



UNIVERSITÀ
DEGLI STUDI
DI PADOVA



ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Biomedical Science

*Interuniversity Bachelor's degree programme
in Biology of Human and Environmental Health*

Final dissertation

**siRNA-mediated Silencing of OPA1, NME4, and RCC1L:
Effects on Proliferation in KP4 Pancreatic Cancer Cells**

Supervisor
Professor Francesco Chemello

Candidate *Lora Pavlova*
Student ID number: 2112419

Contents

1. Introduction

- 1.1. Pancreatic ductal adenocarcinoma (PDAC)
- 1.2. The role of mitochondria in cancer
- 1.3. OPA1, NME4, RCC1L
- 1.4. Preliminary data
- 1.5. Aim of the study

2. Materials and methods

- 2.1. KP4 and PANC1
- 2.2. Proliferation assay using siRNA
- 2.3. Colony formation assay using siRNA
- 2.4. Fluorescent microscopy

3. Results

- 3.1. Proliferation assay
- 3.2. Colony formation assay

4. Discussion

5. Conclusion

6. Bibliography

Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains one of the deadliest and most aggressive malignancies worldwide, characterized by late diagnosis, limited therapeutic options, and extensive metabolic reprogramming. While oncogenic KRAS signaling extensively rewires PDAC metabolism towards glycolysis, pancreatic cancer cells remain actively reliant on mitochondrial oxidative phosphorylation (OXPHOS) and generally on mitochondrial function for survival and proliferation. Mitochondrial dynamics and *cristae* organization are therefore important processes in the adaptation and progression of cancer cells.

This study investigated the contribution of three interconnected mitochondrial proteins, optic atrophy 1 (OPA1), mitochondrial nucleoside diphosphate kinase (NME4), and regulator of chromatin

condensation 1 like (RCC1L), to the proliferative capacity of PDAC cells. OPA1 is a key regulator of the inner mitochondrial membrane fusion and *cristae* architecture, while NME4 and RCC1L are proposed to support OPA1 function through local nucleotide metabolism and guanine nucleotide exchange, respectively. However, their functional contribution to PDAC cell proliferation remains insufficiently characterized.

The effects of transient siRNA-mediated silencing of OPA1, NME4, and RCC1L were investigated in the human PDAC cell line KP4 using two different proliferation assays. Moreover, the effect of NME4 silencing on clonogenic capacity was additionally evaluated by colony formation assays using both KP4 and PANC1 cells.

The results demonstrated differential effects of the three targets on KP4 proliferation. Both OPA1 and NME4 silencing produced variable effects across independent experiments, While RCC1L silencing displayed the most consistent trend towards reduced proliferation across experiments. However, none of the observed differences between silenced and control conditions reached statistical significance. In contrast, the colony formation assays showed a consistent qualitative increase in crystal violet staining following NME4 knockdown in both KP4 and PANC1 cells, suggesting that NME4 depletion did not impair clonogenic growth under the experimental conditions.

Overall, these findings suggest that OPA1, NME4, and RCC1L may have differential contributions to PDAC cell growth, with RCC1L showing the strongest and most consistent trend towards growth inhibition following depletion.

1. Introduction

1.1. Pancreatic ductal adenocarcinoma (PDAC)

Pancreatic ductal adenocarcinoma is one of the deadliest cancers worldwide. Its mortality rate is projected to surpass that of colorectal cancer in less than two decades, placing it right behind lung cancer mortality. This is largely attributable to the absence of reliable biomarkers for detection of early-stage tumors and the inaccessible location of the pancreas, making it difficult to perform routine screening. Most patients present with advanced, unresectable disease at diagnosis, which is why only about 12% of them are expected to live beyond 5 years.[1] The three most common symptoms are loss of appetite, indigestion and change in bowel habits. In patients who present with tumors starting at the head of the pancreas, the symptoms observed are biliary obstruction that leads to dark urine,

appetite and weight loss, jaundice, and exocrine pancreatic insufficiency. Conversely, those presenting with body and tail pancreatic cancer exhibit symptoms of abdominal and back pain together with appetite and weight loss and fatigue. Another sign of PDAC is progression or new onset of diabetes. Amongst the risk factors are smoking, chronic pancreatitis, alcohol use, obesity, diabetes, diets high in saturated fats, processed meats and high-fructose beverages. The disease is increasingly observed in younger individuals, parallel with the increasing incidence of obesity and diabetes, which are considered potentially modifiable risks.[2]

Given that most patients present with advanced disease at diagnosis, treatment strategies are largely determined by disease stage, tumor resectability, vasculature involved, and the patient's performance status and overall health.[1] Most commonly, PDAC metastases to the liver, lymph nodes and lungs and less often to the peritoneum and the bones. As mentioned earlier, there are no reliable biomarkers for diagnosis of PDAC, but the most widely used one, although with limited sensitivity, is the carbohydrate antigen 19-9 (CA 19-9). Elevated levels of this antigen are observed in the plasma prior to PDAC diagnosis. However, there can be a lot of false positives which is why screening of the general population is not advisable. Instead, it should be used as a biomarker in high-risk cohorts only together with imaging-based surveillance. [2]

Surgery is an option only for patients with resectable tumors that have not yet invaded major vascular structures, whereas chemotherapy is used in borderline resectable, locally advanced, and metastatic disease. FOLFIRINOX is one of the standard first-line chemotherapy regimens and is a combination of 5-fluorouracil (5-FU), leucovorin, irinotecan, and oxaliplatin.[1]

PDAC originates in the exocrine pancreatic tissue. Both acinar and ductal pancreatic cells can accumulate mutations in oncogenes and/or lose the function of tumor suppressor genes, leading to the development of the disease. [Fig.1] This process is a multi-step progression and usually spans over many years, giving a chance for early detection and further highlighting the need for more sensitive and specific biomarkers. [1] Pancreatic intraepithelial neoplasms (PanINs) are the most common microscopic precancerous lesions. Even though low-grade PanINs are common, the likelihood of them becoming malignant is still unclear. Another very common type of precursor lesions are the intraductal papillary mucinous neoplasms (IPMNs) that can be observed in the branch or the main pancreatic duct. [Fig.1] [1,2]

During their progression towards invasive PDAC, PanINs and IPMNs accumulate genetic alterations such as activating mutations in the KRAS oncogene and deletions or inactivating alterations in the tumor suppressor genes CDKN2A, TP53, and SMAD4. These alterations support

PDAC development and progression through dysregulation of growth signaling and inflammatory processes. [1,2]

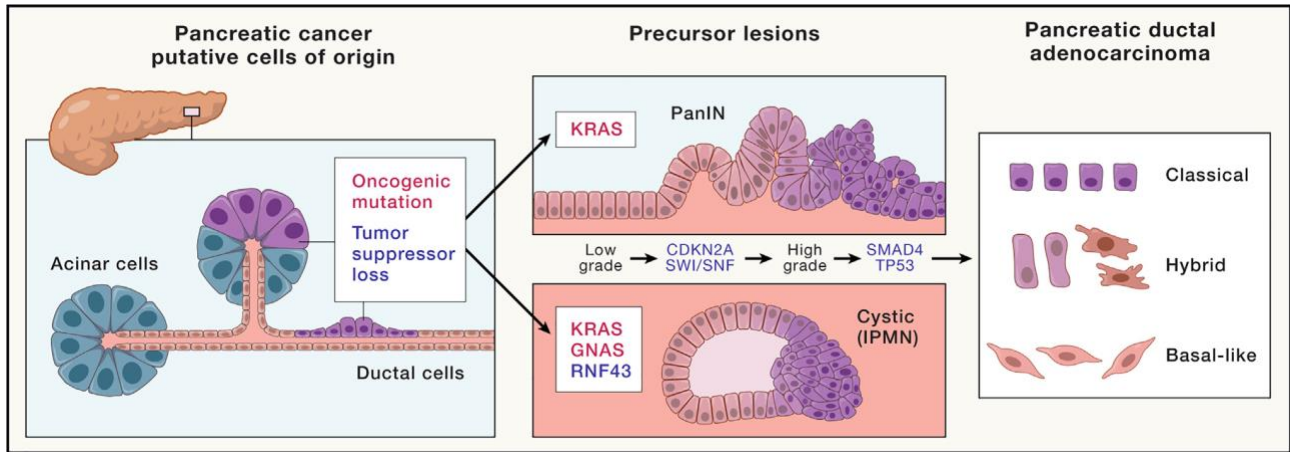


Fig.1: Initiation and progression of pancreatic cancer

The figure shows the progression of the two main types of precursor lesions, PanINs and IPMNs, to PDAC through a stepwise accumulation of genetic alterations. There are many different classifications of PDAC subtypes, an example of which is shown in this figure – it suggests a subdivision into classical, hybrid and basal-like pancreatic cancer through RNA transcriptional analyses. [1] It is yet to be discovered how efficient it is to choose a therapy based on the subtype of PDAC.[2]

Aside from the above-mentioned acquired somatic mutations, between 4-10% of PDAC patients also possess germline gene variants that are pathogenic and increase susceptibility to PDAC – these variants are most often observed in genes that are part of the DNA damage repair (DDR). Most common variants include BRCA1/2 (hereditary breast and ovarian cancer syndrome) and ATM (ataxia and telangiectasia syndrome).[2]

The tumor microenvironment (TME) of PDAC consists of cytokines, immune cells, fibroblasts, metabolites, and desmoplastic stroma which is rich in hyaluronan. It suppresses the host's immunity against cancer thus favoring carcinogenesis. Furthermore, this accounts for the resistance to some single-agent therapy regimes.[2] The predominant cell type are fibroblasts which accounts for the characteristic physiology of PDAC due to their role in the deposition of the extracellular matrix. Activation of KRAS in PDAC cells contributes to the immunosuppressive TME by inducing the granulocyte-macrophage colony-stimulating factor GM-CSF production, a cytokine involved in the regulation of myeloid cells. In turn, GM-CSF promotes the recruitment of myeloid-derived

suppressor cells (MDSCs), which suppress the anti-tumor immune response, favoring tumor progression.[1]

The physical characteristics of PDAC tumors' TME lead to local hypoxia and dysregulation of metabolism and nutrient supply. PDAC cells ensure their survival, adaptation and metastasis by KRAS-promoted upregulation of glycolysis, reprogramming of organelles and mitophagy. Another important mechanism is the downregulation of the major histocompatibility complex class I (MHC-I), which allows the cells to escape the immune system's defense mechanisms.[1]

Although oncogenic KRAS signaling extensively shifts PDAC metabolism towards glycolysis and anabolic pathways, pancreatic cancer cells retain a great reliance on mitochondrial respiration for survival and proliferation. [1] Indeed, previous work from the host laboratory demonstrated that KRAS-mutated PDAC cell lines, such as PANC1, show significantly higher oxygen consumption rates compared to non-malignant cells, underscoring their active dependence on oxidative phosphorylation and mitochondrial metabolism to meet their bioenergetic and biosynthetic demands.

1.2. The role of mitochondria in cancer

According to the endosymbiotic theory, more than 1.5 billion years ago a prokaryotic cell was engulfed by a larger host cell. Instead of being digested, the engulfed prokaryote established a symbiotic relationship with its host through a long process of evolutionary changes, eventually giving rise to the mitochondrion. In the context of the host cell's environment, this endosymbiotic relationship gave rise to fusion and fission of mitochondria and the remodeling of *cristae*, which are all crucial processes for the survival of modern eukaryotes.[3]

Mitochondria are double-membrane-bound organelles composed of an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM) that folds inward, forming structures called *cristae*. The OMM serves as both a protective barrier and an exchange surface. The IMM can be divided into three distinct zones: inner boundary membrane (IBM), *cristae* junctions (CJs) and *cristae*. The IBM runs closely along the OMM, and forms specialized contact sites with it at multiple locations. These contact sites are involved in communication between the two mitochondrion membranes, including the transport of metabolites and the organization of protein complexes. Along the IMM we can find *cristae*, which are continuous invaginations of the inner membrane. The narrow openings of the *cristae* are called *cristae* junction and control the passage of molecules between the intracristal space and the inter membrane space (IMS). [3]

Mitochondria dynamically respond to signals from the cytosol through processes called mitochondrial dynamics, which consist of fusion, fission and remodeling. These are morphological changes and modulations that aim at cell survival and adaptation to the current needs and environment. [3]

Fusion and fission are two coordinated processes that are closely linked to mitochondrial metabolic activity and allow mitochondria to adapt to changing cellular demands. There are three main dynamin-related GTPases involved in mitochondrial dynamics: dynamin-related protein 1 (DRP1), mitofusin 1 and 2 (MFN1/MFN2), and optic atrophy protein 1 (OPA1). DRP1 plays a key role in mitochondrial fission, while MFN1/MFN2 and OPA1 mediate OMM fusion and IMM fusion, respectively. Mitochondrial fusion occurs through two coordinated steps of fusion of the OMM followed by fusion of the IMM. [4]

Although increased mitochondrial fission and a more fragmented morphology are frequently observed in cancer cells, the relationship between mitochondrial morphology and metabolic phenotype is highly context-dependent.[4]

The morphology of *cristae* can vary considerably in terms of length, width, orientation, shape and rigidity. *Cristae* are highly dynamic structures that can undergo great changes in response to alterations in the cellular environment, such as changes in their number, the spacing between the *cristae* membranes, and the degree of opening of the CJs. [3] The *cristae* membrane has a higher potential and contains much of the respiratory chain machinery. Folded, narrow *cristae* facilitate oxidative phosphorylation (OXPHOS), as *cristae* organization is closely associated with the positioning and organization of the respiratory chain complexes and ATP synthase. [3,4]

Apart from participating in IMM fusion, OPA1 is also particularly important for the organization and remodeling of *cristae*, together with the ATP synthase dimers/oligomers and mitochondrial contact site and *cristae* organizing system-sorting and assembly machinery (MICOS-SAM) complexes. [3,4]

OPA1 exists in two main forms, long OPA1 (L-OPA1) and short OPA1 (S-OPA1). [3] L-OPA1 is anchored in the IMM and extends towards the IMS, where it forms oligomeric complexes within the *cristae* membrane, right below the CJs. S-OPA1 is generated from L-OPA1 through proteolytic cleavage and is in the intracristal space. The cleavage is mediated primarily by OMA1, which is a zinc metalloendopeptidase, and YME1L, an ATP-dependent i-AAA protease. The balance between L- and S-OPA1 is crucial for mitochondrial morphology. In normal physiological conditions, YME1L constitutively processes a portion of L-OPA1 into S-OPA1, which are both crucial for IMM fusion.

However, under stress and loss of mitochondrial membrane potential, OPA1 increases its activity, which results in higher OPA1 processing rate, loosening of the *cristae*, and mitochondrial fragmentation. [4]

Deficiency of OPA1 leads to *cristae* widening, while its overexpression promotes tighter *cristae* organization. Complete loss of OPA1 results in loss of most of the *cristae* structures.[4] This is important since OPA1 is contributing not only to fusion and *cristae* integrity, but also sequestration of cytochrome c and regulation of apoptotic cascade. During apoptosis, disassembly of OPA1 oligomers and widening of the *cristae* junctions both facilitate the release of cytochrome c, promoting activation of the apoptotic cascade. These functions highlight the potential relevance of OPA1 in cancer biology, as dysregulation of apoptotic pathways is a hallmark feature of cancer cells.[5]

Mitochondrial function is associated with cancer-cell proliferation, since mitochondria provide both energy and biosynthetic intermediates required for cellular growth. In cancer cells, the tricarboxylic acid (TCA) cycle contributes not only to oxidative metabolism, but also to the production of metabolic intermediates needed for biosynthetic processes.[5]

In the context of PDAC, mitochondrial dynamics (the continuous processes of fusion, fission, and *cristae* remodeling) have been closely linked to tumor progression. These morphological transformations allow tumor cells to adapt their metabolic architecture in response to environmental stress and nutrient availability. [4,5] Oncogenic KRAS signaling has been shown to promote mitochondrial fission through DRP1 activation, altering the metabolism in favor of the tumor progression and growth. Conversely, experimentally induced fusion in pancreatic cancer cells via DRP1 inhibition and overexpression of MFN2 results in increased mitophagy and reduced oxidative phosphorylation (OXPHOS). [4]

These findings underline the importance of mitochondrial dynamics and metabolism in PDAC, urging the investigation of proteins involved in mitochondrial structure and function.

1.3. OPA1, NME4, RCC1L

As already mentioned, OPA1 is one of the key regulators of *cristae* morphology. However, there are two other important proteins supporting its function: NME4 and RCC1L.

NME4, also known as mitochondrial nucleoside diphosphate kinase (NDPK-D), is part of the NDPK-NME family of nucleoside diphosphate kinases. In humans, NME4 consists of 187 amino acids and is the only NME protein containing a typical N-terminal mitochondrial targeting sequence

and is predominantly localized to mitochondria. NME4 exists as a homohexamer and is associated with the IMM, primarily oriented towards the intermembrane space, largely through electrostatic interactions between a specific surface-exposed motif (R89-R90-K91) and cardiolipin (CL), which is an anionic phospholipid enriched in the IMM. This association gives NME4 its dual function as phosphotransferase and as a protein that is part of lipid transfer processes, including pathways that are essential for mitophagy and apoptosis. It catalyzes the reversible transfer of phosphate groups between nucleoside diphosphates and triphosphates. [6] Within the intermembrane space, when bound only to the IMM, NME4 can generate GTP from ATP and GDP, providing a direct local source of GTP to OPA1 and supporting its function in mitochondrial dynamics and *cristae* organization. [6,7] When it fully binds CL, its phosphotransferase activity is almost fully inhibited. Instead, it links the IMM and the OMM, facilitating the transfer of CL from the IMM to the OMM. [7] Under cellular stress or mitophagic stimuli, NME4 facilitates the intermembrane transfer and exposure of CL to the OMM. This exposed CL serves as an elimination signal for the microtubule-associated protein 1 light chain 3 (LC3)-mediated mitophagy or as an anchor for pro-apoptotic machinery, demonstrating the importance of NME4 in the quality control of mitochondria and elimination of damaged ones. [6]

RCC1L (regulator of chromatin condensation 1 like), also known as WBSR16, is a mitochondrial protein encoded by the *RCC1L* gene. [8] There have been identified three isoforms (RCC1L^{v1-3}) in humans, generated through alternative splicing and all connected to mitochondria, with RCC1L^{v1} being the predominant transcript. [8] RCC1L predominantly localizes to the outer face of the IMM facing the intermembrane space, placing itself in direct contact with the OPA1 GTPase domain. [9] It has been established experimentally that it also plays an important role in mitochondrial ribosome biogenesis, with different isoforms contributing to the assembly of the mitochondrial large and small ribosomal subunit. [8] More importantly, it is an OPA1-specific guanine nucleotide exchange factor (GEF). [9] Experiments show that it interacts physically with OPA1 within intact cells, directly catalyzing GTP loading onto OPA1 in a dose-dependent manner, acting as a rate-limiting driver of OPA1 GTP hydrolysis. Its GEF activity is highly specific to OPA1 and does not extend to outer membrane GTPases such as MFN1/2 or mitoribosomal translation factors. RCC1L drives IMM fusion through the OPA1-MFN1 functional axis. While its overexpression induces a hyperfused mitochondrial network in *Mfn2*^{-/-} cells, it fails to promote fusion in the absence of OPA1 or MFN1. On the other hand, genetic ablation of RCC1L leads to mitochondrial network collapse and fragmentation, impaired total GTP hydrolysis, apoptotic induction, and cell cycle arrest. [9] It has also

been established, through proximity ligation assays (PLA), that NME4 and RCC1L are closely associated, suggesting a potential functional relationship between the two proteins. [10]

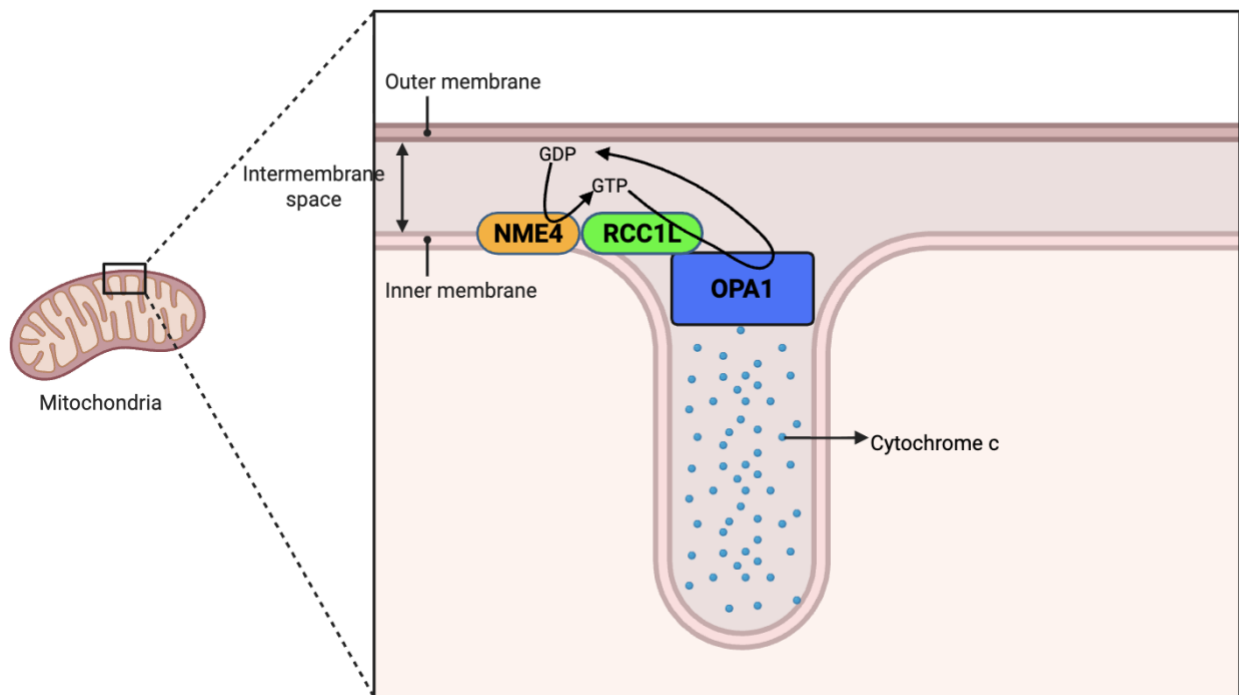


Fig.2: The NME4-OPA1-RCC1L localized machinery at the *cristae* junction

Schematic representation of the localized nucleotide exchange and GTPase activation complex at the IMM. NME4 functions as a local phosphotransferase, utilizing ATP to generate GTP from GDP within the intermembrane space. RCC1L acts as an OPA1-specific guanine nucleotide exchange factor (GEF), promoting the release of bound GDP from OPA1 to allow rapid binding of fresh GTP. Active, GTP-bound OPA1 oligomerizes at the CJs to stabilize *cristae* architecture and sequester intracristal cytochrome c.

Together, these findings suggest that NME4 and RCC1L may regulate OPA1 through complementary mechanisms, with NME4 contributing to the local GTP availability and RCC1L facilitating its exchange onto OPA1. [Fig.2] However, while their individual roles in mitochondrial dynamics have been investigated, their functional relationship and contribution to mitochondrial homeostasis in PDAC remain not fully understood. This provides a rationale for investigating NME4, RCC1L and OPA1 in PDAC cells and determining how they may affect cellular proliferation, more specifically through their depletion.

1.4. Preliminary data

The host laboratory was interested in investigating the role of mitochondria in pancreatic cancer progression. To better understand this, a mitochondrial proteomic screening was conducted in two different cancer cell lines, as a model for advanced PDAC, compared with human pancreatic ductal epithelial cells harboring the KRAS^{G12D} mutation, as a model of the earliest stages of carcinogenesis. In both cancer cell lines, they found NME4 and RCC1L to be up-regulated, suggesting that, together with OPA1, these three proteins may play a role in promoting cancer progression by regulating mitochondrial dynamics and structure and thus cancer metabolism.

1.5. Aim of the study

This study investigates the role of the mitochondrial proteins OPA1, NME4 and RCC1L in pancreatic ductal adenocarcinoma (PDAC) cells. Specifically, we evaluated whether silencing these genes using small interfering RNA (siRNA) affects the proliferative capacity of KP4 and PANC1 pancreatic cancer cell lines, with the broader objective of investigating their potential contribution to mitochondrial function and PDAC progression.

2. Materials and methods

2.1. KP4 and PANC1

KP4 and PANC1 are human pancreatic cancer cell lines used as *in vitro* models for PDAC. Both were grown and maintained in 10 cm petri dishes in DMEM high glucose medium supplemented with 10% fetal bovine serum, 1% glutamine and 1% penicillin-streptomycin solution, which is the standard medium. At 80-85% confluence they were split and transferred to a new plate according to the following protocol:

1. Aspirate the medium fully.
2. Wash the plate twice with 3-4 mL of PBS.
3. Incubate the plate with 1 mL of Trypsin for about 5 minutes at 37 °C.
4. Take the plate out and check under the microscope if all cells have detached. If needed, detach manually as well.

5. Dilute the trypsin with PBS (about 4 mL) and wash the plate well.
6. Collect everything in a 15 mL tube and repeat the wash with 3-4 mL more of PBS.
7. Again, collect the rest of the cell-PBS solution and centrifuge at 300g for 5 minutes.
8. Remove the supernatant and add 3-4 mL of DMEM high glucose medium.
9. Resuspend the cell pellet very well.
10. Take 10 μ L and load them into the Bürker chamber and count under the microscope.
11. To obtain the number of cells in 1 mL of the cell-medium solution, count 3 out of 9 squares of the chamber and add them together. Compute the mean and multiply the number obtained by 10 000.
12. Label a new plate along its borders appropriately: write the name of the cell line, the passage, date and dilution ratio or number of cells plated, or both. The final volume in the new dish should be at least 7 mL. According to how many cells we want to plate or in what ratio, we first put the medium in the new dish and then add the right amount of cell suspension to reach the final volume.
13. To distribute them evenly, we gently slide the plate a few times as if following the outline of either a cross or the sign for infinity.
14. Check if they are evenly distributed under the microscope and then incubating at 37°C 5% CO₂, repeating the previously mentioned movements to make sure they are well-spread.

This protocol was universally used for both cell lines.

2.2. Proliferation assay using siRNA

Proliferation assay (PA) using small interfering RNA (siRNA) is an experiment used to evaluate cells' proliferative capacity. It consists of the seeding of cells in two distinct 12-well plates in 3-4 replicates for each condition, including a control. One plate was labeled 'DAY 0' and the other one contained the 3 replicates for all the conditions, namely siRNA control (siCTRL), siOPA1, siNME4 and siRCC1L. On the first day of the experiment, the cells were plated in triplets for each condition in one plate and in four wells in the 'DAY0' one. On the second day, which is counted as 'day 0', we counted the four wells and obtained a mean cell proliferation number over 1 day. This number represent the basal proliferation rate for cells in normal conditions. We then transfected the other plate with the corresponding siRNAs. These wells were also counted two days afterwards,. These

numbers were then normalized on the 'DAY0' number, which gave us a relative cell growth of each silenced protein.

PA was conducted four times using only KP4 cells. The first experiment investigated the proliferation only of silenced OPA1, the next one of silenced NME4 and RCC1L and the last two included all three proteins.

The protocol used was the following:

1. Label two 12-well plates appropriately with the name of the experiment, name of the cell line, date and number of seeded cells, which is 35 000 in each well.
2. For each condition, prepare 3 replicates and fill each well with 1 mL of DMEM high glucose medium. For the 'DAY0' plate, use 4 wells.
3. Add the cells and check under the microscope if they are evenly distributed and then gently slide them in the cross/infinity sign directions as described before. Then place in the incubator at 37°C 5% CO₂ overnight.

On 'day 0'

4. Transfect the plate with all the different conditions using the appropriate siRNA.
 - a. First, change the medium in all the wells with Opti-MEM, which is a reduced-serum medium. Add 800 µL instead of 1 mL.
 - b. Dilute the target-specific siRNA and RNAiMAX (a type of commercial lipofectamine (Invitrogen, 13778030) which is a lipid-based transfection reagent used to facilitate the entry of the siRNA into the cell) in Opti-MEM. Start by preparing and labeling 3 Eppendorf tubes with 'R1', 'R2' and 'R3', which stands for replicate 1, 2 and 3 and are called master mix. In each, add 100 µL of Opti-MEM together with 1 µL of RNAiMAX for each condition. If we have two conditions for example, we will add 200 µL of Opti-MEM together with 2 µL of the lipofectamine to each tube. Label and prepare 3 tubes per condition by adding 100 µL Opti-MEM and 0,01 nmol of the specific siRNA.
 - c. Distribute the master mix with the RNAiMAX drop by drop across all the conditions and in the appropriate replica number. For example, 'R1' gets distributed among all replicates labeled with number 1 and so on. Incubate at room temperature for 20 minutes for the complexes to form. The final volume in each tube must be 200 µL.

- d. Add each tube to the corresponding well, again drop by drop.
 - e. Incubate the plate at 37°C and in 6 hours, change the medium with regular DMEM high-glucose medium.
5. Take the 'DAY0' plate out of the incubator and count the cells in each well. In the end, compute the average and normalize the other counts from the silenced conditions on it in the end.
- a. Remove the medium and wash each well twice with 800 μ L – 1 mL of PBS.
 - b. Add 200 μ L of Trypsin and incubate for 4 minutes.
 - c. Take the plate and check under the microscope if all the cells have been detached.
 - d. Add 800 μ L of PBS and resuspend very well.
 - e. Take up 10 μ L and count each well using the Bürker chamber.
 - f. Calculate the mean number of cells per 1 mL.

48 hours later

6. Calculate the mean number of cells per condition using the same protocol for detaching and counting used for the 'DAY 0' plate.
7. Normalize these numbers on the mean cell proliferation number from the 'DAY 0' plate.
8. Use this information to make a graph and test for statistical significance.

2.3.Colony formation assay using siRNA

Colony formation assay (CFA) is an experiment that is similar to the PA one – it is also used to examine the proliferative capacities of silenced cells against a control group- but which is used also to evaluate cells' clonogenic capacity. We performed two CFAs including only siCTRL and siNME4 – once using KP4 cell line only with two replicates and the other time using both KP4 and PANC1 with 3 replicates for each, using one and two 6-well plates, respectively. Each time, we seeded different numbers of cells, adjusting it accordingly to previous experiments.

The protocol is as follows:

1. Label a 6-well plate properly (name of experiment, number of cells, name of the cell line and the date) and seed cells doing 2-3 replicates for each condition. One should be siCTRL and the other one is one of the three mitochondrial genes (in our case NME4).

2. Prepare a number of silencing master mixes equal to the number of replicates per condition. For each replicate, for all conditions, add 300 μ L together with 3 μ L of the RNAiMAX in each mix. Label and prepare also one tube for each condition, adding 150 μ L of Opti-MEM together with 0,015 nmol of the target-specific siRNA as many times as the number of replicates per condition.
3. Add the master mixes as in the PA – equally distributed between all replicates for all conditions, drop by drop. The total volume in each tube should be 300 μ L. Incubate at room temperature for 20 minutes.
4. Add the mixes in the appropriate well drop by drop in a final volume of 1,5 mL and incubate at 37°C 5% CO₂.
5. Change the medium 6 hours later and put back in the incubator.
6. Incubate for 48 hours.

Fixing and staining of the cells:

We removed the medium and washed three times with PBS. We fixed the cells with a 4% paraformaldehyde (PFA), incubating for 15-20 minutes. Then we removed the PFA and washed once with PBS. We added the 0,1% crystal violet solution and incubated for 20 minutes at room temperature, covering with aluminum foil. Lastly, we aspirated the crystal violet and washed thrice with milli Q water. The plate was left outside, facing down to dry very well.

2.4.Fluorescent microscopy

Fluorescent microscopy was used in PA III as an alternative to counting cells manually using the Bürker chamber – we seeded cells as per usual in a 12-well plate, following the aforementioned protocol and adding cover slips in each well. Then we fixed and stained them before mounting them on the glass slides, following this protocol:

1. Remove the medium and wash three times with PBS.
2. Fix the cells using a 4% PFA solution, incubating for 10 minutes.
3. Wash thrice with PBS.
4. Permeabilize the cells with a 0,1% PBS-TritonX-100 solution for 10minutes.
5. Wash thrice with PBS.

6. Add 1 mL of Hoechst 33342 (1:10000 in PBS) in all wells of the plate, incubating for no longer than 5-10 minutes.
7. Remove the dye and wash twice with PBS and one time with milli Q water.
8. On the glass slide, put a small drop of the Prolong mounting medium. Since it is very viscous, it is recommended to cut the tip of the pipet wider.
9. Carefully take the glass slip out of the well and place it on the mounting medium with the cells facing down.
10. Remove all bubbles and the excess medium by pushing it away and aspirating it.
11. Leave to dry very well in the fridge covered with aluminum foil.
12. Seal the edges with transparent nail polish.

Then, we took ten random images per cover slip, which were later loaded into Mitochondria Analyzer, an in-house generated software for counting mitochondria. We changed the parameters to answer to the size of the stained nuclei and ran the images through the software. In the end, we calculated the mean proliferation rate for each condition.

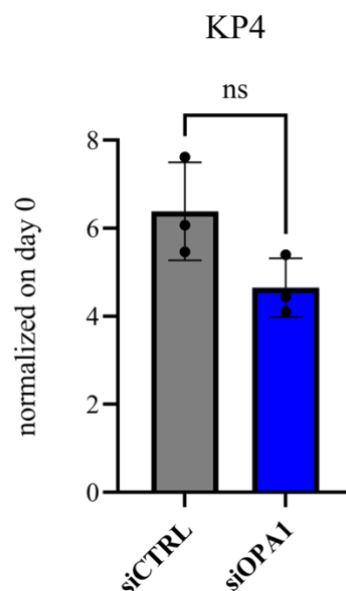
3. Results

3.1. Proliferation assay

PROLIFERATION ASSAY I

‘DAY 0’ plate average = 4,94

CONDITION	MEAN CELL NUMBER	NORMALIZED ON DAY 0*
siCTRL	37,67	7,63
	30	6,1
	27	5,47
siOPA1	26,67	5,4
	22	4,45
	20,3	4,11

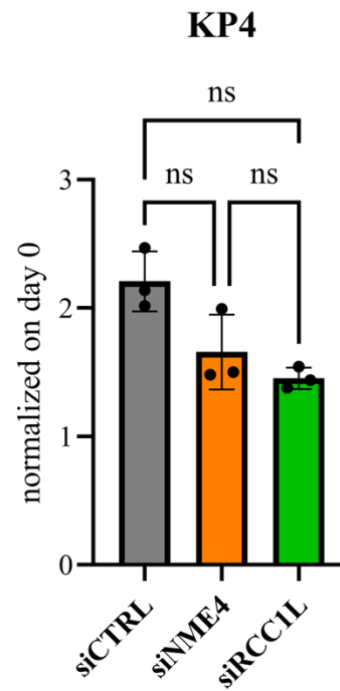


*for the normalization, we divided the mean cell number of each condition by the one of the ‘DAY0’ plate

PROLIFERATION ASSAY II

‘DAY 0’ plate average = 5,41

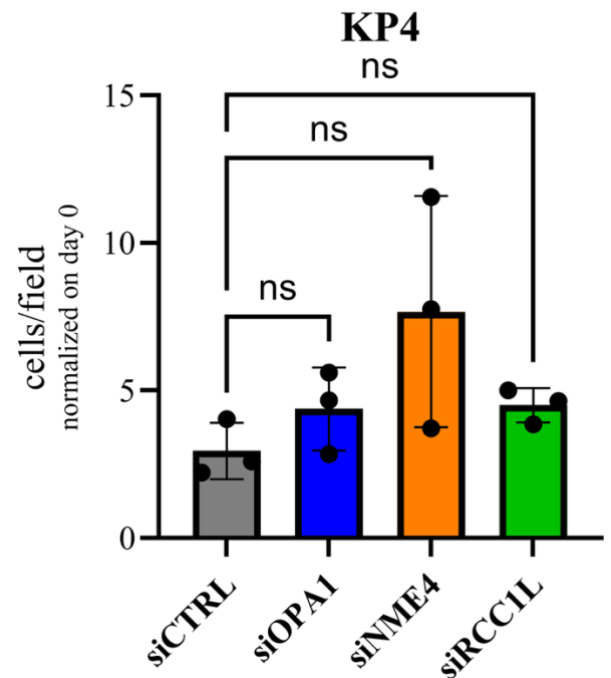
CONDITION	MEAN CELL NUMBER	NORMALIZED* ON DAY 0
siCTRL	10,89	2
	13,33	2,47
	11,56	2,14
siNME4	8,11	1,5
	10,78	1,99
	8	1,48
siRCC1L	7,78	1,44
	7,44	1,38
	8,33	1,54



PROLIFERATION ASSAY III (using fluorescent microscopy images for counting)

‘DAY 0’ plate average = 10,03

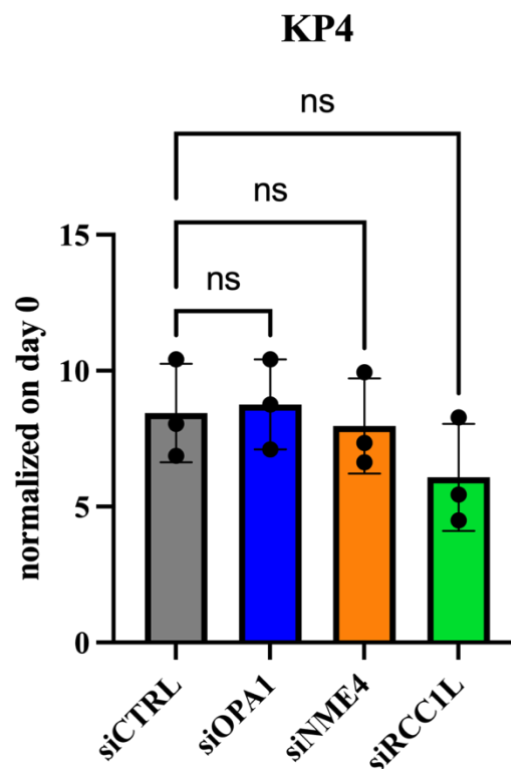
CONDITION	MEAN CELL NUMBER	NORMALIZED* ON DAY 0
siCTRL	22,3	2,22
	26	2,59
	40,5	4,03
siOPA1	56,3	5,61
	46,9	4,67
	28,5	2,84
siNME4	37,3	3,72
	77,8	7,75
	115,9	11,55
siRCC1L	50,18	5
	46,6	4,64
	38,7	3,86



PROLIFERATION ASSAY IV

‘DAY 0’ plate average = 1,41

CONDITION	MEAN CELL NUMBER	NORMALIZED* ON DAY 0
siCTRL	14,67	10,42
	9,67	6,86
	11,33	8,05
siOPA1	12,33	8,76
	14,67	10,42
	10	7,10
siNME4	10,33	7,34
	14	9,94
	9,33	6,63
siRCC1L	7,67	5,45
	6,33	4,5
	11,67	8,29



The four tables above show the numbers we obtained from each PA, while the graphs show the relationship between the numbers of each silencing condition against the control. We evaluated the statistical significance using a two-tailed unpaired t-test which showed no statistically significant differences between any of the silencing conditions and the control group ($p > 0.05$).

PA I shows that silencing of OPA1 seems to have an effect on the proliferation by reducing it compared to the control cells. However, this trend was not maintained across subsequent assays, where OPA1-depleted cells exhibited proliferation rates comparable to or slightly higher than the control.

NME4 knockdown in PA II and PA IV resulted in reduced proliferation relative to the control cells.

Notably, silencing of RCC1L produced the most consistent trend of decreased proliferation across all the assays.

The most different results were obtained in PA III, where cell numbers were quantified automatically from fluorescent microscopy images. [Fig. 3] Under these conditions, control cells displayed the lowest proliferation values, and NME4 knockdown showed no inhibitory effect on KP4 cell growth.

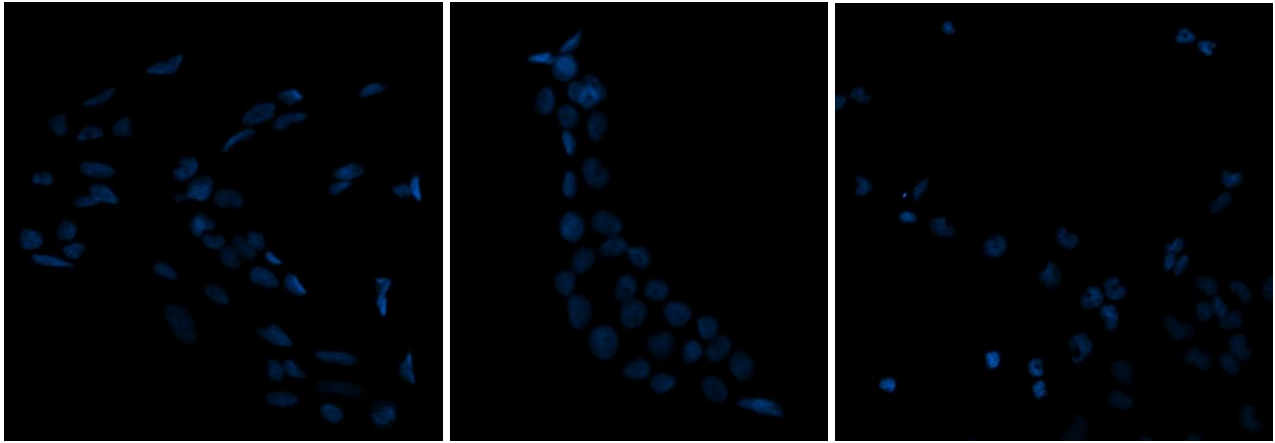


Fig.3: Fluorescent microscopy images of Hoechst-stained KP4 cells

Cells were fixed and stained with Hoechst and imaged using fluorescent microscopy. Nuclear fluorescence was used for subsequent automated cell counting.

3.2.Colony formation assay

CFAs were evaluated qualitatively by visual assessment of the crystal violet staining intensity across siCTRL and siNME4-knockdown conditions in both KP4 and PANC1 pancreatic cancer cells. Individual colony counting was not performed. Qualitative comparison revealed a noticeable increase in crystal violet staining intensity and total colony coverage in siNME4-treated wells compared to control wells across both cell lines. [Fig.4-5] These findings indicated that NME4 depletion does not exert an inhibitory effect on the growth and colony formation.

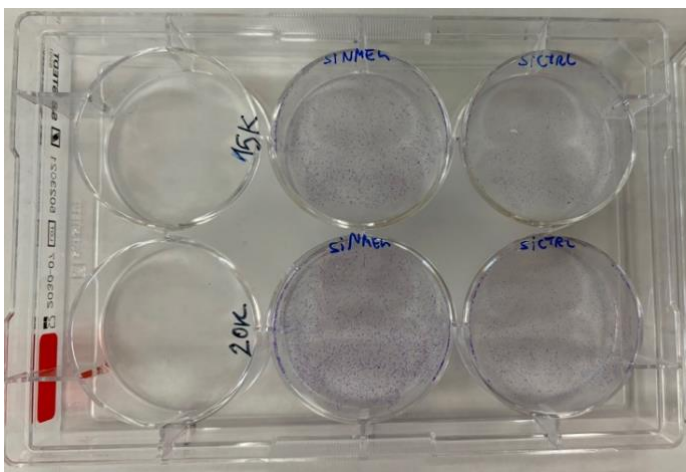


Fig.4: Qualitative colony formation assay of KP4 cells following NME4 knockdown

The image shows a 6-well plate stained with crystal violet following siRNA transfection. We seeded 15 000 and 20 000 cells/well in just one well per condition for both cell numbers. The wells depleted of NME4 demonstrate increased crystal violet staining density relative to the siCTRL, which indicates sustained clonogenic capacities.

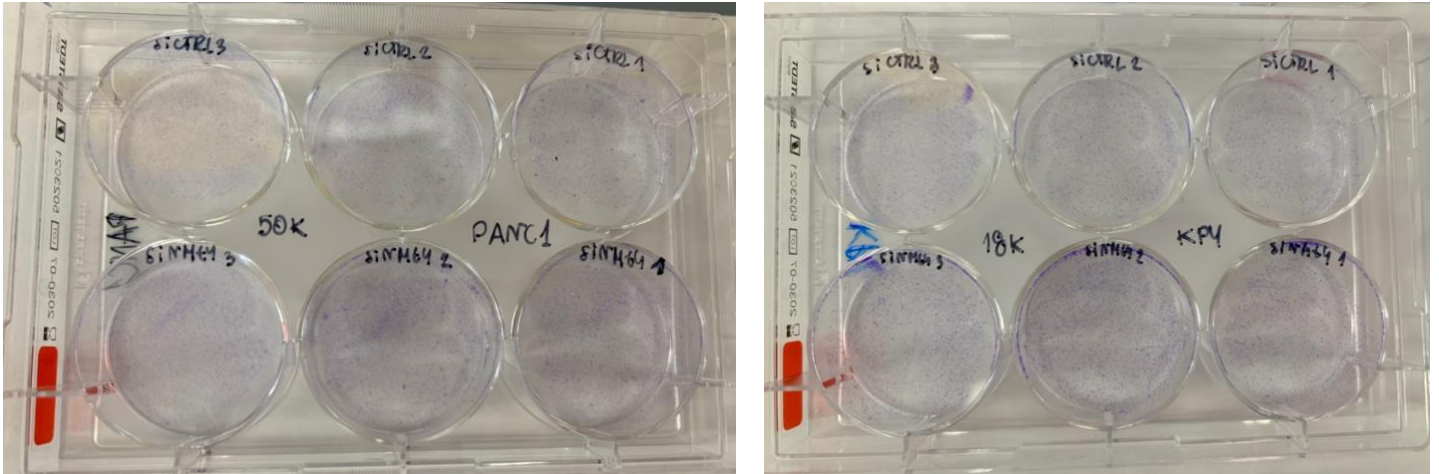


Fig. 5: Qualitative colony formation assays of both KP4 and PANC1 cells following NME4 knockdown

Both images show siCTRL and siNME4 plated in triplets. The left image represents a 6-well plate with 50 000 PANC1 cells/well, which were transfected with siRNAs and subsequently stained with crystal violet. The right image shows another 6-well plate using KP4 cells with only 18 000 cells/well. In both cases, it is visible that NME4 has higher cell density compared to the control wells, regardless of the cell line or the number of cells seeded.

Overall, siRNA-mediated silencing of the mitochondrial triad exhibited differential impacts on the proliferative capacities of KP4 cells in PAs, while the CFAs showed a robust trend in the clonogenic capacities of NME4 against the control in both KP4 and PANC1.

4. Discussion

The primary goal of this study was to investigate the functional contribution of three key mitochondrial proteins – OPA1, NME4 and RCC1L – to the proliferation of PDAC cells. By utilizing transient siRNA-mediated silencing in KP4 and PANC1 human pancreatic cancer cell lines, this work evaluated how targeting proteins from the IMM affects cancer cell growth dynamics.

Even though these three proteins are known to function in close physical and catalytic proximity at the CJ to maintain IMM fusion and *cristae* architecture and morphology, the knockdown experiments showed different outcomes.

Among the three targeted genes, RCC1L silencing produced the most consistent inhibitory trend on cell proliferation across multiple PAs. This gene acts as an OPA1-specific guanine nucleotide exchange factor (GEF), facilitating the rate-limiting exchange of bound GDP for fresh GTP on OPA1.

The heightened sensitivity of KP4 cells to RCC1L depletion suggests that its loss may trigger disruption of OPA1-driven *cristae* junction stability.

In contrast, silencing of OPA1 resulted in modest and somewhat variable effects on proliferation. Although PA I indicated a trend towards reduced growth upon OPA1 knockdown, the rest of the PAs showed comparable proliferation rates with respect to control cells. While complete loss of OPA1 leads to severe *cristae* widening, cytochrome c release, and mitochondrial network collapse, silencing of the protein may leave a sufficient pool of residual L-OPA1/S-OPA1 oligomers to maintain CJs. This suggests that partial OPA1 loss may not immediately impair proliferation under nutrient-rich *in vitro* conditions.

One of the most interesting observations came from the CFAs with silencing of NME4. While two of the PAs showed a minor downward trend in KP4 cell numbers upon silencing of NME4, CFAs revealed a marked, qualitative increase in crystal violet density in both KP4 and PANC1 cell lines. NME4 silencing did not yield the expected results – the siNME4 wells were noticeably denser compared to the siCTRL ones.

It is also important to acknowledge that the divergence observed across the PA results, more specifically between PA I, II and IV versus PA III, might be due to the different methods used for cell counting. Manual cell counting with the Bürker chamber (PA I, II and IV) gave quite different results compared to the ones obtained with the automated cell counting (PA III). Furthermore, minor variations in manual handling, such as cell seeding, resuspension, and chamber counting, may have additionally contributed to the observed inter-assay variability. Overcoming these technical limitations is essential for obtaining robust and reproducible results. Future studies should establish optimized protocols and automated quantification pipelines tailored to each specific cell line, accounting for the inherent differences in growth rates and survival capabilities.

5. Conclusion

In summary, this study provides insight into the role of three important interconnected mitochondrial proteins - OPA1, NME4 and RCC1L – in pancreatic ductal adenocarcinoma cells. Among the triad components, RCC1L shows to be the most vulnerable target upon siRNA-mediated silencing, consistently demonstrating the strongest trend towards growth inhibition. Deepening our understanding of the exact metabolic and structural dependencies of this mitochondrial machinery

will shed more light on organellar adaptation in pancreatic cancer and help evaluate whether these genes have the potential of being promising therapeutic targets.

6. Bibliography

1. Halbrook CJ, Lyssiotis CA, Pasca di Magliano M, Maitra A. Pancreatic cancer: Advances and challenges. *Cell*. 2023 Apr 13;186(8):1729-1754. doi: 10.1016/j.cell.2023.02.014. PMID: 37059070; PMCID: PMC10182830.
2. Park W, Chawla A, O'Reilly EM. Pancreatic Cancer: A Review. *JAMA*. 2021 Sep 7;326(9):851-862. doi: 10.1001/jama.2021.13027. Erratum in: *JAMA*. 2021 Nov 23;326(20):2081. doi: 10.1001/jama.2021.19984. PMID: 34547082; PMCID: PMC9363152.
3. Pernas L, Scorrano L. Mito-Morphosis: Mitochondrial Fusion, Fission, and Cristae Remodeling as Key Mediators of Cellular Function. *Annu Rev Physiol*. 2016;78:505-31. doi: 10.1146/annurev-physiol-021115-105011. Epub 2015 Nov 19. PMID: 26667075.
4. Kawano I, Bazila B, Ježek P, Dlasková A. Mitochondrial Dynamics and Cristae Shape Changes During Metabolic Reprogramming. *Antioxid Redox Signal*. 2023 Oct;39(10-12):684-707. doi: 10.1089/ars.2023.0268. Epub 2023 Sep 19. Erratum in: *Antioxid Redox Signal*. 2023 Nov;39(13-15):1025. doi: 10.1089/ars.2023.0268.correx. PMID: 37212238.
5. Grasso D, Zampieri LX, Capelôa T, Van de Velde JA, Sonveaux P. Mitochondria in cancer. *Cell Stress*. 2020 May 11;4(6):114-146. doi: 10.15698/cst2020.06.221. PMID: 32548570; PMCID: PMC7278520.
6. Lacombe, ML., Tokarska-Schlattner, M., Boissan, M. *et al.* The mitochondrial nucleoside diphosphate kinase (NDPK-D/NME4), a moonlighting protein for cell homeostasis. *Lab Invest* **98**, 582–588 (2018). <https://doi.org/10.1038/s41374-017-0004-5>
7. Schlattner U, Tokarska-Schlattner M, Ramirez S, Tyurina YY, Amoscato AA, Mohammadyani D, Huang Z, Jiang J, Yanamala N, Seffouh A, Boissan M, Epand RF, Epand RM, Klein-Seetharaman J, Lacombe ML, Kagan VE. Dual function of mitochondrial Nm23-

- H4 protein in phosphotransfer and intermembrane lipid transfer: a cardiolipin-dependent switch. *J Biol Chem.* 2013 Jan 4;288(1):111-21. doi: 10.1074/jbc.M112.408633. Epub 2012 Nov 13. PMID: 23150663; PMCID: PMC3537004.
8. Reyes A, Favia P, Vidoni S, Petruzzella V, Zeviani M. RCC1L (WBSCR16) isoforms coordinate mitochondrial ribosome assembly through their interaction with GTPases. *PLoS Genet.* 2020 Jul 31;16(7):e1008923. doi: 10.1371/journal.pgen.1008923. PMID: 32735630; PMCID: PMC7423155.
 9. Huang, G., Massoudi, D., Muir, A. M., Joshi, D. C., Zhang, C. L., Chiu, S. Y., & Greenspan, D. S. (2017). WBSCR16 Is a Guanine Nucleotide Exchange Factor Important for Mitochondrial Fusion. *Cell Reports*, 20(4), 923–934. <https://doi.org/10.1016/j.celrep.2017.06.090>
 10. Proust B, Radić M, Vidaček NŠ, Cottet C, Attia S, Lamarche F, Ačkar L, Mikulčić VG, Tokarska-Schlattner M, Četković H, Schlattner U, Bosnar MH. NME6 is a phosphotransfer-inactive, monomeric NME/NDPK family member and functions in complexes at the interface of mitochondrial inner membrane and matrix. *Cell Biosci.* 2021 Nov 17;11(1):195. doi: 10.1186/s13578-021-00707-0. PMID: 34789336; PMCID: PMC8597243.

ACKNOWLEDGEMENTS

I would like to specifically thank my supervisor, Professor Chemello, for suggesting me to contact Professor Carrer. And I am also grateful to Professor Carrer for accepting me into his laboratory!

I had the best internship experience I could ever imagine! I am grateful for the new friendships I made and, of course, all the new knowledge and hands-on experience I gained.

This thesis is the result of pure interest for the topic, that was sparked by Francesca Bozzetto, who was extremely patient with me, even when my questions would repeat or when they were basically rhetorical and containing the answer. And I would also like to thank everyone at Carrer's lab for always being so kind and ready to help! You all made me feel accepted and like part of a real team.

It was a real pleasure spending all this time with you at the laboratory.

Thank you!