IL-17-driven response. This establishes a potential avenue to combine these strategies with immune checkpoint inhibitors, which have exhibited promise in other cancer models (Zhang, Chandra et al. 2020, Pelly, Moeini et al. 2021). Moreover, therapeutic interventions targeting SRC may hold potential for human PDAC tumors displaying low Rab27a expression.

Regarding the Rab27a gene in human tumors, no mutations have been reported to account for the loss of Rab27a expression in PDAC patients. In fact, our analysis of TCGA data in PDAC samples identified only one somatic mutation in a single sample. Furthermore, the gradual decline in Rab27a expression across different PDAC stages suggests the involvement of an epigenetic mechanism. Our work primarily focused on a methylation-mediated mechanism, as treatment with demethylating agents prompted an increase in Rab27a expression in PDAC cell lines. However, it is important to note that Rab27a expression might also be regulated by additional mechanisms. Several studies have highlighted the role of non-coding RNAs, particularly microRNAs (miRNAs) and long non-coding RNAs, in regulating gene expression [26]. Indeed, various miRNAs such as miR-134-3p, miR-124, and miR-582-5p have been demonstrated to suppress Rab27a expression in different models [27-29]. Exploring these non-coding RNAs and others could yield valuable insights into the modulation of exosomes communication in the context of PDAC.

SMC3, an integral component of the cohesin complex, has emerged as a prime candidate for influencing Rab27a expression. However, it is important to note that other proteins, particularly transcription factors, might also undergo differentially methylation in PDAC, therefore impacting Rab27a expression. For instance, microphthalmia-associated transcription factor (MITF) has been described to bind to two E-boxes within the Rab27a promoter, leading to enhanced transcriptional activity in melanocytes [30]. The identification of additional transcription factors that not only regulate Rab27a expression but also modulate the expression of other proteins involved in EVs biogenesis could significantly enrich our understanding of EVs biology and their functional role in cancer.

We postulate that SMC3 selectively binds to the Rab27a gene conferring a conformational change that facilitates interactions between the promoter and enhancer regions, thereby stimulating transcriptional activity. This postulation is further corroborated by insights from existing Hi-C data. To further confirm this, the use of cell lines engineered to express mutant variants of SMC3, exhibiting compromised binding to the motifs present within the Rab27a gene, would greatly enhance our understanding of this regulatory mechanism.

In summary, our work underscores that PDAC tumors characterized by compromised cEVs secretion exhibit enhanced sensitivity to therapies designed to modulate the immune response. In this context, we have identified TSP1 and Rab27a as immune modulators in PDAC. Our preclinical results illustrate a novel approach to tackle PDAC by modulating the immune response. Clinical evaluation and implementation of our findings needs to focus in individuals with diminished Rab27a expression, particularly those associated with an inflammatory microenvironment. Future clinical studies should determine the benefit of an anti-inflammatory or anti-Th17 therapy, administered alone or in combination with the current standard of care, in controlled randomized clinical trials.

PART V – CONCLUSION

Within the tumor microenvironment, cooperative interactions facilitated by cEVs have long been implicated in propelling cancer progression and fostering resistance to therapies. Yet, the intricate dynamics of this communication throughout disease development and its impact on natural disease advancement have not been explored. The work in this thesis unveils a contrasting facet of cEVs, uncovering their tumor suppressive role and influence on the efficacy of immunotherapeutic strategies. This work has described for the first time that:

- Rab27a-dependent cEVs secretion impairment in PDAC is associated with an unfavorable prognosis exclusively in an immunocompetent context.
- Impaired release of TSP1⁺ cEVs promotes angiogenesis and favors MRP8⁺ myeloid cells recruitment.
- PDAC with compromised Rab27a-dependent cEVs secretion is supported by inflammation and a Th17 response.
- Immunomodulatory treatments present notable efficacy in PDAC marked by impaired Rab27a-dependent cEVs secretion.
- SMC3-mediated epigenetic silencing constrains Rab27a expression in PDAC.

Overall, our work underscores the necessity to comprehend the multifaceted role of EVs in the cancer context. We demonstrate that cEVs, dependent on Rab27a, harbor the capacity to exert a tumor suppressive influence via the modulation of angiogenesis and immune responses. This indicates that exploiting cEVs communication as a therapeutic avenue in cancer needs to be explored with great caution. Intriguingly, within the context of impaired cEVs communication, an inflammatory immune response ensues, sensitizing PDAC tumors to immunomodulatory therapies. Using Rab27a expression status and SMC3 methylation profile as stratification markers for patients suited to such therapeutic interventions bears great promise in PDAC patients care.

Future perspectives

The work in this thesis opens new avenues in the EVs field and in pancreatic cancer biology. The establishment of a conditional Rab27a knockout model extends the purview of Rab27a and EVs research, encompassing not only diverse cancer types but also physiological contexts. The revelation of a specific context wherein PDAC presents sensitivity to immunomodulatory therapies stimulates the field to identify alternative patient subsets or novel strategies to enhance tumor responsiveness to immunotherapy. Below, we delineate pressing questions stemming from this thesis that hold promise as relevant research opportunities.

- Do all types of EVs present the same function in PDAC progression?

Here we describe for the first time a tumor suppressive role for EVs derived from cancer cells. As mentioned before, it is important to clarify that Rab27a is involved in the late stages of the endocytic pathway mediating the release of a subset of EVs, called exosomes (Ostrowski, Carmo et al. 2010). The development of additional GEMMs that target other steps of EVs biogenesis of endosomal origin or even other subsets of EVs would allow to answer this question. This line of research would potentially allow to identify subset of EVs that have an oncogenic role and others that present a tumor suppressive function, in a similar fashion to what recently was described in the cancer-associated fibroblasts field (Özdemir, Pentcheva-Hoang et al. 2014, McAndrews, Chen et al. 2022). In addition, studies that would focus in understanding the role of EVs derived from other populations of the tumor microenvironment, such as cancer-associated fibroblasts and immune cells, in a natural setting of PDAC development would be of great value.

- How are TSP1⁺ cEVs mediating MRP8⁺ myeloid cells recruitment/expansion?

We identified TSP1⁺ cEVs as regulators of angiogenesis and MRP8⁺ myeloid cells recruitment/expansion. The role of TSP1 in inhibiting angiogenesis is well defined. TSP1 either binds directly to VEGF, inhibiting its angiogenic function, or suppresses endothelial cells proliferation and migration through CD36 and CD47 (Bornstein 2009, Lawler and Lawler 2012). However, how TSP1 can modulate immune cells recruitment/expansion is not so well established. Contradictory reports either describe the ability of TSP1 to inhibit immune cells' function or to promote their migration and activity (Kaur, Bronson et al. 2021). In order to fully comprehend if the increase in intratumoral MRP8⁺ myeloid cells that occurs upon loss of Rab27a and TSP1⁺ EVs is simply a bypass effect of the increase in angiogenesis and/or dependent on TSP1 direct interaction with MRP8⁺ myeloid cells, additional experiments need to be performed. *In vivo* treatments with anti-angiogenic drugs

would allow to suppress angiogenesis in Rab27a impaired tumors and verify if the same alterations in immune cells repertoire is present. In addition, *ex vivo* treatments of MRP8⁺ myeloid cells with TSP1⁺ EVs would allow to assess if these cells become more proliferative and capable of migrating.

- What other players could be involved in Th17 differentiation in Rab27a-impaired tumors?

Despite loss of TSP1⁺ cEVs being identified as a major contributor to the phenotype observed in Rab27a-impaired tumors, no alterations in the Th17 response was observed upon loss of TSP1 in PDAC GEMMs. With this being said, we cannot exclude that other proteins present in cEVs and additional cargo, such as nucleic acids, could also play a role. Further proteomic and transcriptomic analysis could allow us to identify further molecular effectors relevant to the phenotype observed. In addition, Rab27a role in regulating protein exocytosis was also already described (Bobrie, Krumeich et al. 2012). Secretome analysis of Rab27a KO cells could potentially identify additional alterations relevant to immune cells activation and differentiation.

- How is SRC signaling mechanistically activated in cancer cells?

In Rab27a-impaired tumors we demonstrate that SRC signaling is activated in cancer cells which fuels their proliferative capacity. In addition, we validate that this activation is dependent on inflammation and a Th17 response. However, we did not describe what are the molecular effectors involved in SRC activation. We propose that IL-17 could be a critical player involved in this phenotype through IL-17RA and IL-17RC activation. To further access this, co-culture experiments of MRP8⁺ myeloid cells or Th17 T cells with cancer cells in the presence of blocking antibodies targeting molecules such as IL-17 would be needed. Identification of the molecular players involved in SRC activation could be crucial to pinpoint novel therapeutic targets to tackle the inflammatory immune response and SRC signaling present in Rab27a-impaired tumors.

- Are Rab27a-impaired tumors responsive to conventional immunotherapy?

Immunotherapy efficacy is highly dependent on the content of the tumor immune microenvironment. Thus far, PDAC has been mainly characterized as a “cold tumor” due to the lack of immune cell infiltrate that, ultimately, hinders immunotherapy success. Here, we demonstrate that loss of Rab27a and cEVs promotes immune cells recruitment and activation, specifically MRP8⁺ myeloid cells and Th17 T cells. First, it would be interesting to analyze if the expression of immune regulatory and exhaustion molecules are altered in Rab27a-impaired tumors. This type of analysis could indicate if the immune cell repertoire

in these tumors is activated and/or being regulated by checkpoint molecules which can be critical to determine immunotherapy efficacy.

We also describe an increase in angiogenesis in Rab27a KO tumors which partially mediates MRP8⁺ myeloid cells recruitment. With this being said, infiltration of activated immune cells to the tumor milieu could be facilitated in Rab27a-impaired tumors upon challenge with conventional immunotherapy, ultimately, improving treatment response.

PART VI - MATERIAL AND METHODS

Mice

NOD scid (RRID:IMSR_JAX:001303) mice were orthotopically injected in the pancreas with 1 million EpCAM⁺ cells derived from a non-treated KPC iRab27a mouse. Mice surgery was performed as described previously (Ruivo, Bastos et al. 2022). Tamoxifen treatment was performed in a subset of mice at days 3, 4, 5 and 7 post-surgery through oral gavage (300µL of a 20mg/mL solution). Mice were euthanized when presented severe symptoms. KPC (*Pdx1-Cre*; *LSL-Kras*^{G12D/+}; *LSL-Trp53*^{R172H/+}) and PKT (*Ptf1a-CRE*; *LSL-Kras*^{G12D/+}; *Tgfbr2*^{loxP/loxP}) alleles were purchased from Jackson Laboratory.: B6.FVB-Tg(*Pdx1-cre*)6Tuv/J (RRID:IMSR_JAX:014647); B6.129S4-Krastm4Tyj/J (RRID:IMSR_JAX:008179); 129S-Trp53tm2Tyj/J (RRID:IMSR_JAX:008652); Ptf1atm1(*cre*)Hnak/RschJ (RRID:IMSR_JAX:023329); B6;129-Tgfbr2tm1Karl/J (RRID:IMSR_JAX:012603). *Rab27a*^{Frt/Frt} allele was developed in collaboration with Cyagen as described previously (Ruivo, Bastos et al. 2022). The *R26*^{LSL-FLPoERT2/+} was a kind gift of David Goodrich, Roswell Park Cancer Institute, United States of America (Goodrich, Talhouk et al. 2018). The *Pdx1-Flp* allele was kindly provided by Dr. Dieter Saur, Technische Universität München, Germany (Schönhuber, Seidler et al. 2014). PKT iRab27a and PKT iRab27a *Rag2*^{-/-} (*Ptf1a-CRE*; *LSL-Kras*^{G12D/+}; *Tgfbr2*^{loxP/loxP}; *R26*^{LSL-FLPoERT2/+}; *Rab27a*^{Frt/Frt}; *Rag2*^{-/-}) and KPC iRab27a (*Pdx1-Cre*; *LSL-Kras*^{G12D/+}; *LSL-Trp53*^{R172H/+}; *R26*^{LSL-FLPoERT2/+}; *Rab27a*^{Frt/Frt}) developed spontaneous PDAC tumors in a similar way to the PKT and KPC mice, respectively. Tamoxifen treatments to induce recombination were given to pups via lactation through oral gavage of the mother with 6mg of tamoxifen diluted in corn oil (20mg/mL Sigma-Aldrich T5648) at days 0, 1, 2 and 4 post-birth. *Rag2*^{-/-} mice were a kind gift from Dr. James Di Santo, Institute Pasteur, France (Colucci, Soudais et al. 1999). Treatments with dexamethasone (Sigma-Aldrich D2915) were performed in PDAC GEMMs. PKT and PKT iRab27a mice were treated everyday (drinking water, 1mg/kg/day) starting at 21 days and euthanized at 35 days. Additionally, treatments with a depleting antibody for CD4 (Bio X Cell Cat# BE0003-1, RRID:AB_1107636) were performed in PDAC GEMMs. PKT and PKT iRab27a mice were treated weekly starting at 21 days (300µg first treatment, 100µg in the following weeks) and euthanized at 52 days. Tumor growth was monitored by ultrasound (Micro Ultrasound Vevo 2100, RRID:SCR_015816). PKT mice were orthotopically injected with 1x10¹¹ GCU adeno-associated virus 8 (AAV8) GFP-shSCR (Vector Biolabs Cat.No: 7044) or GFP-shTSP1 (Vector Biolabs Cat.No: shAAV-274145) at 21 days of age. Surgery protocol was as previously described (Ruivo, Bastos et al. 2022). Mice were euthanized when presented severe symptoms. Blood glucose levels were

measured at euthanasia using a glucose meter (Accu-Check). Facial vein was used to collect blood sample. Regarding the KPC mouse model, a cross-sectional study was performed. Mice were euthanized at different timepoints of disease progression (8 weeks, 16 weeks and HEP).

All mice were housed under standard housing conditions at the i3S animal facility, and all animal procedures were reviewed and approved by the i3S Animal Welfare and Ethics Body. Animal protocol was approved by DGAV “Direção Geral de Alimentação e Veterinária” (ID 015225).

Cell culture

The following cell lines were used in our study: human pancreatic ductal adenocarcinoma (PDAC) cell lines - MIA PaCa-2 (ATCC Cat# CRL-1420, RRID:CVCL_0428), PANC-1 (ATCC Cat# CRL-1469, RRID:CVCL_0480), BxPC-3 (ATCC Cat# CRL-1687, RRID:CVCL_0186), and T3M4 (RCB Cat# RCB1021, RRID:CVCL_4056 kindly provided by Dr. Christoph Kahlert, Universitätsklinikum Carl Gustav Carus an der Technischen Universität Dresden, Germany), mouse PDAC cell lines - KPC (Ximbio Cat# 153474), pancreas epithelial cell line - hTERT-HPNE (ATCC Cat# CRL-4023, RRID:CVCL_C466) and HEK293T (ATCC Cat# CRL-3216, RRID:CVCL_0063). Primary cell cultures of cancer cells (EPCAM⁺) and cancer-associated fibroblasts derived from KPC tumors were established and cultured. Briefly, tumors were minced and digested in digestion buffer (0,012% Dispase II Sigma-Aldrich D4693, 0,012% Collagenase Sigma-Aldrich C7657 in HBSS 1X) for 20 min at 37°C in agitation. Next, blocking buffer (FBS 10% in HBSS 1X) was added to inhibit the digestion process. Finally, cells were filtered (70µm strainer, Falcon) and incubated with Red Blood Cell lysis buffer for 5 min at 37°C. Single-cell suspension was then added to a cell culture plate. Differential trypsinization was performed as previously described to obtain enriched pool of cancer-associated fibroblasts and cancer cells (Jones 2008). All cells were tested for mycoplasma during our study, and cells derived from ATCC were STR profiled. All PDAC cell lines and HEK293T were cultured in RPMI-1640 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). Primary cultures derived from mouse tumors and hTERT-HPNE were cultured in RPMI-1640 medium supplemented with 20% (v/v) FBS 100 U/mL penicillin and 100 µg/mL streptomycin. All cell lines were kept at 5% CO₂ and 37°C in a humidified atmosphere. Cell lines were STR profiled and tested for mycoplasma.

Azacitidine and decitabine treatments

2×10^5 cells of MIA PaCa-2, PANC-1 and hTERT-HPNE were plated in 6-well plates. On the next day, cells were washed and treated with either azacitidine (Sigma-Aldrich) or decitabine (Sigma-Aldrich) for the following 96h. During the treatment period, cells were washed daily and fresh medium containing the appropriate concentration of the demethylating agent was used. In azacitidine (Sigma-Aldrich) and decitabine (Sigma-Aldrich) treatments, PANC-1 were treated with a final concentration of $5 \mu\text{M}$ and MIA PaCa-2 and hTERT-HPNE were treated with a final concentration of $1 \mu\text{M}$. Vehicle-treated cells (water/acetic acid 1:1 v/v) were used as control for both azacitidine (Sigma-Aldrich) and decitabine (Sigma-Aldrich) treatments. Technical triplicates were used in each experiment.

ChIP-qPCR

Cross-linking ChIP in MIA PaCa-2 was performed using 5×10^6 cells per immunoprecipitation. Cells were fixed using 1% formaldehyde for 10min at room temperature with gentle shaking and, afterwards, glycine was added for quenching (125 mM, incubated for 2min at room temperature). Then, cells were washed twice with cold PBS 1X, prior to scrapping. To obtain a soluble chromatin extract, cells were resuspended in 10 mL LB1 (50 mM HEPES-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 10% glycerol; 0.5% NP-40; 0.25% Triton X-100; 1x Complete protease inhibitor) and incubated in rotation at 4°C for 10min. Samples were then centrifuged and resuspended in 10 mL LB2 (10 mM Tris-HCl, pH 8; 200 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 1x Complete protease inhibitor), and incubated in rotation at 4°C for 5min. Finally, samples were centrifuged and resuspended in 3 mL LB3 (10 mM Tris-HCl, pH 8; 100 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 0.1% Na-deoxycholate, 0.5% N-lauroylsarcosine; 1x Complete protease inhibitor). Chromatin extracts were sonicated for 18 cycles of 30s ON/OFF, at high power with a Diagenode Bioruptor Plus sonicator. 1% Triton X-100 was added to the sonicated lysates and 1% was kept as whole-cell extract (WCE). The lysates were incubated with $8 \mu\text{g}$ anti-SMC3 (Abcam Cat# ab9263, RRID:AB_307122) or $2 \mu\text{g}$ anti-rabbit IgG (Thermo Fisher Scientific Cat# 10500C, RRID:AB_2532981) antibodies bound to 100 μl protein G Dynabeads (Invitrogen) and incubated overnight at 4°C . Magnetic beads were washed with RIPA buffer (140 mM NaCl; 0.1% SDS; 1% Triton X-100; 1 mM EDTA; 0.5 mM EGTA; 10 mM Tris-HCl, pH 8; 0.1% sodium deoxycholate) for 4 to 5 times, then washed with TBS. After centrifugation, DNA was eluted (Tris-EDTA, pH 8) and reverse cross-linked (200mM NaCl; 0.5% SDS and

Proteinase K) for at least 5h at 65°C. The eluate was then treated with RNase for 30min at 37°C. Finally, DNA was cleaned and purified with Zymo Research Clean and concentrator kit (Zymo Research). ChIP-qPCR was performed using Power SYBR green PCR master mix (Applied Biosystems). Primers sequences used were as follows:

SMC3 promoter region

Forward - CCTCTGTCGGAAGAAACCTG

Reverse - GAACTTGGCTGCCTCTGAGT

SMC3 enhancer region

Forward - TGGTTTCCATTGCTTCATCA

Reverse - TACCGGCCAGTCTGAAATGT

SMC3 Next to enhancer region

Forward - CAAGAGGATGTATTGTTCCCATT

Reverse - TCCAAATGGCCTTTAAGTGG

SMC3 Negative region

Forward - TGCCATGCGTTGAAAATATCC

Reverse - TGCTTTCTGAAGTTGCCAAGC

% Input was calculated by dividing the enrichment of each region in SMC3-IP samples by the WCE. Negative control region was used to validate the specificity of the enrichment in the analyzed regions.

CRISPR

1x10⁶ HEK293T cells were plated in a T25 flask for viral particles production. On the next day, cells were washed and transfected with the plasmids psPAX2 packaging (RRID:Addgene_12260), VSV/G envelope (RRID:Addgene_8454) and lentiCRISPR v2 (RRID:Addgene_52961) containing the sgRNA sequence of interest using Lipofectamine 2000 (Thermo Fisher Scientific) using the manufacturer's instructions. After 72h, cell culture medium containing viral particles was centrifuged at 2000g for 10min to remove cell debris and consequently filtered through a 200nm strainer (GE Healthcare). Then, the resultant culture medium supplemented with polybrene (10 µg/mL) was used to culture MIA PaCa-2

cells previously plated in 6-well plates. After infection, puromycin (1 µg/mL, Sigma-Aldrich) was used to enrich the cell culture in transduced cells.

Sg sequences used were as follows:

Control: GGATCAGAGTCAAGAATACGTGG

Enhancer 1: TTGTCACAGGGCTAACAACATC

Enhancer 2: GAGGCACTCATGTAACGTAGTGG

Enhancer 3: CAGATAAGCGACAATATATGAGG

Next to enhancer 1: TGGAAATGCATTATAATTAA

Transfection and luminescence assay

2.5x10⁵ MIA PaCa-2 cells were plated in 6-well plates. On the following day, cells were either non-transfected or transfected with shSMC3 in a pLKO1 vector (TRCN0000160156, sh sequence - CGAGTAGAGACTTATCTCAAT). After 72h, cells were harvested and lysed for western blot. 5x10⁴ MIA PaCa-2 cells were plated in 24-well plates. On the next day, cells were either non-transfected or transfected with empty pGL3-Promoter plasmid (Promega, Cat # E1761), pGL3-Promoter Sense Control, pGL3-Promoter Anti-Sense Control, pGL3-Promoter Sense SMC3 binding site, pGL3-Promoter Anti-Sense SMC3 binding site using Lipofectamine 2000 (Thermo Fisher Scientific) using the manufacturer's instructions. Cloning was done in collaboration with GenScript. After 48h, cells were harvested and plated in triplicates in 96-well plates. On the following day, Dual-Luciferase[®] Reporter Assay System (Promega) was used according to the manual to detect luciferase activity. To detect luminescence signal Synergy Mx (BioTek) was used. Representative picture of luciferase activity using IVIS Lumina iii (Perkin Elmer IVIS Spectrum In-Vivo Imaging System (RRID:SCR_020397)) was taken. Luminescent image mode was used to detect luminescence signal. Luminescence intensity is depicted by a multicolor scale ranging from blue (least intense) to red (most intense).

Immunohistochemistry and histological analysis

Tissue samples were fixed in 10% formalin followed by paraffin embedding. 4µm sections were cut using a Microm HM335E microtome (Thermo Fisher Scientific). Immunohistochemistry was performed as described previously (Ruivo, Bastos et al. 2022).

Heat-mediated antigen retrieval for 40 min using a citrate buffer pH 6 solution (Vector Laboratories) was used for Rab27a, KI67, α SMA, CD11b, Amilase and Insulin while TRIS-EDTA pH 9 solution was used for CD4, T-bet, ROR γ T, CD68, MRP8, CD31 and pSRC. Antibody dilutions used were: anti-Rab27a 1:200 (Sigma-Aldrich Cat# HPA001333, RRID:AB_1079730), anti-KI67 1:500 (Abcam Cat# ab15580, RRID:AB_443209), α SMA 1:1000 (Sigma-Aldrich Cat# A2547, RRID:AB_476701), CD11b 1:4000 (Abcam Cat# ab8878, RRID:AB_306831), Amilase 1:400 (Abcam Cat# ab21156, RRID:AB_446061), Insulin 1:100 (Cell Signaling Technology Cat# 4590, RRID:AB_659820), CD4 1:200 (Abcam Cat# ab183685, RRID:AB_2686917), T-bet 1:200 (Abcam Cat# ab91109, RRID:AB_2050371), ROR γ T 1:100 (Abcam Cat# ab207082, RRID:AB_2889310), CD68 1:50 (Abcam Cat# ab31630, RRID:AB_1141557), MRP8 1:1000 (Abcam Cat# ab92331, RRID:AB_2050283), CD31 1:50 (Abcam Cat# ab28364, RRID:AB_726362), pSRC Tyr416 1:200 (Cell Signaling Technology Cat# 2101, RRID:AB_331697). Percentage of Rab27a-expressing cells in the pancreas of the Pdx1 Rab27aKO model was calculated using FIJI (Fiji, RRID:SCR_002285) according to the following formula: Percentage of Rab27a-expressing cells = Area of Rab27a-expressing cells/ Total analyzed area of the pancreas * 100. Rab27a score in the KPC mouse model and human samples was performed based on cytoplasmatic staining and the percentage of cells for each score. Cytoplasm staining intensity was determined for Rab27a. Score 0, 1, 2 or 3 corresponds to the presence of negative, weak, intermediate or strong staining. The number of cells for each staining intensity based on the percentage of the area occupied by these cells was also associated with a score, 1 - 0-25%, 2- 25-50%, 3- 50-75%, 4- >75%. The formula applied was as follows: Rab27a score = Score intensity x Area percentage score. A score between 0-12 was given to each slide. The number of intratumoral CD4⁺, T-bet⁺, ROR γ T⁺, CD68⁺, MRP8⁺ and CD11b⁺ cells were determined in at least 5 random fields (10x – CD4, T-bet, ROR γ T, CD68 and 20x – MRP8 and CD11b). The number of pSRC⁺ ductal cells was determined in at least 5 random fields (10x). Quantification of CD31⁺ vessels was performed in at least 5 random fields (10x). α -SMA score was determined in representative sections as previously described (Özdemir, Pentcheva-Hoang et al. 2014). KI67 staining was quantified using the average of the percentage of nuclear KI67⁺ cells in at least 5 random fields (40x). Amilase and insulin staining intensity was determined using CellProfiler (RRID:SCR_007358) in at least 5 random fields (40x). Insulin intensity was normalized to the number of cells present in the Islet of Langerhans analyzed. Representative H&E sections were used for histopathological scoring based on the percentage of the different morphological stages of the pancreas: normal, pancreatic intraepithelial neoplasia (PanIN) and pancreatic ductal adenocarcinoma (PDAC). All analyses were performed in a blinded fashion.

Flow cytometry

Tumors and spleen derived from PKT and PKT iRab27a mice were collected for analysis. Tumors were chopped and digested in digestion buffer (0,012% Dispase II Sigma-Aldrich D4693, 0,012% Collagenase Sigma-Aldrich C7657 in HBSS 1X) for 20 min at 37°C in agitation. After, blocking buffer (FBS 10% in HBSS 1X) was added to stop digestion and cells were centrifuged at 600g for 5 min. Then, cells were filtered through a 70µm strainer (Falcon) and incubated with Red Blood Cell lysis buffer for 5 min at room temperature (RT). Spleen was directly filtered through a 70µm strainer (Falcon) using a syringe plunger and incubated with Red Blood Cell lysis buffer for 5 min at RT. HBSS 1X was added in excess to both pancreas and spleen suspensions to stop the reaction and cells were centrifuged as previously described to obtain a single-cell suspension. Prior to staining, a blocking step using blocking buffer was performed for 15min at RT. Finally, antibody staining was performed in FACS buffer (FBS 2% in HBSS 1X) for 30 min at 4°C. Antibodies used were: TCRβ PE 1:400 (Thermo Fisher Scientific Cat# 12-5961-83, RRID:AB_466067), CD4 PerCP eFluor710 1:1000 (Thermo Fisher Scientific Cat# 46-0041-82, RRID:AB_11150050) and CD8 BV650 1:400 (BioLegend Cat# 100741, RRID:AB_11124344). In addition, viable dye was used to identify dead cells (Fixable Viability Dye eFluor 780 1:100000 eBioscience 65-0865-14).

Immunofluorescence and microscopy imaging

2x10⁴ primary CAFs derived from a PKT tumor were plated in a coverslip inside a 24-well plate. On the next day, cell culture media was removed and coverslips were washed with ice cold PBS 1X. CAFs were fixed using 4% paraformaldehyde in PBS 1X for 15min at RT. After washing steps with PBS 1X, cells were permeabilized with 0.1% Triton-X100 in PBS 1X for 10min at RT. Coverslips were then rinsed for three times with PBS 1X and then blocked for 1 hour at RT in 10% BSA 0.5%Tween20 in PBS 1X. Incubation with anti-TLR4 1:50 (Novus Biologicals Cat# NB100-56566, RRID:AB_2205129) in PBS 1X 2% BSA 0.5%Tween20 at 4°C overnight. Next, incubation with anti-mouse Alexa Fluor 594 1:100 (Abcam Cat# ab150108, RRID:AB_2732073) for 1h at RT, after several washing steps with PBS 1X 0.2%Tween20. Overnight incubation with anti-αSMA-FITC 1:300 (Sigma-Aldrich Cat# F3777, RRID:AB_476977) in PBS 1X 2% BSA 0.5%Tween20 at 4°C was performed. After washing steps, a Hoescht (Invitrogen) 1:5000 solution in PBS 1X 0.01% Triton-X100 was used to label nuclei. Coverslips were mounted in glass slides with VECTASHIELD mounting medium (Vector Laboratories). 4µm paraffin sections were used to perform immunofluorescence staining. Protocol used is the same as the one performed for

immunohistochemistry until primary antibody incubation step. Incubation with primary antibody anti-mCherry 1:500 (Abcam Cat# ab205402, RRID:AB_2722769), MRP8 1:1000 (Abcam Cat# ab92331, RRID:AB_2050283), CD31 1:50 (Abcam Cat# ab28364, RRID:AB_726362), CD11b 1:2000 (Abcam Cat# ab8878, RRID:AB_306831) was performed at 4°C, overnight. After washing steps with PBS 1X/0.1% Tween 20, incubation with secondary antibody was performed for 1h at RT. Secondary antibodies dilutions were as follow: anti-chicken Alexa-Fluor® 633 (Sigma-Aldrich Cat# SAB4600127, RRID:AB_2916287), anti-rabbit Alexa-Fluor® 488 (Jackson ImmunoResearch Labs Cat# 711-545-152, RRID:AB_2313584), anti-rabbit Alexa-Fluor® 488 (Abcam Cat# ab150081, RRID:AB_2734747) and anti-rabbit Alexa-Fluor® 594 (Abcam Cat# ab150084, RRID:AB_2734147) at a dilution of 1:500. After incubation, the slides were washed with PBS 1X/0.1% Tween 20. In sequential antibody incubations an additional blocking step was performed before incubation with the second primary antibody. The nuclei were stained with Hoechst solution (1:5000, Thermo Scientific) for 10 min at RT. Finally, slides were mounted with VECTASHIELD mounting medium (Vector Laboratories). A Leica TCS SP5 inverted confocal system with a HCL PL APO CS 40x/ N.A. 1.3 or a HCL PL APO CS 63x/ N.A. 1.4 oil objective was used to acquire immunofluorescence images. Sequential acquisition was used to acquire each fluorescent protein (FITC/Alexa Fluor 488, Alexa Fluor 594 and Alexa Fluor 633) laser lines used were 488, 561, 633, respectively. Presented images were max z-projected and contrast was optimized in FIJI (Fiji, RRID:SCR_002285).

Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS)

Protein digestion

Extracellular vesicles samples were digested as previously described (Ruivo, Bastos et al. 2022). Whole tumor specimens were collected at euthanasia and snapfrozen immediately. After thawing, tumor samples were lysed in a 100mM Tris pH 8.5 solution containing 1% sodium deoxycholate (Sigma-Aldrich), 10 mM tris(2-carboxyethyl)phosphine (TCEP, Sigma-Aldrich), 40 mM chloroacetamide (Sigma-Aldrich) and supplemented with cComplete (Roche) using a TissueRuptor II (Qiagen). Next, samples were incubated for 10min at 1000RPM (ThermoMixer, Eppendorf). Finally, samples were centrifuged at 17000RPM for 30min at 4°C. Protein quantification was performed using DC™ Protein Assay (Bio-Rad) and 100µg were used for consequent analysis.

Liquid chromatography and mass spectrometer analysis

Extracellular vesicles samples analysis was performed as previously described (Ruivo, Bastos et al. 2022). Whole tumor samples were processed for proteomic analysis following the solid-phase-enhanced sample-preparation (SP3) protocol and enzymatically digested with Trypsin/LysC as previously described (Osório, Silva et al. 2021). Protein identification and quantitation was performed by nanoLC-MS/MS. This equipment is composed of an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Five hundred nanograms of peptides of each sample were loaded onto a trapping cartridge (Acclaim PepMap C18 100 Å, 5 mm × 300 µm i.d., 160454, Thermo Scientific, Bremen, Germany) in a mobile phase of 2% ACN, 0.1% FA at 10 µL/min. After 3 min loading, the trap column was switched in-line to a 50 cm × 75 µm inner diameter EASY- Spray column (ES903, PepMap RSLC, C18, 2 µm, Thermo Scientific, Bremen, Germany) at 250 nL/min. Separation was achieved by mixing A: 0.1% FA and B: 80% ACN, 0.1% FA with the following gradient: 5 min (2.5% B to 10% B), 120 min (10% B to 30% B), 20 min (30% B to 50% B), 5 min (50% B to 99% B), and 10 min (hold 99% B). Subsequently, the column was equilibrated with 2.5% B for 17 min. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.11 software (Thermo Scientific, Bremen, Germany). The mass spectrometer was operated in the data-dependent (dd) positive acquisition mode alternating between a full scan (m/z 380-1580) and subsequent HCD MS/MS of the 10 most intense peaks from a full scan (normalized collision energy of 27%). The ESI spray voltage was 1.9 kV. The global settings were as follows: use lock masses best (m/z 445.12003), lock mass injection Full MS and chrom. peak width (FWHM) of 15 s. The full scan settings were as follows: 70 k resolution (m/z 200), AGC target 3×10^6 , maximum injection time 120 ms; dd settings: minimum AGC target 8×10^3 , intensity threshold 7.3×10^4 , charge exclusion: unassigned, 1, 8, >8, peptide match preferred, exclude isotopes on, and dynamic exclusion 45 s. The MS2 settings were as follows: microscans 1, resolution 35 k (m/z 200), AGC target 2×10^5 , maximum injection time 110 ms, isolation window 2.0 m/z , isolation offset 0.0 m/z , dynamic first mass, and spectrum data type profile.

Raw data alignment and analysis

Raw files were processed with MaxQuant (v 1.6.17.0) using the standard settings against either a mouse protein database (TrEMBL /Swiss-Prot, 53,449 sequences) or a human database (Swiss-Prot, 20,373 sequences). Carbamidomethylation of cysteines was set as a fixed modification whereas oxidation of methionines and protein N-term acetylation were set as variable modifications. Minimal peptide length was set to 7 amino acids and a

maximum of two tryptic missed-cleavages were allowed. Results were filtered at 0.01 FDR (peptide and protein level). Afterwards, the “proteinGroups.txt” file was loaded in Prostar using the LFQ intensity values for further statistical analysis (Wieczorek, Combes et al. 2017). Briefly, proteins with less than two valid values in at least one experimental condition were filtered out. Missing values were imputed using the algorithms SLSA for partially observed values and DetQuantile for values missing on an entire condition (Bø, Dysvik et al. 2004). Differential analysis was done using the empirical Bayes statistics Limma. Proteins with a p.value < 0.01 and a log2 ratio >1 or <-1 were defined as regulated. The FDR was estimated to be below 4.48% by Benjamini-Hochberg.

Data availability

The mass spectrometry data has been deposited to the MetaboLights platform with the dataset identifier MTBLS7563 (www.ebi.ac.uk/metabolights/MTBLS7563) (Haug, Cochrane et al. 2020).

Cytokine Array

150µg of protein isolated from whole tumors was used to perform cytokines quantification according to the manufacturer’s instruction (R&D Systems, Cat# ARY006). Protein isolation was performed according to the mass spectrometry section.

Ex vivo culture assays

4x10⁴ cells of primary CAFs derived from a PKT tumor were plated in a 24-well plate. Then, cells were cultured in serum-free RPMI medium supplemented with 1X insulin–transferrin–selenium A (Thermo Fisher Scientific, Cat# 51500056) for 1 day. Afterwards, CAFs were further incubated in the presence or absence of 5 µg/mL recombinant mouse MRP8 protein (Abcam, Cat# ab108120). In figure 4E, 1 day prior to MRP8 stimulation, cells were transfected with a siRNA SRC or siRNA TLR4 (50nM, Thermo Fisher Scientific, Cat# s75206) using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. After 72h, cells were collected for RNA extraction. Co-cultures with CD4⁺ T cells (1 × 10⁵ cells per well) were performed using primary CAFs derived from a PKT tumor incubated in the presence or absence of 5 µg/mL recombinant mouse MRP8 protein (Abcam, Cat# ab108120) for 36h. CD4⁺ T cells were isolated from the spleen of a WT mouse using a CD4⁺ T cells isolation kit according to the manufacturer’s instructions (Miltenyi Biotec, Cat# 130-104-454). CD4⁺ T cells were activated in the presence of anti-CD3 (10 µg/mL, BioLegend

Cat# 100302, RRID:AB_312667) and anti-CD28 (8 µg/mL, BioLegend Cat# 102102, RRID:AB_312867) monoclonal antibody for 2 h prior to co-culture with CAFs. For IL-6 blocking, 2 µg/mL anti-IL-6 monoclonal antibody (R&D Systems Cat# MAB406, RRID:AB_2233899) was added into the co-culture. After 72h, CD4⁺ T cells were collected for RNA extraction.

Size-exclusion chromatography - SEC

MIA PaCa-2 and MIA PaCa-2 Tet-ON shRab27a EVs isolated by ultracentrifugation were purified according to (Kugeratski, Hodge et al. 2021). In Figures 2E and 2F, a qEV original column 35 nm (Izon SP5) was used to separate EVs preparation in 25 fractions. Fractions 7–10, corresponding to EVs fractions, and fractions 11–25, corresponding to nV fractions, were pooled together and spun down by ultracentrifugation at 100 000 *g* for 3 hours. In Figure S3A and S3B, according to the updated Izon software, a qEV original column 35 nm (Izon SP5) was used to separate EVs preparation in 13 fractions. Each individual fraction was ultracentrifuged at 100 000 *g* for 3 hours. NanoSight NS300 (Nanoparticle Tracking Analysis, RRID:SCR_014239) analysis was performed to confirm EVs enrichment in the SEC isolated fractions. Western blot was also performed using pre-casted polyacrylamide gradient gels (Bolt 4%–12% Bis Tris Plus, Invitrogen) and proteins were transferred using a semidry system (Trans-Bolt turbo, Biorad) into methanol activated polyvinylidene difluoride (PDVF Transblot turbo, Biorad) membrane. EVs markers syntenin-1 (1:5000, Abcam Cat# ab133267, RRID:AB_11160262) and Alix (1:1000, Cell Signaling Technology Cat# 2171, RRID:AB_2299455) were used and anti rabbit-horseradish peroxidase (HRP) (1:5000, Cell Signaling Technology Cat# 7074S RRID:AB_2099233) and anti mouse-HRP (1:2000, R&D Systems Cat# HAF007 RRID:AB_357234) secondary antibodies, respectively, allowed primary antibody detection.

Patient tissue specimens

Tumor paraffin blocks from PDAC patients were obtained from *Hospital Beatriz Ângelo*, Lisboa, Portugal, *Centro Hospitalar de São João (CHSJ)*, Porto, Portugal, *Centro Hospitalar Tondela-Viseu*, Viseu, Portugal and from *Carl Gustav Carus University Hospital, Dresden, Germany*. Informed consent was obtained from all patients included in this study in both Hospitals. The use of paraffin blocks was approved by the ethical committee of *Centro Hospitalar de São João (CHSJ)*, Porto, Portugal (ID number CES 253_2022), ethical committee of *Hospital Beatriz Ângelo*, Lisboa, Portugal (ID number 1372/2015_CMOEB),

ethical committee of *Centro Hospitalar Tondela-Viseu*, Viseu (ID number 02/07/2018), and ethical committee of *Carl Gustav Carus University Hospital, Dresden, Germany*. Clinical information was age, sex and tumor stage. Human studies were conducted in accordance with the Declaration of Helsinki ethical guidelines. Blinding is not relevant for our study.

Extracellular vesicles isolation

Extracellular vesicles isolation was performed as previously described (Ruivo, Bastos et al. 2022). Briefly, PDAC cells were cultured in RPMI medium supplemented with extracellular vesicles-free FBS for 72h. Afterwards, medium was centrifuged at 2500RPM for 10 min and at 4000RPM for 5 min. Prior to ultracentrifugation, medium was filtered using a 200nm filter (GE Healthcare) and ultracentrifuged at 100000g for 3h at 4°C. On the following day, supernatant was discarded, and extracellular vesicles pellet was resuspended in a solution of 8M/2.5% SDS-Urea (Sigma Aldrich) for LC-MS/MS and western-blot or 100µL of PBS 1X for NanoSight NS300 (Nanoparticle Tracking Analysis, RRID:SCR_014239) analysis.

PCR

Rab27a recombination in the pancreas of tamoxifen-treated mice was assessed by PCR as previously described (Ruivo, Bastos et al. 2022). PCR primers sequences were as follows:

Rab27a Forward - CCAGGGCACTTCCTAAAGATAAG

Rab27a Reverse 1 - CACGCTGCTTCAATGAATAAGGTG

Rab27a Reverse 2 – CATCCACAAAGCTCCAGATTCCAC

Quantitative reverse transcription-PCR

CAFs and CD4⁺ T cells RNA was isolated using PureLink™ RNA Mini Kit (Invitrogen) according to the manufacturer's instructions and RNA isolation from human cell lines was performed using TRIzol (Thermo Fisher Scientific) as described in the manual. cDNA synthesis was performed using the NZY First-Strand cDNA Synthesis Kit (NZYTech) according to the manual. Power SYBR green PCR master mix (Applied Biosystems) was used for the quantification PCR. The following primers were used to quantify expression levels.

Oligonucleotide sequences used were:

Mouse IL-6 forward: GAGGATACCACTCCCAACAGACC

Mouse IL-6 reverse: AAGTGCATCATCGTTGTTTCATACA

Mouse IL-17 forward: ATCCCTCAAAGCTCAGCGTGTC

Mouse IL-17 reverse: GGGTCTTCATTGCGGTGGAGAG

Mouse β -actin forward: CTGTCCCTGTATGCCTCTG

Mouse β -actin reverse: ATGTCACGCACGATTTCC

Human Rab27a forward: AGCTTTGGGAGACTCTGGTG

Human Rab27a reverse: TGTGTCCCATAACTGCAGGT

Human SMC3 forward: GGAGGGCAGTCAGTCTCAAG

Human SMC3 reverse: AGCAAGGGCTACCAAGGATT

Human β -actin forward: GAGCACAGAGCCTCGCCTTT

Human β -actin reverse: ACATGCCGGAGCCGTTGTC

Western blot

Western blot was performed as previously described (Ruivo, Bastos et al. 2022). Briefly, 30 μ g of protein derived from cell lines, whole tumors or extracellular vesicles samples was used for western blot analysis. Primary antibody incubation was performed overnight at 4°C. Antibody dilutions used were: anti-Rab27a 1:500 (Abnova Cat# H00005873-M02, RRID:AB_519010), anti-IL-17 1:500 (Abcam Cat# ab79056, RRID:AB_1603584), anti-IL-6 1:500 (R&D Systems Cat# AF-406-NA, RRID:AB_354478), anti-total SRC 1:1000 (Cell Signaling Technology Cat# 2108, RRID:AB_331137), anti-pSRC Tyr416 1:1000 (Cell Signaling Technology Cat# 2101, RRID:AB_331697), anti-CD63 1:500 (BD Biosciences Cat# 564221, RRID:AB_2738677), anti-Alix 1:1500 (Cell Signaling Technology Cat# 2171, RRID:AB_2299455) and anti-SMC3 1:1000 (Abcam Cat# ab9263, RRID:AB_307122). After washing steps, membranes were incubated with the respective HRP-conjugated secondary antibody at 1:5000 dilution for 1h at RT (Anti-rabbit IgG HRP-linked antibody Cell Signaling Technology Cat# 7074, RRID:AB_2099233, Anti-mouse IgG HRP-linked antibody Advansta Cat# R-05071-500, RRID:AB_10718209, Anti-Rat IgG HRP-linked Abcam Cat# ab6734, RRID:AB_955450, Anti-Goat HRP-linked Abcam Cat# ab6741, RRID:AB_955424). β -actin (Sigma-Aldrich Cat# A3854, RRID:AB_262011) was used as loading control.

In vivo imaging system - IVIS

KPF CD63-mCherry and PKT tumors orthotopically injected with AAV shSCR or AAV shTSP1 were collected and imaged on the IVIS Lumina iii (Perkin Elmer IVIS Spectrum In-Vivo Imaging System (RRID:SCR_020397)). The 465 laser/GFP emission filter was used to detect GFP signal and the 535 laser/DsRed emission filter was used to detect mCherry signal. Fluorescence intensity is depicted by a multicolor scale ranging from red (least intense) to yellow (most intense). IVIS Living image Software was used to determine images signal intensity over gray scale reference photographs. Pancreas from WT mice were used as negative control.

Statistical analysis

For all analyses, significance was determined at $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, $p < 0.0001^{****}$ represent significance between conditions. All analyses were performed in GraphPad Prism® (GraphPad Prism, RRID:SCR_002798).

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APPENDIX I

Original Publication

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RESEARCH

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SMC3 epigenetic silencing regulates Rab27a expression and drives pancreatic cancer progression

Nuno Bastos^{1,2}, Stéphanie A. Castaldo¹, Bárbara Adem^{1,2}, José C. Machado^{1,3,4}, Carlos A. Melo⁵ and Sonia A. Melo^{1,3,4*} 

Abstract

Background Pancreatic ductal adenocarcinoma (PDAC) is expected to soon surpass colorectal cancer as a leading cause of cancer mortality in both males and females in the US, only lagging behind lung cancer. The lethality of PDAC is driven by late diagnosis and inefficient therapies. The complex biology of PDAC involves various cellular components, including exosomes that carry molecular information between cells. Thus, recipient cells can be reprogrammed, impacting tumorigenesis. Rab27a is a GTPase responsible for the last step of exosomes biogenesis. Hence, dissecting the mechanisms that regulate the expression of Rab27a and that control exosomes biogenesis can provide fundamental insights into the molecular underpinnings regulating PDAC progression.

Methods To assess the mechanism that regulates Rab27a expression in PDAC, we used PDAC cell lines. The biological significance of these findings was validated in PDAC genetically engineered mouse models (GEMMs) and human samples.

Results In this work we demonstrate in human PDAC samples and GEMMs that Rab27a expression decreases throughout the development of the disease, and that Rab27a knockout promotes disease progression. What is more, we demonstrate that Rab27a expression is epigenetically regulated in PDAC. Treatment with demethylating agents increases Rab27a expression specifically in human PDAC cell lines. We found that SMC3, a component of the cohesin complex, regulates Rab27a expression in PDAC. SMC3 methylation is present in human PDAC specimens and treatment with demethylating agents increases SMC3 expression in human PDAC cell lines. Most importantly, high levels of SMC3 methylation are associated with a worse prognosis in PDAC. Mechanistically, we identified an enhancer region within the Rab27a gene that recruits SMC3, and modulates Rab27a expression.

Conclusion Overall, we dissected a mechanism that regulates Rab27a expression during PDAC progression and impacts disease prognosis.

Keywords Pancreatic cancer, Rab27a, Exosomes, SMC3

*Correspondence:

Sonia A. Melo
 smelo@i3s.up.pt

Full list of author information is available at the end of the article



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Background

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive and lethal malignancy with a 94% mortality rate [1, 2]. The lethality of PDAC is associated with late diagnosis and lack of effective therapeutic options, urging the necessity to better understand PDAC biology [3, 4]. The development and progression of PDAC involves complex cellular interactions and signaling pathways, and recent studies have demonstrated that exosomes play a critical role in cancer progression and metastasis [5–8]. Exosomes are extracellular vesicles of endosomal origin released by cells that contain a variety of bioactive molecules (proteins, lipids, RNA and DNA) and, in cancer, have been shown to mediate intercellular communication and reprogram the tumor microenvironment [5, 6, 9, 10]. One of the main players involved in exosomes biogenesis and secretion is the Rab GTPase family of proteins. Rab GTPases regulate vesicle trafficking and membrane fusion events in cells, including exosomes release [11]. Among the many Rab GTPases, Rab27a has been shown to regulate the late stages of the endocytic pathway mediating exosomes release from cancer cells, including in PDAC [10, 12]. Several studies have demonstrated that Rab27a is critical for efficient exosomes-mediated communication modulating different cancer related processes such as cancer cells proliferation and dissemination, as well as remodeling of the tumor microenvironment [13–15]. Understanding the regulation of expression of Rab GTPases, such as Rab27a, and their impact on exosomes biogenesis and secretion throughout cancer progression could provide important insights into the biology of PDAC and unravel novel opportunities for the development of targeted therapies aimed at modulating intercellular communication mediated by exosomes. Here we dissected the mechanism that regulates Rab27a expression in PDAC and that may condition communication mediated by exosomes during the course of the disease.

Results

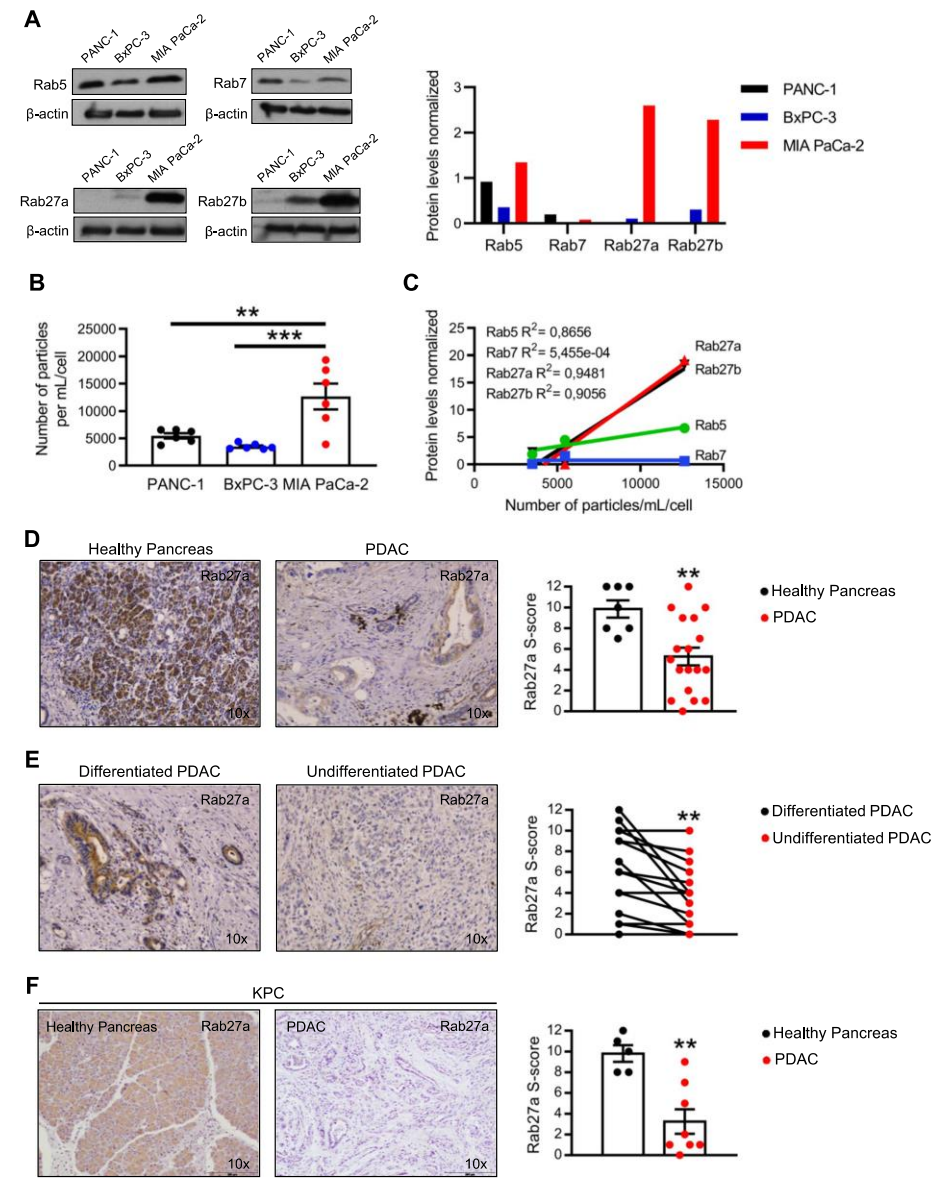
Rab27a protein expression decreases throughout PDAC progression

In order to determine which is the best Rab GTPase to analyze as a surrogate marker for secretion of exosomes by cancer cells, we screened for four Rab GTPases (Rab5, Rab7, Rab27a and Rab27b) using three human PDAC cell lines (PANC-1, BxPC-3 and MIA PaCa-2). Each Rab GTPase corresponds to a specific step in exosomes biogenesis, from early endosomes to exocytosis of exosomes. We demonstrated that all Rabs are differentially expressed between the PDAC cell lines analyzed, being this difference more striking for Rab27a and Rab27b (Fig. 1A). MIA PaCa-2 is the human PDAC cell line that expresses the higher amounts of Rab27a and 27b proteins (Fig. 1A). As expected, and in agreement with the Rab27a and 27b expression levels, MIA PaCa-2 is also the cell line that secretes the highest number of vesicles in comparison with PANC-1 and BxPC-3 (Fig. 1B). Most importantly, amongst the four Rab GTPases analyzed, Rab27a protein levels is the one that correlates the best with the number of secreted vesicles by cancer cells in a linear regression analysis (Fig. 1C). In addition, previous work also demonstrates that Rab27a expression is important for exosomes secretion [12, 13, 15]. Based on our results and previously published data, we have focused our analysis on Rab27a and Rab27a-dependent exosomes communication in PDAC.

We have started by evaluating Rab27a protein expression by immunohistochemistry (IHC) during PDAC progression in a cohort of human PDAC samples (n=18). We observed a significant decrease in Rab27a protein levels in PDAC tissue in comparison to adjacent healthy pancreas (Fig. 1D). The same holds true when comparing differentiated to undifferentiated tumor lesions (Fig. 1E). Most importantly, we have recapitulated the results observed in human samples using the KPC mouse model (*LSL-Kras^{G12D}/+*; *LSL-Tp53^{R172H}/+*; *Pdx1-Cre*), a genetically engineered mouse model (GEMM) that

(See figure on next page.)

Fig. 1 Rab27a protein levels correlate the best with the number of extracellular vesicles released in PDAC and its expression decreases throughout disease progression. **A** Western blot of Rab5, Rab7, Rab27a and Rab27b (left) and respective quantification (right) in PANC-1, BxPC-3 and MIA PaCa-2 cells. β -actin was used as loading control. **B** Number of particles per cell and per mL released by PANC-1, BxPC-3 and MIA PaCa-2 cells (n=6). One-way anova **p<0.01, ***p<0.001. **C** Correlation between the normalized Rab5 (green), Rab7 (blue), Rab27a (red) and Rab27b (black) protein levels and the number of particles secreted per cell and per mL in PANC-1, BxPC-3 and MIA PaCa-2 cells. Linear regression. **D** Representative Rab27a IHC images (10x, left) and quantification (right) in histologically healthy pancreas (n=7) and PDAC (n=18) tissues from human specimens (Unpaired t-test ** p<0.01). **E** Representative Rab27a IHC images (10x, left) and quantification (right) in differentiated and undifferentiated PDAC tissues from the same human specimen (n=15, Paired t-test ** p<0.01). **F** Representative Rab27a IHC images in histologically healthy pancreas (n=5) and PDAC (n=8) in KPC mice (left, 10x) and quantification (right). Unpaired t-test **p<0.01. Data are Mean $\pm$ SEM



spontaneously develops PDAC (Fig. 1F) [16]. We demonstrate that PDAC tissue in KPC mice show significantly lower expression levels of Rab27a in comparison to healthy pancreas (Fig. 1F).

Taken together, using human PDAC samples and a well-established PDAC GEMM we demonstrated that Rab27a protein expression is lost during the course of the disease.

Rab27a behaves as a tumor suppressor gene in PDAC and its expression is epigenetically regulated

In order to better understand the role of Rab27a in PDAC development and progression, we generated the PKT Ashen model. The PKT (*Ptf1a-Cre; LSL-Kras^{G12D/+}; Tgfbr2^{loxP/loxP}*) is a GEMM that develops PDAC in a spontaneous manner, recapitulating the different stages of the human disease [17]. This is a fast progression model with a more homogeneous progression of the disease when compared to the KPC, which takes several months to develop PDAC. In combination with the Ashen mouse, a constitutional Rab27a knockout, we obtain a GEMM that develops PDAC in a Rab27a knockout background (PKT Ashen, Fig. 2A) [18]. Rab27a knockout was validated by immunohistochemistry (IHC) in tumors of PKT Ashen mice (Fig. 2A). Interestingly, we observed a significant increase in tumor burden in the PKT Ashen (Rab27a KO) in comparison with the PKT (Rab27a WT) reflected in a significant increase in the number of liver macro-metastasis in the Rab27 KO mice (Fig. 2B, C). These results indicate that loss of Rab27a expression in cancer cells represents an advantage for disease progression in PDAC.

Expression of Rab27a in human and mouse samples decreases during the course of the disease. In addition, loss of Rab27a expression accelerates disease in a PDAC GEMM. In sum, Rab27a gene behaves as a tumor suppressor in PDAC. Therefore, we went on to look for the mechanism that regulates Rab27a expression in these tumors. Genetic and epigenetic mechanisms are the top candidate processes to regulate gene expression. We have queried the TCGA (Cancer Genome Atlas Program) for somatic mutations across the Rab27a gene in human PDAC samples and we have not identified any mutations that could explain the observed loss of expression of Rab27a in PDAC. Therefore, we went on and tested if epigenetic regulation could be the mechanism by which Rab27a expression is lost in PDAC. Methylation is frequently observed in human cancer and targets many genes involved in the progression of the disease. To test this hypothesis, we treated two human PDAC cell lines (PANC-1 and MIA PaCa-2) with the demethylating agents, azacitidine and decitabine. Rab27a expression levels significantly increase in cancer cells treated

with any of the two demethylating agents (Fig. 2D, E). In addition, an increase in Rab27a protein levels was validated in PANC-1 cells treated with decitabine (Fig. 2F). In stark contrast, treatments with the same demethylating agents did not alter Rab27a expression in a healthy pancreas cell line (hTERT-HPNE, Fig. 2G). Hence, our data shows that an epigenetic regulatory mechanism of Rab27a expression occurs specifically in cancer. We went on and interrogated the DNMIIVD platform for Rab27a methylation levels in human PDAC tissue (n=185) and healthy pancreas (n=10) [19]. The average levels of methylation across the Rab27a gene were not significantly different between PDAC patients and healthy tissue (Fig. 2H). Therefore, we could conclude that Rab27a expression is indirectly regulated by an epigenetic-driven mechanism.

Epigenetic regulation of SMC3 modulates Rab27a expression in PDAC

We next tested the hypothesis that epigenetic regulation of transcription factors that bind Rab27a gene could control its expression and explain our observations. Thus, we screened the genome browser to identify transcription factors that bind the promoter region of Rab27a. We have also filtered for those transcription factors that, besides binding to Rab27a, are also methylated in human PDAC samples (Fig. 3A). SMC3 emerged as one of the best candidates because it has two binding regions in the Rab27a gene (one in the promoter region and a second one next to a potential enhancer region). SMC3 is part of the cohesin complex, which mediates sister chromatid cohesion, double-stranded DNA break repair and regulates gene expression [20]. We demonstrate that SMC3 methylation levels are increased in PDAC tissue in comparison to healthy pancreas using the DNMIIVD platform (Fig. 3B) [19]. Most importantly, high levels of SMC3 methylation are associated with worse prognosis in PDAC (Fig. 3C) [19]. Thus, we went on to validate that SMC3 is regulated by methylation and determine if the expression of Rab27a is dependent on the expression of SMC3. In agreement with our previous observations, treatment with demethylating agents, azacitidine and decitabine, led to a significant increase in SMC3 expression and protein levels in PDAC cell lines, suggesting that this gene is commonly methylated in PDAC (Fig. 3D–F). Next, to understand if Rab27a expression is dependent on SMC3, we transfected MIA PaCa-2 cells with a short hairpin RNA (shRNA) targeting SMC3 and evaluated Rab27a protein levels. Upon SMC3 knockdown we observed a decrease in Rab27a protein levels suggesting that Rab27a could be regulated by SMC3 (Fig. 3G). Notably, SMC3 expression levels positively correlate with

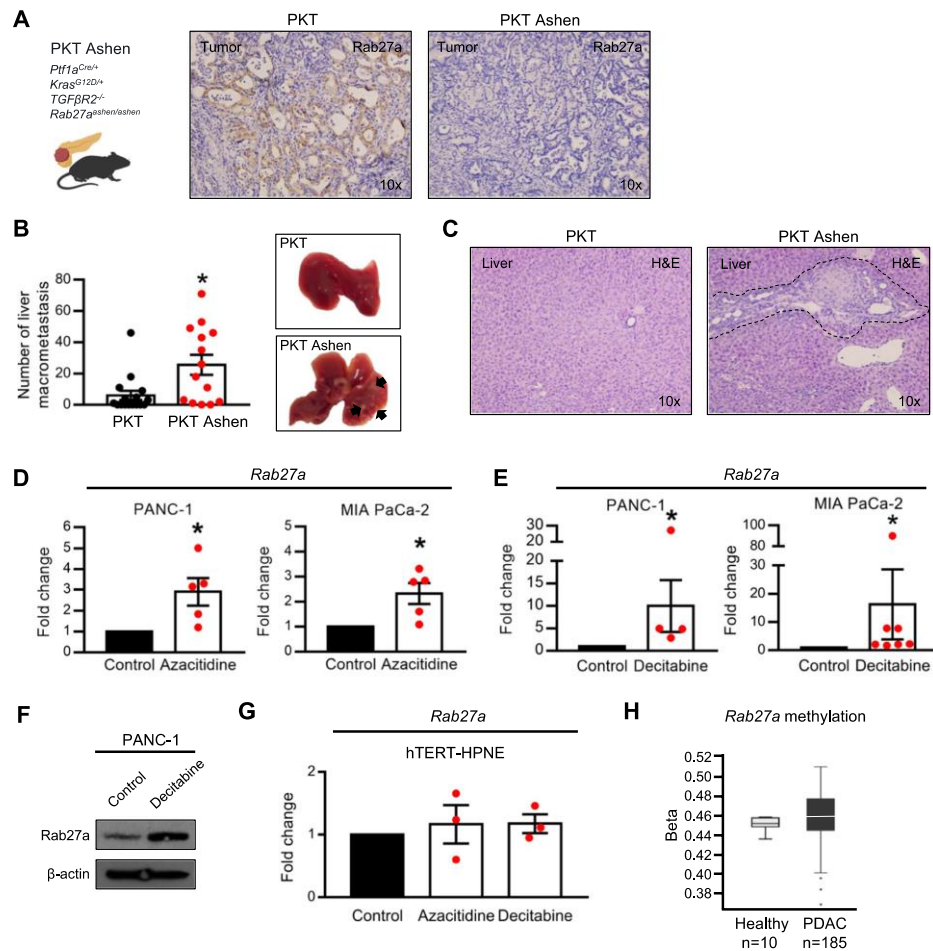


Fig. 2 Rab27a expression is regulated via an indirect epigenetic mechanism. **A** Representative Rab27a IHC in PKT and PKT Ashen tumors (10x). **B** Representative liver images (right) and number of liver macrometastasis (left) in PKT (n = 16) and PKT Ashen (n = 14) mice. Arrows indicate sites of metastasis. Unpaired t-test *p < 0.05. **C** Representative liver H&E of PKT and PKT Ashen mice (10x). Dashed lines indicate metastatic lesion. **D** Fold change of Rab27a expression determined by qPCR in PANC-1 and MIA PaCa-2 treated with azacitidine (5 μM—PANC-1, 1 μM—MIA PaCa-2). N = 5, unpaired t-test, *p < 0.05. **E** Fold change of Rab27a expression determined by qPCR in PANC-1 and MIA PaCa-2 treated with decitabine (5 μM—PANC-1, 1 μM—MIA PaCa-2). N = 4 in PANC-1, n = 7 in MIA PaCa-2, unpaired t-test, *p < 0.05. **F** Western blot of Rab27a in PANC-1 treated with vehicle (control) or decitabine (5 μM). β-actin was used as loading control. **G** Fold change of Rab27a expression determined by qPCR in hPNE-hTERT treated with azacitidine (1 μM) or decitabine (1 μM) (n = 3). **H** Rab27a DNA methylation beta values in healthy pancreas and PDAC in human samples. Data extracted from DNMTVD platform. Data are Mean ± SEM

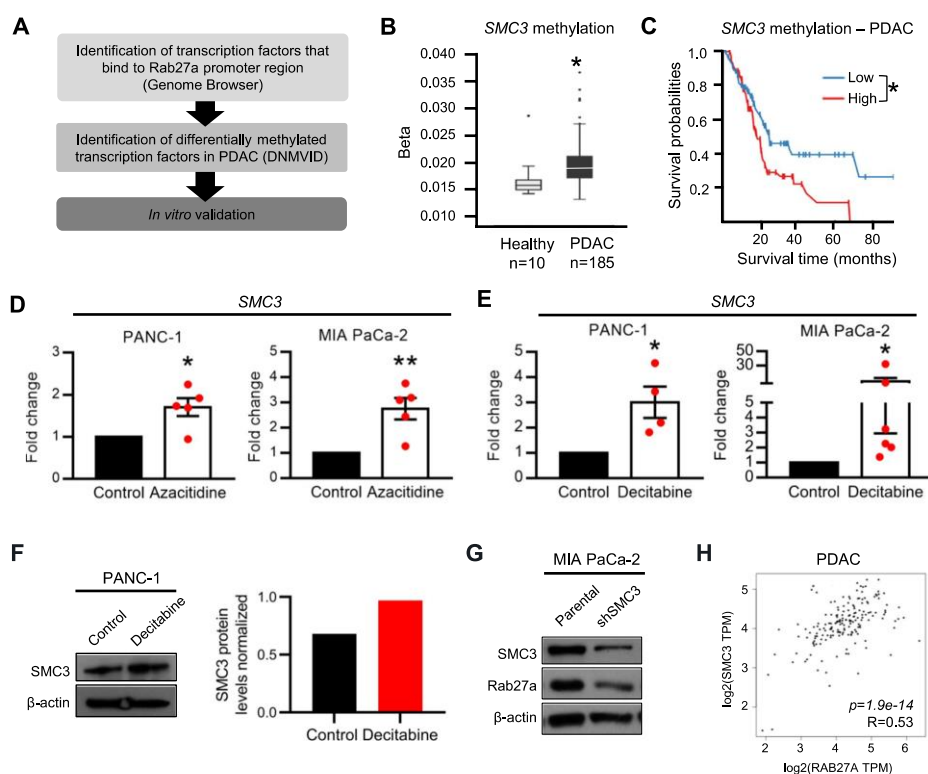


Fig. 3 SMC3 methylation is associated with worse prognosis in PDAC and its expression correlates with Rab27a expression. **A** Pipeline to determine potential targets that regulate Rab27a expression in PDAC. **B** SMC3 DNA methylation beta values in healthy pancreas and PDAC in human samples. Data extracted from DNMIIVD platform. Unpaired t-test, * $p < 0.05$. **C** Kaplan–Meier curve of PDAC patients with low (blue) or high (red) levels of SMC3 methylation. Data extracted from DNMIIVD platform. Log-rank test, * $p < 0.05$. **D** Fold change of SMC3 expression determined by qPCR in PANC-1 and MIA PaCa-2 treated with azacitidine (5 μ M – PANC-1, 1 μ M – MIA PaCa-2). N = 5, unpaired t-test, * $p < 0.05$, ** $p < 0.01$. **E** Fold change of SMC3 expression determined by qPCR in PANC-1 and MIA PaCa-2 treated with decitabine (5 μ M—PANC-1, 1 μ M—MIA PaCa-2). N = 4 in PANC-1, n = 6 in MIA PaCa-2, unpaired t-test, * $p < 0.05$. **F** Western blot of SMC3 (left) and respective quantification (right) in PANC-1 treated with vehicle (control) or decitabine (5 μ M). β -actin was used as loading control. **G** Western blot of SMC3 and Rab27a in MIA PaCa-2 parental cell line or transfected with a short hairpin that targets SMC3. β -actin was used as loading control. **H** RNA expression correlation of SMC3 and Rab27a in human PDAC samples. $R = 0.53$, $p = 1.9 \times 10^{-14}$. Data extracted from TCGA. Data are Mean $\pm$ SEM

Rab27a expression in human PDAC samples (analysis of TCGA data using the GEPIA platform; Fig. 3H) [21].

SMC3 mediates Rab27a expression by supporting promoter-enhancer interactions

SMC3 within the cohesin complex regulates gene expression through 3D genome organization facilitating promoter and enhancer contacts [22]. Using the UCSC genome browser chromatin-immunoprecipitation

sequencing (ChIP-seq) tracks for SMC3, we verified that SMC3 is able to bind the promoter region of Rab27a gene as well as two other regions within close proximity (Fig. 4A) [23]. Based on the presence of high histone 3 lysine 4 mono-methylation and histone 3 lysine 27 acetylation marks present in one of the regions, we defined these locations as enhancer (E) and next to enhancer (NTE) regions (Fig. 4A). We postulated that NTE serves as an anchor region to bring Rab27a enhancer and

promoter regions together. Indeed, we have used available databases to study in situ chromatin interactions by means of Hi-C chromatin structure analysis [24]. We showed that NTE, where SMC3 binds with high affinity, contacts with the promoter region of Rab27a (Fig. 4A). To support our hypothesis, we validated through ChIP-qPCR the binding of SMC3 to these three regions. In agreement with what is observed in SMC3 ChIP-seq datasets, we observed high SMC3 binding to the promoter and NTE regions in a human PDAC cell line (Fig. 4B). In order to confirm that the region defined as an enhancer, and specifically the SMC3 binding site, has the ability to promote gene expression, we cloned this sequence into a pGL3-Promoter luciferase reporter plasmid. Strikingly, both the sense and anti-sense sequences of the SMC3 binding site in the enhancer region led to an increase in the luminescence signal, indicating that these sequences have enhancer function (Fig. 4C). Next, to determine the impact on Rab27a expression of the enhancer and NTE regions, we designed different CRISPR sgRNA sequences to target both regions (Fig. 4D). Small-guided RNA sequences E1, E2 and E3 were designed to target the enhancer region of Rab27a (Fig. 4D). Additionally, sgRNA sequence NTE1 was designed to target the SMC3 binding site in the NTE region (Fig. 4D). We show that all sgRNA sequences led to a decrease in Rab27a protein levels (Fig. 4E). The combination of three sgRNA sequences targeting the enhancer region and the single sgRNA targeting the NTE region were the conditions that most impacted Rab27a protein levels (Fig. 4E). These results demonstrate that the enhancer and the NTE regions are important for Rab27a expression. In addition, the NTE region also appears to be a crucial anchor point, through a SMC3-dependent spatial gene organization mechanism (Fig. 4F).

Discussion

Our work focused on understanding how Rab27a expression changes throughout the different stages of PDAC progression and how this expression is regulated. We described that Rab27a expression decreases upon malignant transformation and is further silenced in undifferentiated PDAC lesions. More importantly, impairment of Rab27a expression leads to an increase in metastatic liver burden in a PDAC GEMM. We identified a novel epigenetic mechanism mediated by SMC3 that regulates Rab27a expression. Methylation of SMC3 inhibits its binding to Rab27a resulting in gene silencing, driving disease progression.

Progressive loss of gene expression from healthy pancreas to undifferentiated cancer tissue suggests a tumor suppressive role for Rab27a. In addition, we demonstrate that in a Rab27a knockout context an increase in the metastatic burden occurs in PDAC GEMMs, which further strengthens this observation. Thus far, the majority of reports associate Rab27a and exosomes mediated communication with an oncogenic function by promoting tumor growth and dissemination [8, 14, 15]. However, the majority of mouse studies were performed using orthotopic models in immunodeficient backgrounds. Interestingly, a report on pancreatic cancer demonstrated for the first time a dual role for Rab27a in the metastatic potential of cancer cells [25]. Loss of Rab27a compromises efficient outgrowth of pancreatic cancer metastatic lesions, however, it also provides an advantage at the first steps of metastasis establishment, through upregulation of different genes associated with epithelial-to-mesenchymal transition [25]. Together with our findings, this indicates that in a setting of natural development of the disease loss of Rab27a expression could be more advantageous rather than detrimental in the metastatic process. However, since the Ashen mouse is a constitutional Rab27a knockout, we cannot exclude a

(See figure on next page.)

Fig. 4 SMC3 binding to Rab27a gene is crucial for gene expression. **A** Hi-C dataset analysis for the study of the binding of SMC3 protein in the Rab27a gene using the H1-hESC cell line. Dashed lines highlight the region of the Rab27a gene. **B** ChIP-qPCR of SMC3 for different binding sites in the Rab27a gene (Promoter, Enhancer and Next to Enhancer regions) in MIA PaCa-2 cells (n = 4). IgG antibody was used as control so that we could identify regions of specific binding for Rab27a. Negative region for SMC3 binding was used to confirm specificity of the enrichment (Neg). One-way anova **p < 0.01, ****p < 0.0001. **C** Luminescent imaging (left) and quantification (right) of MIA PaCa-2 cells non-transfected (1), transfected with pGL3-Promoter empty vector (2), pGL3-Promoter vector with a control sequence (sense, 3), pGL3-Promoter vector with a control sequence (anti-sense, 4), pGL3-Promoter vector with SMC3 binding site in the enhancer region of Rab27a gene (sense, 5) or pGL3-Promoter vector with SMC3 binding site in the enhancer region of Rab27a gene (anti-sense, 6). N = 6, two-way anova, *p < 0.05, ****p < 0.0001. **D** Genome browser panel highlighting regions in the Rab27a gene targeted by sgE1, sgE2, sgE3 and sgNTE1. Sg1 and 3 target regions of the Rab27a enhancer. SgE2 targets SMC3 binding site in the Rab27a enhancer region and sgNTE1 targets SMC3 binding site in a region next to the Rab27a enhancer. Dashed lines identify the enhancer region in the Rab27a gene. **E** Western blot of Rab27a (top) and quantification (bottom) in MIA PaCa-2 cells transduced with a control sg (intron region of Rab27a gene), a sg for Rab27a enhancer region 1, a sg for Rab27a enhancer region 2, a sg for Rab27a enhancer region 3, a combination of sg for different regions of the Rab27a enhancer (1 + 2 + 3) or a sg for next to enhancer region 1. β -actin was used as loading control. **F** Schematic representation of the proposed mechanism of SMC3 binding to the Rab27a gene. Data are Mean $\pm$ SEM

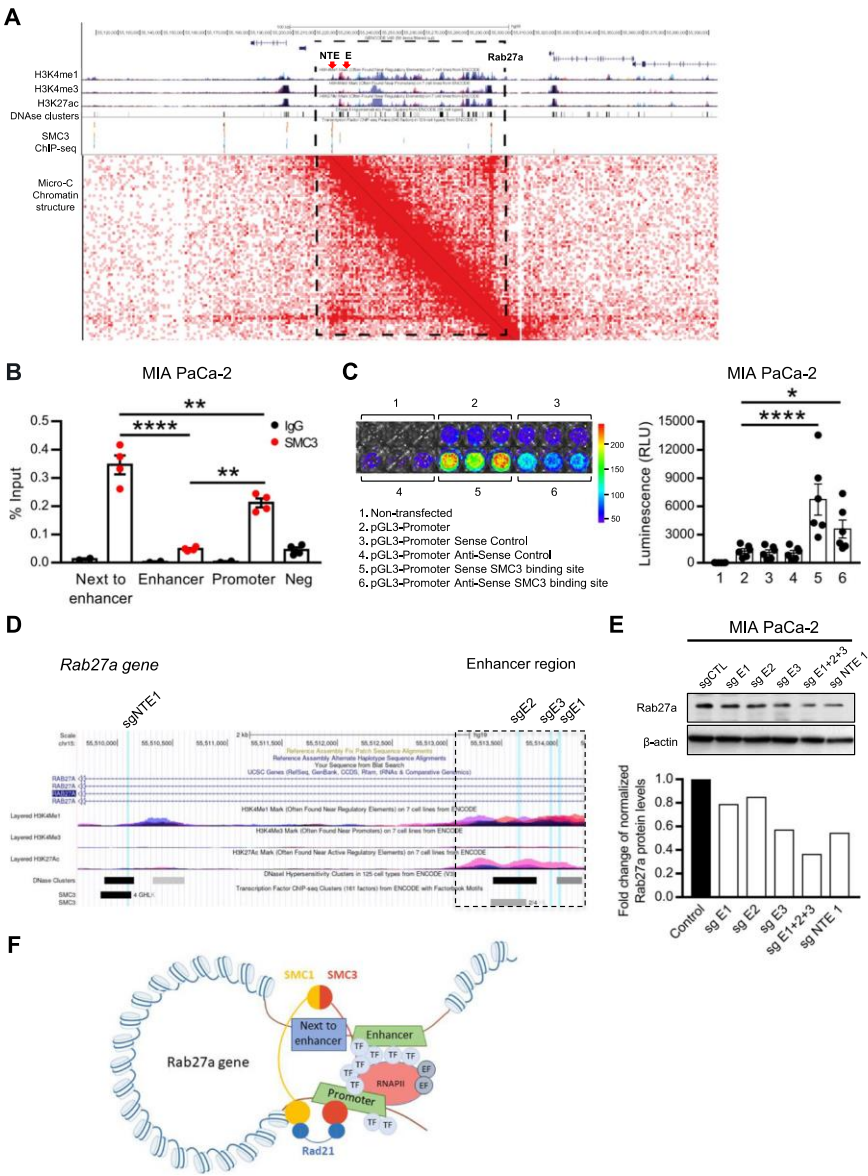


Fig. 4 (See legend on previous page.)

role of Rab27a impairment in other cells of the organism in PDAC progression.

Rab27a mutations have not been described, thus far, in the context of cancer. In fact, when performing TCGA data analysis in PDAC samples we only identified one somatic mutation in one sample which further indicates that epigenetic regulation could play a role in Rab27a expression. Here, we focused on a mechanism mediated by methylation since upon treatment with demethylating agents we observed an increase in Rab27a expression in PDAC cell lines. Nonetheless, Rab27a expression could also be regulated by additional mechanisms. Several studies have demonstrated the ability of non-coding RNAs, in particular microRNAs (miRNAs) and long non-coding RNAs, in controlling gene expression [26]. In fact, different miRNAs such as, miR-134-3p, miR-124 and miR-582-5p have been demonstrated to impair Rab27a expression in different models [27–29]. The study of these non-coding RNAs and others could also be relevant to better understand how modulation of exosomes communication occurs in PDAC.

We propose and have shown it using Hi-C data, that SMC3 binds to the Rab27a gene preferentially in the promoter and next to enhancer regions conferring a conformational change that facilitates interaction between the promoter and enhancer driving transcriptional activity.

Conclusion

In sum, we describe an epigenetic mechanism that regulates Rab27a expression indirectly through SMC3. We have for the first time described an enhancer region within the Rab27a gene. Based on our findings, this mechanism can promote disease progression in the context of PDAC. Targeting of SMC3 expression and activity could potentially be used to modulate Rab27a-dependent exosomes communication and improve patients' outcome.

Methods

Patient tissue samples

Tumor paraffin blocks from PDAC patients were obtained from *Centro Hospitalar Tondela-Viseu*, Viseu, Portugal and from *Carl Gustav Carus University Hospital, Dresden, Germany*. Informed consent was required from all patients. Clinical information provided included age, sex and tumor stage.

Mice

KPC (*Pdx1-Cre; LSL-Kras^{G12D/+}; LSL-Trp53^{R172H/+}*) and PKT (*Ptf1a-Cre; LSL-Kras^{G12D/+}; Tgfb^{2loxP/loxP}*) mouse models alleles were purchased from Jackson Laboratory. B6.FVB-Tg(*Pdx1-Cre*)6Tuv/J (RRID:IMSR_JAX:014647);

B6.129S4-Krastm4Tyj/J (RRID:IMSR_JAX:008179); 129S-Trp53tm2Tyj/J (RRID:IMSR_JAX:008652); Ptf1atm1(Cre)Hnak/RschJ (RRID:IMSR_JAX:023329); B6;129-Tgfb^{2tm1Karl/J} (RRID:IMSR_JAX:012603).

Rab27a^{ash/ash} allele was kindly provided by Doctor Miguel Seabra, CEDOC, NOVA Medical School, Lisbon, Portugal [18].

PKT Ashen (*Ptf1a-Cre; LSL-Kras^{G12D/+}; Tgfb^{2loxP/loxP}; Rab27a^{ash/ash}*) developed PDAC in a spontaneous manner in a similar way to the PKT mouse model.

Regarding the KPC mouse model, a cross-sectional study was performed. Mice were euthanized at different timepoints of disease progression (8 weeks, 16 weeks and HEP - humane end point). PKT and PKT Ashen mice were euthanized when presented severe symptoms.

All mice were housed under standard housing conditions at the i3S animal facility.

Immunohistochemistry

4 µm sections were used for immunohistochemistry staining. Prior to antibody incubation, heat-mediated antigen retrieval was performed for 40 min using a citrate buffer pH 6 solution (Vector Laboratories) followed by an incubation for 30 min at RT with a protein block solution (Dako). Overnight incubation at 4°C was performed for the primary antibody anti-Rab27a 1:200 (Sigma-Aldrich Cat# HPA001333, RRID:AB_1079730). After washing steps, incubation for 30 min at RT with anti-Rabbit/Mouse HRP Dako REAL EnVision Detection System, Peroxidase/DAB (Dako) was performed. Finally, incubation with DAB solution was performed for 1 min. Rab27a score in mouse and human samples was performed based on staining intensity and the percentage of cells present for each intensity. Score 0, 1, 2 or 3 corresponds to negative, weak, intermediate or strong staining, respectively. The percentage of cells for each staining intensity within the slide was also associated with a score, 1—0–25%, 2—25–50%, 3—50–75%, 4— >75%. The final formula applied was: Rab27a score = Score intensity x Area percentage score. A score between 0–12 was given to each tumor slide.

Cell culture

The following cell lines were used: MIA PaCa-2 (ATCC Cat# CRL-1420, RRID:CVCL_0428), PANC-1 (ATCC Cat# CRL-1469, RRID:CVCL_0480), BxPC-3 (ATCC Cat# CRL-1687, RRID:CVCL_0186), hTERT-HPNE (ATCC Cat# CRL-4023, RRID:CVCL_C466) and HEK293T (ATCC Cat# CRL-3216, RRID:CVCL_0063).

All cells were tested for mycoplasma and STR profiled for our study. All PDAC cell lines (MIA PaCa-2, PANC-1 and BxPC-3) and HEK293T were cultured in RPMI-1640 medium (Gibco) supplemented with 10% (v/v)

fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). hTERT-HPNE was cultured in RPMI-1640 medium supplemented with 20% (v/v) FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cell lines were cultured at 5% CO₂ and 37 °C in a humidified incubator.

Azacitidine and decitabine treatments

2 × 10⁵ cells of MIA PaCa-2, PANC-1 and hTERT-HPNE were plated in 6-well plates. On the next day, cells were washed and treated with either azacitidine (Sigma-Aldrich) or decitabine (Sigma-Aldrich) for the following 96 h. During the treatment period, cells were washed daily and fresh medium containing the appropriate concentration of the demethylating agent was used. In azacitidine (Sigma-Aldrich) and decitabine (Sigma-Aldrich) treatments, PANC-1 were treated with a final concentration of 5 µM and MIA PaCa-2 and hTERT-HPNE were treated with a final concentration of 1 µM. Vehicle-treated cells (water/acetic acid 1:1 v/v) were used as control for both azacitidine (Sigma-Aldrich) and decitabine (Sigma-Aldrich) treatments. Technical triplicates were used in each experiment.

Quantitative reverse transcription-PCR

RNA isolation was performed using TRIzol (Thermo Fisher Scientific) as described in the manual. cDNA synthesis was performed using the NZY First-Strand cDNA Synthesis Kit (NZYTech) according to the manufacturer's instructions. For qPCR analysis, Power SYBR green PCR master mix (Applied Biosystems) was used.

Oligonucleotide sequences used for quantification were:

Rab27a forward: AGCTTTGGGAGACTCTGGTG.

Rab27a reverse: TGTGTCCCATAACTGCAGGT.

SMC3 forward: GGAGGGCAGTCAGTCTCAAG.

SMC3 reverse: AGCAAGGGCTACCAAGGATT.

β-actin forward: GAGCACAGAGCCTCGCCTTT.

β-actin reverse: ACATGCCGGAGCCGTTGTC.

2^{-ΔΔCT} method was used to calculate the fold change between conditions.

ChIP-qPCR

Cross-linking ChIP in MIA PaCa-2 was performed using 5 × 10⁶ cells per immunoprecipitation. Cells were fixed using 1% formaldehyde for 10 min at room temperature with gentle shaking and, afterwards, glycine was added for quenching (125 mM, incubated for 2 min at room temperature). Then, cells were washed twice with cold PBS 1X, prior to scrapping.

To obtain a soluble chromatin extract, cells were resuspended in 10 mL LB1 (50 mM HEPES-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 10% glycerol; 0.5% NP-40; 0.25% Triton X-100; 1 × Complete protease inhibitor) and incubated in rotation at 4 °C for 10 min. Samples were then centrifuged and resuspended in 10 mL LB2 (10 mM Tris-HCl, pH 8; 200 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 1 × Complete protease inhibitor), and incubated in rotation at 4 °C for 5 min. Finally, samples were centrifuged and resuspended in 3 mL LB3 (10 mM Tris-HCl, pH 8; 100 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 0.1% Na-deoxycholate, 0.5% N-lauroylsarcosine; 1 × Complete protease inhibitor). Chromatin extracts were sonicated for 18 cycles of 30 s ON/OFF, at high power with a Diagenode Bioruptor Plus sonicator. 1% Triton X-100 was added to the sonicated lysates and 1% was kept as whole-cell extract (WCE).

The lysates were incubated with 8 µg anti-SMC3 (Abcam Cat# ab9263, RRID:AB_307122) or 2 µg anti-rabbit IgG (Thermo Fisher Scientific Cat# 10500C, RRID:AB_2532981) antibodies bound to 100 µl protein G Dynabeads (Invitrogen) and incubated overnight at 4 °C. Magnetic beads were washed with RIPA buffer (140 mM NaCl; 0.1% SDS; 1% Triton X-100; 1 mM EDTA; 0.5 mM EGTA; 10 mM Tris-HCl, pH 8; 0.1% sodium deoxycholate) for 4 to 5 times, then washed with TBS. After centrifugation, DNA was eluted (Tris-EDTA, pH 8) and reverse cross-linked (200 mM NaCl; 0.5% SDS and Proteinase K) for at least 5 h at 65 °C. The eluate was then treated with RNase for 30 min at 37 °C. Finally, DNA was cleaned and purified with Zymo ChIP Clean and concentrator kit (Zymo Research).

ChIP-qPCR was performed using Power SYBR green PCR master mix (Applied Biosystems). Primers sequences used were as follows:

SMC3 promoter region.

Forward—CCTCTGTCGGAAGAAACCTG.

Reverse—GAACCTGGCTGCCTCTGAGT.

SMC3 enhancer region.

Forward—TGTTTCCATTGCTTCATCA.

Reverse—TACCGGCCAGTCTGAAATGT.

SMC3 Next to enhancer region.

Forward—CAAGAGGATGTATTGTTCCCAT.

Reverse—TCCAAATGGCCTTTAAGTGG.

SMC3 Negative region.

Forward—TGCCATGCCTTGAAATATCC.

Reverse—TGCTTCTGAAGTTGCCAAGC.

% Input was calculated by dividing the enrichment of each region in SMC3-IP samples by the WCE. Negative control region was used to validate the specificity of the enrichment in the analyzed regions.

CRISPR

1×10^6 HEK293T cells were plated in a T25 flask for viral particles production. On the next day, cells were washed and transfected with the plasmids psPAX2 packaging (RRID:Addgene_12260), VSV/G envelope (RRID:Addgene_8454) and lentiCRISPR v2 (RRID:Addgene_52961) containing the sgRNA sequence of interest using Lipofectamine 2000 (Thermo Fisher Scientific) using the manufacturer's instructions. After 72 h, cell culture medium containing viral particles was centrifuged at 2000 g for 10 min to remove cell debris and consequently filtered through a 200 nm strainer (GE Healthcare). Then, the resultant culture medium supplemented with polybrene (10 µg/mL) was used to culture MIA PaCa-2 cells previously plated in 6-well plates. After infection, puromycin (1 µg/mL, Sigma-Aldrich) was used to enrich the cell culture in transduced cells.

Sg sequences used were as follows:

Control: GGATCAGAGTCAAGAATACGTGG.

Enhancer 1: TTGTCACAGGGCTAACAACATC.

Enhancer 2: GAGGCACTCATGTAACGTAGTGG.

Enhancer 3: CAGATAAGCGACAATATATGAGG.

Next to enhancer 1: TGGAAATGCATTATAATTAA.

Transfection and luminescence assay

2.5×10^5 MIA PaCa-2 cells were plated in 6-well plates. On the following day, cells were either non-transfected or transfected with shSMC3 in a pLKO1 vector (TRCN0000160156, sh sequence—CGAGTAGAGACTTATCTCAAT). After 72 h, cells were harvested and lysed for western blot.

5×10^4 MIA PaCa-2 cells were plated in 24-well plates. On the next day, cells were either non-transfected or transfected with empty pGL3-Promoter plasmid (Promega, Cat # E1761), pGL3-Promoter Sense Control, pGL3-Promoter Anti-Sense Control, pGL3-Promoter Sense SMC3 binding site, pGL3-Promoter Anti-Sense SMC3 binding site using Lipofectamine 2000 (Thermo Fisher Scientific) using the manufacturer's instructions. Sequences used to clone upstream of the promoter of pGL3-Promoter plasmid are described in Additional file 1: Table S1. Cloning was done in collaboration with GenScript.

After 48 h, cells were harvested and plated in triplicates in 96-well plates. On the following day, Dual-Luciferase® Reporter Assay System (Promega) was used according to the manual to detect luciferase activity. To detect luminescence signal Synergy Mx (BioTek) was used.

Representative picture of luciferase activity using IVIS Lumina iii (Perkin Elmer IVIS Spectrum In-Vivo Imaging System (RRID:SCR_020397)) was taken. Luminescent

image mode was used to detect luminescence signal. Luminescence intensity is depicted by a multicolor scale ranging from blue (least intense) to red (most intense).

Western blot

Western blot was performed as described previously [13]. 30 µg of protein derived from cell lines was used. Primary antibody incubation was performed overnight at 4 °C. Antibody dilutions used were: anti-Rab27a 1:500 (Abnova Cat# H00005873-M02, RRID:AB_519010), anti-SMC3 1:1000 (Abcam Cat# ab9263, RRID:AB_307122). After washing steps, membranes were incubated with the respective HRP-conjugated secondary antibody at 1:5000 dilution for 1 h at RT (Anti-mouse IgG HRP-linked antibody Advanta Cat# R-05071-500, RRID:AB_10718209; Anti-rabbit IgG HRP-linked antibody Cell Signaling Technology Cat# 7074, RRID:AB_2099233). β-actin (Sigma-Aldrich Cat# A3854, RRID:AB_262011) was used for loading control.

Exosomes isolation

Exosomes isolation was performed as previously described [13]. Succinctly, PDAC cells were cultured in RPMI medium with exosomes-free FBS for 72 h. Afterwards, medium was centrifuged at 2500RPM for 10 min followed by a centrifugation at 4000RPM for 5 min to remove cell debris. Next, medium was filtered using a 200 nm filter (GE Healthcare) and ultracentrifuged at 100000 g for 3 h at 4 °C. Finally, supernatant was discarded and exosomes pellet was resuspended in 100 µL of PBS 1X for NanoSight NS300 (Nanoparticle Tracking Analysis, RRID:SCR_014239) analysis.

Statistical analysis

For the analyses performed in this manuscript significance was determined at $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, $p < 0.0001^{****}$ and represent significance between conditions. All analyses were performed using GraphPad Prism® (GraphPad Prism, RRID:SCR_002798).

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12967-023-04448-1>.

Additional file 1: Table S1. pGL3-promoter sequences for luminescence assay.

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Author contributions

Conceptualization: SAM and NB; methodology: NB; CM and SAM; formal analysis: NB, SC; investigation: NB, SC, BA, CM; resources: SAM; Data curation: NB; CM; Writing—original draft: NB and SAM; Writing—Review & Editing: NB, SAM, JCM; visualization: BA and NB; supervision: SAM; funding acquisition: SAM.

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Availability of data and materials

The source data file contains all the source data related to the manuscript and is deposited in the Figshare Data Repository DOI: <https://doi.org/10.6084/m9.figshare.23799390>.

Declarations

Ethics approval and consent to participate

Informed consent was required from all patients that participated in this study. The ethical committee of Centro Hospitalar Tondela-Viseu, Viseu (ID number 02/07/2018), and ethical committee of Carl Gustav Carus University Hospital, Dresden, Germany approved the use of tumor paraffin blocks. Human studies were conducted in accordance with the Declaration of Helsinki ethical guidelines. Animal protocol was approved by DGAV “Direção Geral de Alimentação e Veterinária”.

Consent for publication

Not applicable.

Competing interests

SAM hold patents in the area of exosomes biology that are licensed to Codiak Biosciences, Inc. The other authors declare no competing interests.

Author details

¹I3S - Instituto de Investigação e Inovação Em Saúde, University of Porto, 4200-135 Porto, Portugal. ²Institute of Biomedical Sciences Abel Salazar, University of Porto, 4050-313 Porto, Portugal. ³Department of Pathology, Faculty of Medicine, University of Porto, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal. ⁴Porto Comprehensive Cancer Center (PCCC) Raquel Seruca, Porto, Portugal. ⁵The Gurdon Institute and Department of Pathology, University of Cambridge, Cambridge, UK.

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APPENDIX II

Supplementary spreadsheets are available online in the following link:

<https://figshare.com/s/e814270a22e6c450bca0>.

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