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Dissecting the Role of Selenoprotein P and Thioredoxin Reductase in Pancreatic Cancer Metabolism

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DISSECTING THE ROLE OF SELENOPROTEIN P AND THIOREDOXIN REDUCTASE IN PANCREATIC CANCER METABOLISM

By

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Author Contribution Statement

The studies in this report were conducted by Alyssa Abbas, Xiaohu Tang, Idowu Micah, and Aqsa Ahsan. Preliminary data shown was conducted by Arslan Amer.

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Abstract

Pancreatic ductal adenocarcinoma (PDAC), the fourth leading cause of US cancer-related deaths, has a stark 5-year survival rate of 9%, emphasizing an urgent need for effective therapies. A well-established characteristic of cancer, metabolic dysregulation, presents an opportunity for developing therapies that target specific metabolic susceptibilities inherent in cancer cells. Our recent studies have discovered a susceptibility in PDAC; deprivation of cystine or blocking its uptake by erastin (a cystine transport inhibitor) triggers significant lipid peroxidation, thereby inducing ferroptosis specifically in the mesenchymal subtype of PDAC, but not in the epithelial subtype. This study aims to elucidate the roles of Selenoprotein P (Sepp1) and Thioredoxin Reductase (TrxR) in epithelial PDAC. Using RNAi and CRISPR-CAS9 methodologies, we found that the activity of TrxR2 is responsible for drug resistance in epithelial PDAC, while Sepp1 and TrxR1 are unnecessary. These findings could significantly contribute to novel therapeutic developments.

1 Chapter 1

1.1 Introduction

1.1.1 Biology of Pancreatic Cancer

Pancreatic cancer is one of the deadliest cancers, ranking fourth in the United States for cancer related deaths and is projected to be the second most deadly cancer by 2030 [1-4]. Unlike many other cancers, the incidence rate and the death rate of pancreatic cancer is increasing each year. The American Cancer Society projects that 64,050 people will be diagnosed and 50,550 people will die from pancreatic cancer in the United States during 2023. In fact, the five-year survival rate is still extremely low and remains in the single digits at 9% [4-6]. This is due to two facts: first, pancreatic cancer is difficult to detect in the early stages and second, there are very few effective treatments [3]. Of the ones that might initially be effective, they ultimately stop working because cancerous cells are able to develop resistance to the treatment[7]. This is partially due to increases in DNA mutations and phenotype switching [1, 8]. Current treatment plans for pancreatic cancer depends on the stage of which it is found and how resectable the tumor is [7, 9].

There are two main categories of pancreatic cancer, exocrine pancreatic cancer and neuroendocrine pancreatic cancer. Neuroendocrine pancreatic cancer is rare but includes gastrinomas, insulinomas, and glucagonomas. Exocrine pancreatic cancer is much more common and includes pancreatic adenocarcinoma (PDAC), which makes up 90% of all pancreatic cancer diagnosis [3, 4, 10]. Along with PDAC, exocrine pancreatic cancer also includes squamous cell carcinoma, adenosquamous carcinoma, and colloid carcinoma.

The four most common mutations that lead to pancreatic cancer include KRAS, CDKN2A, TP53, and Smad-4 [3, 11, 12]. As with any cancer, pancreatic cancer comes with many changes within the cell itself, including changes in metabolism.

1.1.2 Metabolic Deregulation is a Hallmark of Cancer

Most cancerous cells undergo metabolic deregulation and continue to do so as they proliferate [13]. For example, the Warburg Effect is a well-known metabolic deregulation of glycolysis[14]. An increase of glucose uptake helps to support cell metabolism and biomass production for cell proliferation. An increase in glycolysis also allows for an increase of products such as NADPH, which is able to combat increasing levels of reactive oxygen species (ROS)[12]. While most cells keep a low base level of ROS, cancerous cells generally have a higher basal level of ROS [13, 15]. If cancer cells are unable to keep ROS levels at an acceptable number, they risk dying due to oxidative stress.

1.1.3 Oxidative Stress in Cancer Progression and Cysteine Metabolism

ROS are broken down metabolites of oxygen including superoxide anions, peroxide, hydrogen peroxide, hydroxyl radicals, and hydroxyl ions[14]. They mostly originate from the electron transport chain in the mitochondria but can also be produced in other areas of the cell such as the endoplasmic reticulum [10, 15]. While the Warburg Effect allows a higher NADPH production to eliminate excess ROS, it also drives phenotype switching in cancerous cells. In normal cells, low levels of ROS are used in cellular signaling.

However, in high concentrations, they can induce a special type of cell death called ferroptosis [16]. An increase of ROS levels will damage DNA by diffusing through the mitochondria and affects redox-sensitive pathways that are associated with cell survival, therapeutic resistance, and disease progression [14, 15]. Due to their high proliferation rates, ROS levels are generally higher in cancerous cells [14]. Because of this, cancer cells have adapted their cellular metabolism to allow for higher ROS levels. Cells are also able to combat ROS using selenoproteins and many of these proteins are upregulated in cancerous cells. Due to ROS being partially responsible for initiation, progression, metastasis, and drug resistance of cancer making it a viable target for new therapies.

1.1.4 Interaction Between Ferroptosis and Cysteine Metabolism

Ferroptosis is a specialized type of cell death that occurs from iron-independent lipid peroxide accumulation [17, 18]. Mediated by the Fenton reaction, stored ferrous iron reacts with hydrogen peroxide to generate ROS. From there, ROS will cause immense damage to the mitochondria and DNA, leading to the induction of ferroptosis. Both Xc- and glutathione peroxidase 4 act as negative regulators of ferroptosis. The Xc- system helps to transport cysteine. This system will import cysteine in exchange for glutamate, causing a decrease in intracellular cysteine.

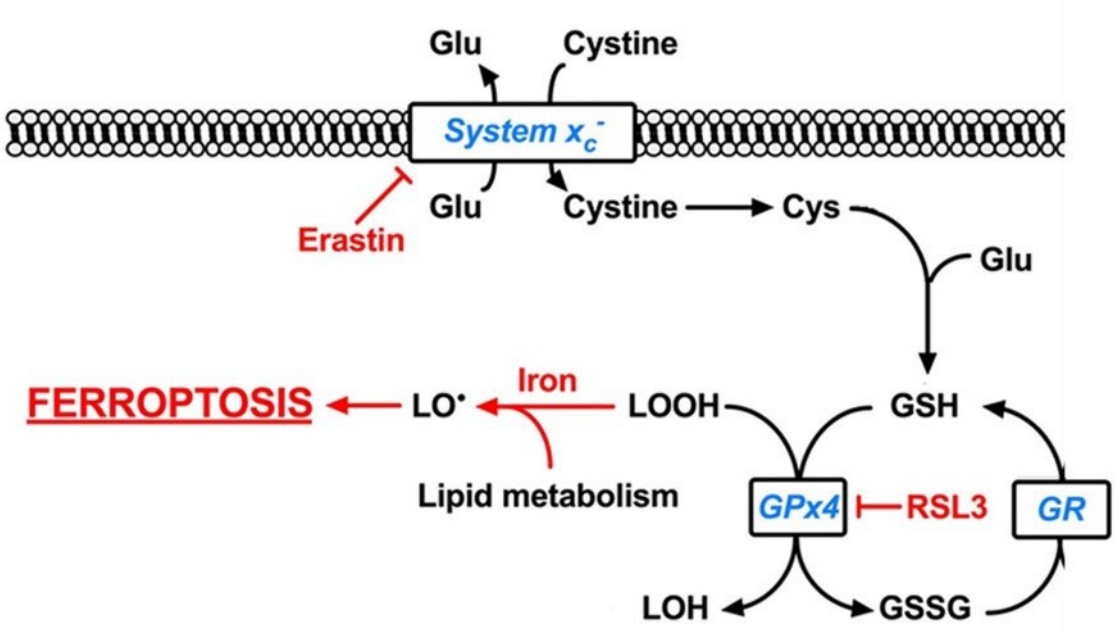

Figure 1: The interplay between cysteine metabolism and lipid peroxidation-induced ferroptosis.

A depletion of cysteine will cause an increase in ROS, inducing ferroptosis (Fig. 1). Once ferroptosis is induced, the mitochondria will shrink and ultimately rupture [17, 18]. One way cells are able to escape death via ferroptosis is by utilizing selenoproteins.

1.1.5 Role of Selenoproteins in Protection of Oxidative Stress

Selenoproteins are a class of twenty-five proteins that mainly function as antioxidant agents [19, 20]. These proteins are different compared to other antioxidant proteins due to having selenocysteine in their active site in place of cysteine. Selenocysteine (Sec) is produced from selenium, a micronutrient needed in the diet. Sec is different from other amino acids as it is coded for by a stop codon (UGA) in the mRNA [19]. This makes the active site of selenoproteins more reactive compared to proteins with cysteine in their active site [20]. However, the production of these proteins is dependent on selenium

levels. Of the twenty-five selenoproteins, glutathione peroxidase 4 (Gpx4) is one of the most studied selenoproteins. This is because it plays a detrimental role in ridding cells of excess ROS and helps to prevent lipid peroxidation [16, 17]. It was also found that the inhibition of Gpx4 triggers ferroptosis in cells [21].

1.1.6 Role of Selenoprotein P

Unlike many other selenoproteins, Sepp1 has two purposes within cells. The first is to work as an antioxidant agent. The other is to transport selenium in the plasma to other cells as it is the precursor to selenocysteine, which will then be used to make other selenoproteins [22]. The only other selenoprotein known to transport selenium is Gpx3. Due to Sepp1 making up 50% of plasma selenium, it is important to keep this protein functional [6, 20, 23]. Sepp1 also differs from other selenoproteins due to having ten residues of selenocysteine in its active site [6, 22]. It is thought that since Sepp1 is a transporter of selenium, it would be a great target for therapies. In addition to targeting Sepp1 due to its transportation abilities, studies have also found that Sepp1 is capable of protecting pancreatic cancer cells from treatments such as gemcitabine, which has been shown to increase ROS levels [6, 8]. If cells are deprived of selenocysteine they will be unable to produce the level of selenoproteins needed to protect cancerous cells from ROS and ferroptosis [23].

1.1.7 Role of Thioredoxin Reductases in Protection from Oxidative Stress

There are three different types of TrxR proteins in mammals including TrxR1, TrxR2, and TrxR3. Each are structurally similar but are expressed in different areas. TrxR1 is localized to the cellular cytosol of cells, TrxR2 is mostly expressed in the mitochondria, and TrxR3 is expressed mainly in the testis [9, 19]. Just as any of its other selenoprotein cousins, TrxRs are known to be reducers. They are known to be the only reducers of thioredoxins and were found to not be downregulated when selenocysteine is depleted [19, 20]. These proteins are also very active in cell signaling pathways by controlling transcription factors such as p53 [19]. TrxR has many anticancer qualities; however, it is upregulated in many types of cancer and are correlated with a poor prognosis [20, 24]. This is most likely due to its ties as an antioxidant agent as ROS levels increase with cancer cell proliferation [25]. TrxR proteins show promise in targeted therapies due to its depletion causing apoptosis and other types of cell death in cancer cells.

1.1.8 CRISPR-Cas9 Gene Editing Technology

Clustered regularly interspaced short palindromic repeats (CRISPR) originates from prokaryotic cell defense [26]. The DNA from these sequences are fragments of previous bacteriophages that infected the cell. The RNA fragments are then used as a template to destroy other bacteriophages by creating double stranded breaks (DSB) in their DNA before they can infect and injure the prokaryotic cells. The discovery of CRISPR in these cells has led to a new and much more precise way to genetically engineer genomes and

better understand gene functions, as the previous gene editing techniques such as using short-hair pin RNAs are known to be less efficient [26-28].

The use of CRISPR-Cas9 has three components. The first of which is Cas9, an endonuclease which is used to cut the targeted DNA. Next two small RNAs are used, one CRISPR RNA (crRNA) that targets specific sequences and one trans-activating crRNA that creates a link between Cas9 and the crRNA [29]. These two RNAs are combined into one single guide RNA (sgRNA) that leads Cas9 to the targeted site. The sgRNA has a 20-nucleotide sequence that points Cas9 to the exact locus and sequence that will be edited. Another component that is essential to CRISPR-Cas9 gene editing is the protospacer adjacent motif (PAM), which is located on the targeted DNA. After creating a DSB, the cell will attempt to repair the DNA. Non-homologous end joining (NHEJ) will lead to either the insertion or deletion of targeted genes while homologous directed repair (HDR) will result in precise repair at the cut site [27]. While CRISPR is known to be much more effective at its job, it is still not 100% efficient and may have off-target hits within other areas of the genome [28, 30]. However, with this study, it was one of the best options.

1.1.9 Aims of the Study

Given both PDAC subtypes' differential sensitivity to cysteine deprivation, our aim is to understand the underlying mechanism and eventually circumvent drug resistance when implementing targeted cysteine therapy. Analyzing gene expression profiles from both PDAC subtypes (Mesenchymal and Epithelial), we noted a substantial decrease in Selenoprotein P (Sepp1) in the mesenchymal, cysteine sensitive PDAC subtype. Sepp1 serves as both an antioxidant and a transporter of selenocysteine. In addition, Thioredoxin

Reductases (TrxR), similarly as selenoproteins, are crucial to Reactive Oxygen Species (ROS) protection and maintaining cellular redox balance. This study has two main objectives:

Aim 1: To assess the role of Sepp1 in epithelial PDAC cells by suppressing its expression using RNAi and CRISPR/CAS9 methodologies.

Aim 2: To elucidate the protective role of TrxR1 and TrxR2 in epithelial PDAC cells by inhibiting their expression using RNAi.

2 Chapter 2

2.1 Results

2.1.1 Exploring the Protective Role of Sepp1 in Lipid Peroxidation Induced Ferroptosis

2.1.1.1 Early Data Shows Sepp1 Plays a Protective Role

Previous data showed that the expression of Sepp1 is significantly downregulated in mesenchymal-type of PDAC, but not in epithelial-type PDAC. It is interesting to examine whether the loss of Sepp1 renders cells sensitive to cysteine-deprivation.

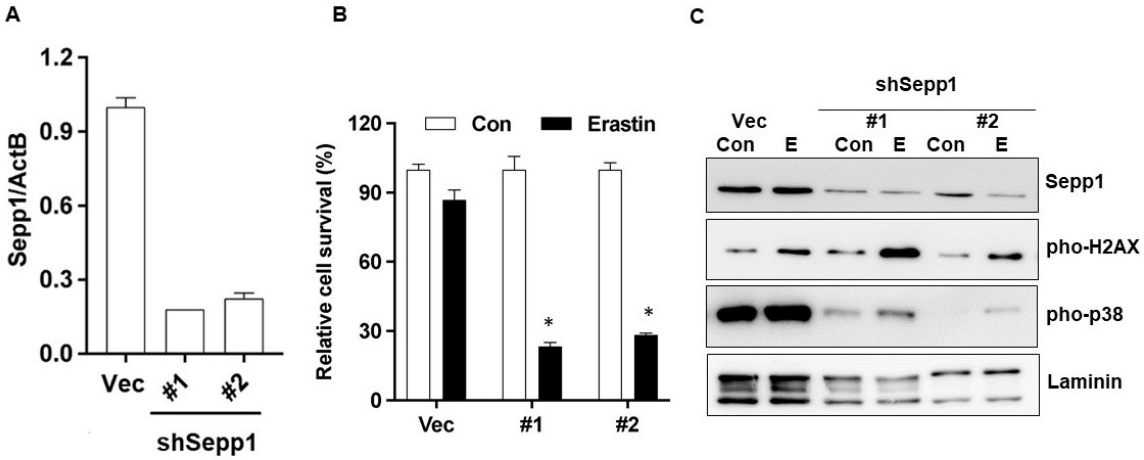

Figure 2: Preliminary data showed that suppression of Sepp1 expression sensitizes cells to cysteine deprivation in CAPAN-2 cells. (A) RT-qPCR analysis of Sepp1 expression in CAPAN-2 vector (Vec) or shSepp1cells. . (B) Relative cell viability of Vec or shSepp1 cells after either control or erastin (2.5 μM) treatment for 96 hours (n=3, *, p<0.0006). (C) Western blot analysis of Sepp1, phosphorylated p38 and pho-H2AX protein expression in CAPAN-2 vector cells and Sepp1 knockdown cells after being treated with either control (C) or erastin (E) for 72 hrs.

By RT-qPCR analysis, it is evident that the knockdown Sepp1 using shRNA was effective (Fig. 2A). The CAPAN2 cells were then treated with erastin to test whether cells were less protected without normal levels of Sepp1. Indeed, it was found that cells that underwent treatment were more susceptible to treatment than cells that did not have a

knockdown of Sepp1 (Fig. 2B). Death marker and death signaling tested by pho-H2AX and pho-p38 antibodies were found only to be induced in Sepp1knockdown cells with the erastin treatment (Fig. 2C). These results show that the absence of Sepp1 by shRNAs led to pancreatic cancer CAPAN2 cells becoming more susceptible to treatments of erastin. With these results, we planned to confirm these observations in an independent PDAC line, ASPC1 cells.

2.1.1.2 Establishment of Sepp1 Knockdown in AsPC1 Pancreatic Cancer Cells

After repeating the process of the knockdown, the results did not match the initial data. We first checked the expression of Sepp1 with Western Blot to ensure the knockdown was efficient, but no bands were formed. This was most likely due to the use of a poor antibody during the procedure. The ATP assay showed the vector line acting very similar to both knockdown lines, with little resistance to erastin (Fig. 3B). The crystal violet stain also yielded the same results showing almost identical susceptibility between the three groups (Fig. 3C). The tests were repeated with similar results, the vector cells not behaving as the parental line.

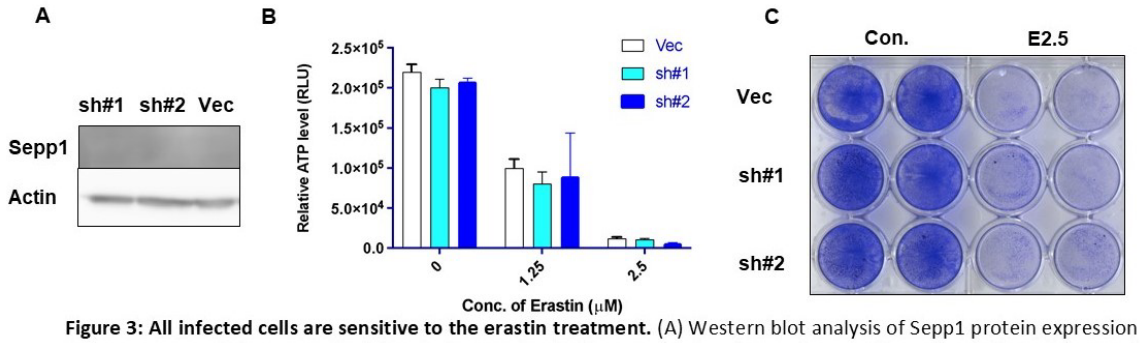

Figure 3: All infected cells are sensitive to the erastin treatment. (A) Western blot analysis of Sepp1 protein expression in ASPC1 Vec and shSepp1 cells. (B) Relative ATP level of vector (Vec) and two shSepp1 lines after indicated doses of erastin treatments. (C) Relative cell survival indicated by the crystal violet staining in cells treated as (B).

The vector line should have been more resistant to treatment than the knockdown lines, indicating something within the cells was not normal. Cell morphology indicated that the cells were under stress and had black spots when viewed under the microscope. We believed the cells were contaminated with mycoplasmas, so the cells were treated accordingly. After mycoplasma treatment for three weeks, cells were tested again with the vector cell now behaving similar to the parental line (Fig. 4A and 4B).

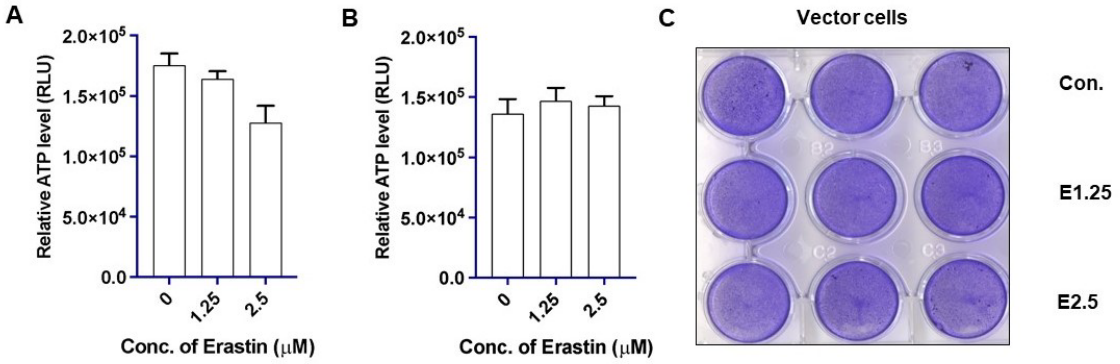

Figure 4: Cells are resistant against erastin after mycoplasma removal. (A) Relative ATP levels in ASPC1 parental cells after 4 days treatments with indicated doses of erastin. (B) Relative ATP levels in ASPC1 vector mycoplasma-free cells treated as (A). (C) Relative cell survival indicated by the crystal violet staining in cells treated as (B).

After mycoplasma treatment, the vector line showed resistance to erastin treatments of varying concentrations with the ATP assay and crystal violet stains showing a high level of resistance (Fig. 4B and 4C). After we had tested the vector line, we treated and retested the two knockdown lines.

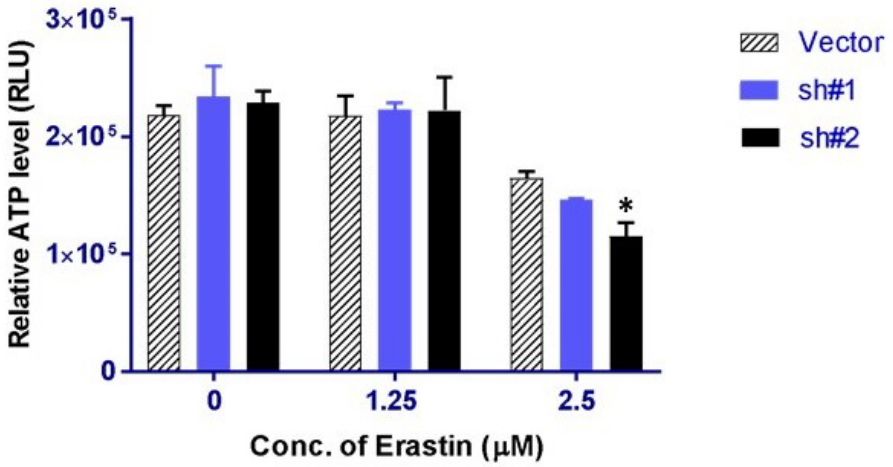

Figure 5: Suppression of Sepp1 expression does not sensitize ASPC1 cells to cysteine deprivation after mycoplasma removal. Relative cell viability of Vec or shSepp1 ASPC1 cells after indicated doses of erastin treatments for 96 hours (n=3, *, p<0.01).

It was found that after mycoplasma treatment, both knockdown lines were also resistant to erastin treatment and showed little to no significant difference when compared to the vector (Fig. 5). These results were not shown in the preliminary data, making us question the technique we were using or if the original cells were also contaminated at the time they were tested. With the knockdown technology not being as efficient as other gene editing protocols, we then decided to use CRISPR-Cas9 to establish the knockout of Sepp1 to see if we could regenerate similar results from the preliminary data.

2.1.1.3 Establishment of Sepp1 Knockout in Pancreatic Cancer Cells using CRISPR-Cas9 Gene Editing Approach

Two different sgRNAs were used for the knockout of Sepp1 to ensure poor results were not generated from off target hits, as this is common within gene editing tools.

After lentivirus knockout, cells were placed into single cell wells where they were allowed to grow into colonies. Once they were placed into 6 well plates, cell survival assays were conducted to find the cells with the least amount of resistance to erastin. Clones from the previous study and the current study were tested. Four cell lines were then chosen to continue the study, two from the previous study and two from our study. Cells with the letter "O" were from the previous study while cells with the letter "N" were from the new knockouts we had done (Table 1).

Table 1: Single cell clone of Sepp1 gRNA-mediated knockout in ASPC1 cells				
Cell clones	**Estimated survival after treatment with 2.5 µM Erastin**			
gSepp1-65 clones	**2d**	**3d**	**4d**	
O1	**30%**	**20%**		
O2	**70%**	**60%**		
O3	**90%**	**90%**		
O4	**40%**		**20%**	
O5			**30%**	
N1	**90%**	**80%**		
N2	**90%**		**90%**	
N3	**90%**		**50%**	**Linear survival cells**
gSepp1-26 clones				
O1	**60%**		**50%**	**Loss**
N1				**Loss**
N2	**Did not do the test, but the clone remained**			

Table 1: Single-cell clones generated by Sepp1 two gRNAs in ASPC1 Cells. The estimated survival rate of Sepp1 gRNA cells after 2.5 µM erastin treatment.

These knockout lines along with the vector were also treated for mycoplasmas due to odd behavior and morphology. After three weeks, the cells were treated with erastin to check for changes in cell survival. Again, it was found that there was not a large difference in resistance compared to the vector line.

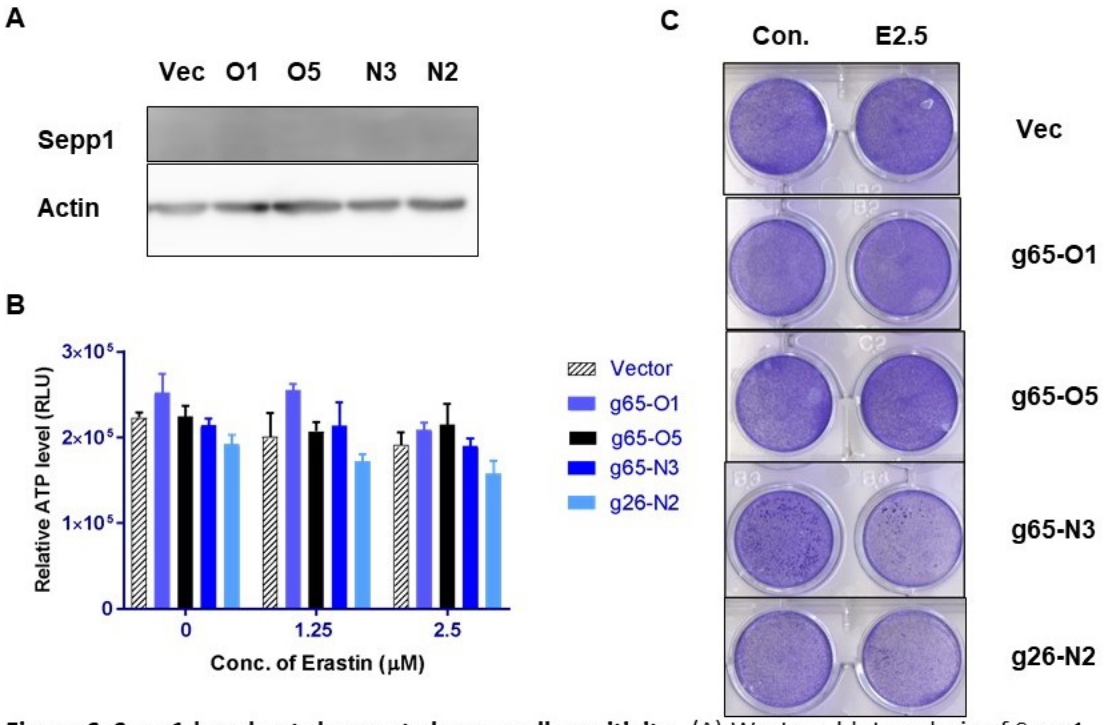

Figure 6: Sepp1 knockout does not change cell sensitivity. (A) Western blot analysis of Sepp1 protein expression in ASPC1 Vec and indicated gSepp1 cell clones. (B) Relative ATP level of vector (Vec) and gSepp1 cell clones after indicated doses of erastin treatments. (C) Relative cell survival indicated by the crystal violet staining in cells treated as (B).

Western Blot analysis was used in both the knockdown and knockout of Sepp1, however; no bands were detected after multiple techniques were used (Fig. 6A). Due to this, we were unable to determine if Sepp1 was truly knocked out in our cells. ATP assays of these lines also did not yield any significant results, as no cell lines' resistivity were substantially affected by Sepp1 knockout (Fig. 6B). The crystal violet stains of each line also did not show any significant difference in susceptibility to treatment (Fig. 6C). After having little success within the ASPC1 line, we went back to the original CAPAN2 line to ensure what was first observed was not specific to that cell line alone.

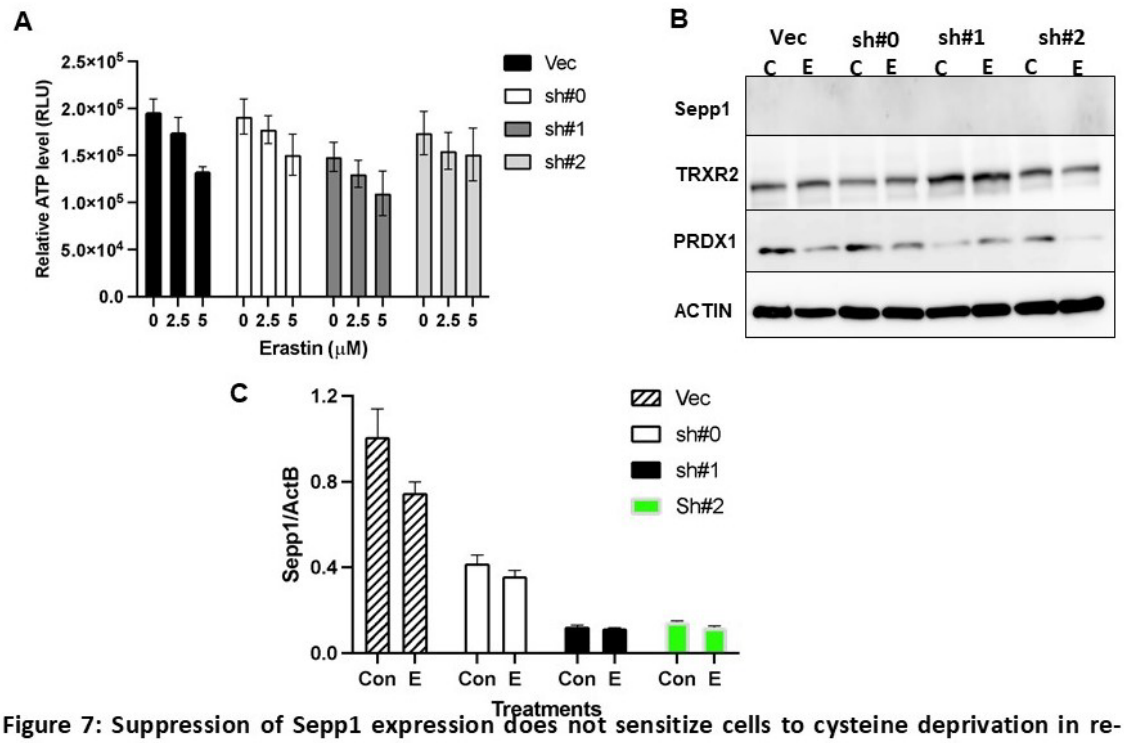

Figure 7: Suppression of Sepp1 expression does not sensitize cells to cysteine deprivation in re-established CAPAN-2 shSepp1 cells. (A) Relative cell viability of Vec or shSepp1 CAPAN-2 cells after indicated doses of erastin treatments for 96 hours. (B) Western blot analysis of indicated protein expressions in Vec and shSepp1 cells treated with either control (C) or erastin (E) for 3 days. (C) Relative Sepp1 expression by RT-qPCR in cells treated as (B).

Since the original data had been generated by knocking down Sepp1, we also went back and knocked the protein out. The knockdown of Sepp1 was done using multiple shRNAs to account for any off targets. An ATP assay was conducted to test general sensitivity after erastin treatment and matched our results showing no significant change between the shRNA lines and the vector (Fig. 7A). Western Blot analysis was performed again with no results for Sepp1 expression (Fig. 7B). Since Western Blot analysis was unable to determine the efficiency of the knockdown, RT-qPCR was then used, showing that Sepp1 was in fact knocked down within the cells (Fig. 7C). These results were similar to our earlier knockdown results leading us to believe that both the knockdown and knockout results were true, despite being unable to use Western Blot. With our results for Sepp1

pointing towards not having a protective role against ferroptosis, we then decided to look further into a different selenoprotein.

2.1.2 Exploring the Protective Role of TrxR in Lipid Peroxidation

2.1.2.1 Establishment of TrxR1 and TrxR2 Knockdown in Pancreatic Cancer Cells

Following the Sepp1 tests, we then wanted to test a different selenoprotein and its effect on pancreatic cancer cells' resistance to treatment. Both TrxR1 and TrxR2 were chosen due to having similar functions in different locations of the cells. Following the knockdown and mycoplasma treatment of TrxR1 and TrxR2 in the ASPC1 line, cells were treated with erastin to find if the absence of these proteins caused the cells to be more susceptible to undergoing ferroptosis.

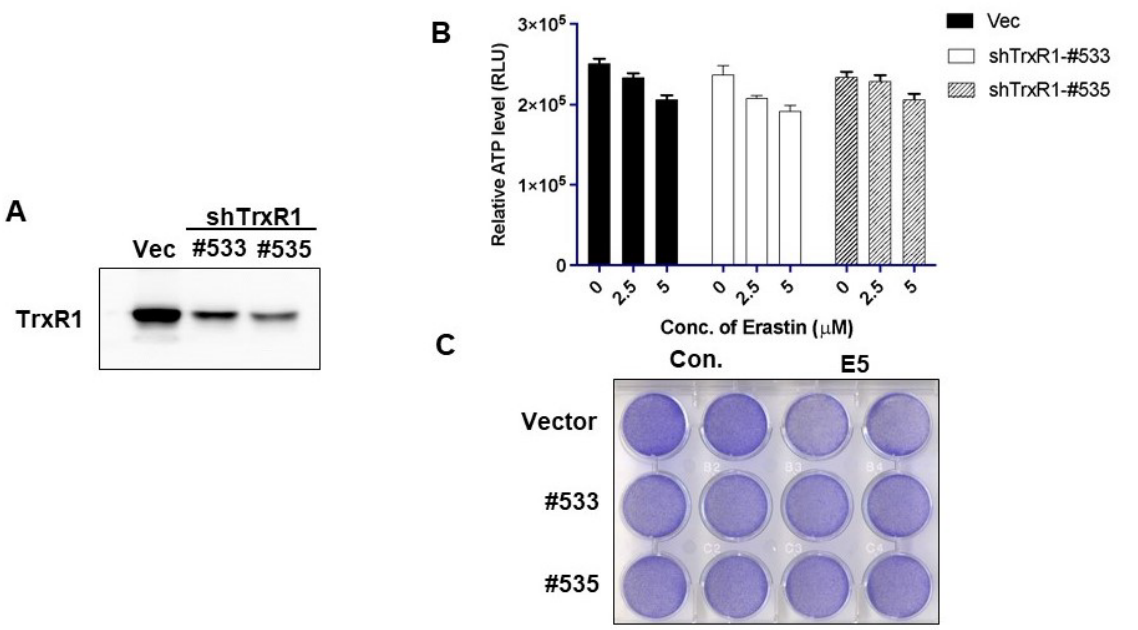

Figure 8: Suppression of TrxR1 expression does not sensitize cells to cysteine deprivation. (A) Western blot analysis of TrxR1 protein expressions in Vec and shTrxR1 ASPC1 cells. (B) Relative cell viability of Vec or shTrxR1 cells after indicated doses of erastin treatments for 96 hours. (C) Relative cell survival indicated by the crystal violet staining in cells treated as (B).

Following Western Blot analysis, it was found that both shRNAs had significantly lessened the expression of TrxR1 (Fig. 8A). However, actin expression was not captured at this time. While we could see that the knockdown of TrxR1 was efficient, the ATP assay results showed that there was no significant change in resistance (Fig. 8B). The crystal violet stain also mirrored the results from the ATP assay with little to no difference in cell density between both the vector and knockdown lines (Fig. 8C). This led us to believe that TrxR1 does not play a significant role in cell protection from cysteine deprivation.

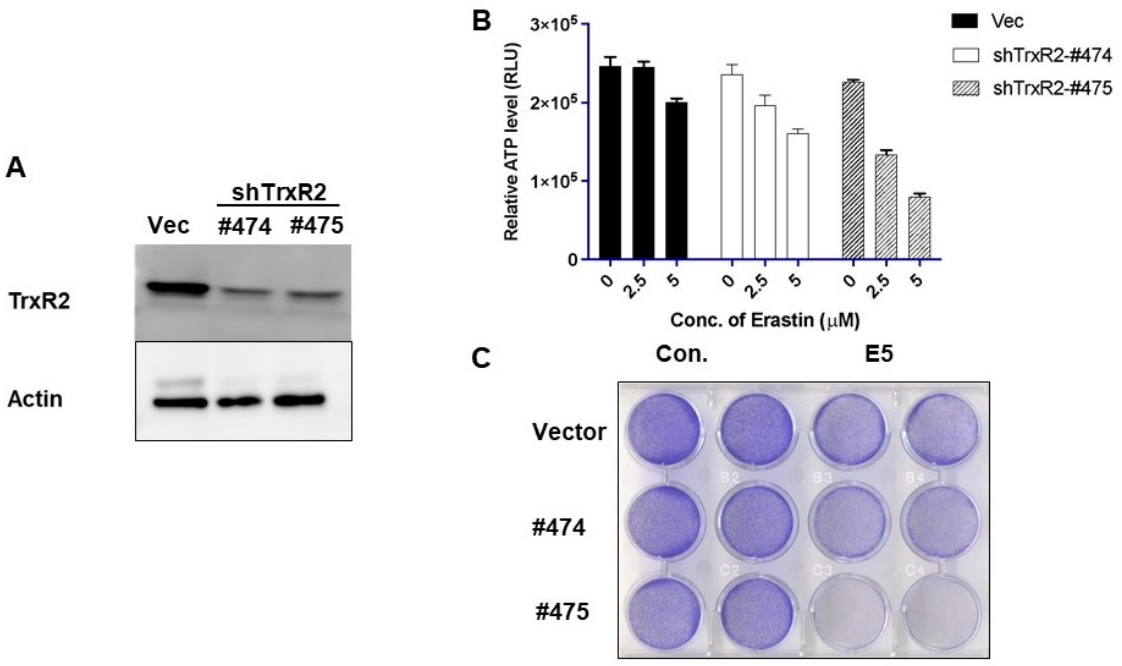

Figure 9: Suppression of TrxR2 expression sensitizes ASPC1 cells to cysteine deprivation. (A) Western blot analysis of TrxR2 protein expressions in Vec and shTrxR1 ASPC1 cells. (B) Relative cell viability of Vec or shTrxR2 cells after indicated doses of erastin treatments for 96 hours. (C) Relative cell survival indicated by the crystal violet staining in cells treated as (B).

The results for TrxR1 were a stark contrast to what we observed in the ASPC1 cells that had a knockdown of TrxR2. We first checked the efficiency of TrxR2 knockdown using Western Blot. It was found that both shRNAs had effectively lessened the expression of TrxR2, with sh#474 having the most effective knockdown when compared to sh#475 (Fig. 9A). Once we had established that TrxR2 was knocked down, ATP assays were done. It was found that there was a significant change in susceptibility to erastin treatment after knockdown with sh#475 yielding the best results (Fig. 9B). This made us wonder if this shRNA had off target hits that were causing the cells to become weaker than the cells that had a knockdown using sh#474. The results of the ATP assay were also mirrored by the results of the crystal violet stain (Fig. 9C).

Once the results pointed towards TrxR2 having a protective role against ferroptosis in the ASPC1 cell line, we repeated our mycoplasma treatment and experiment in the CAPAN2 line.

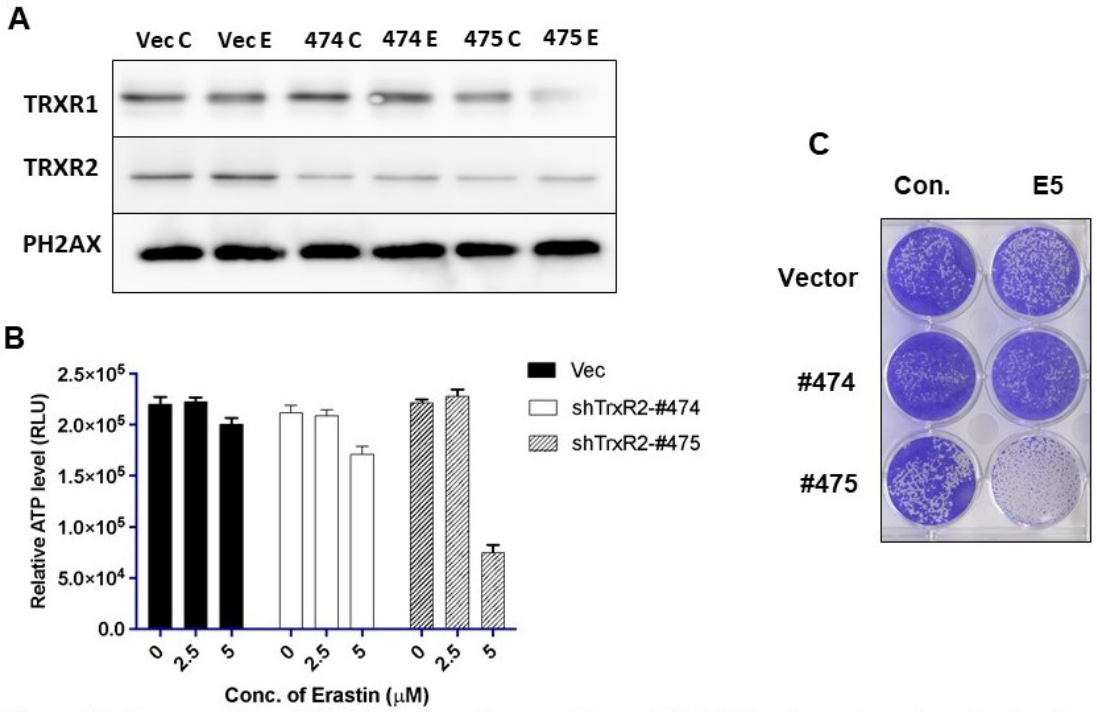

Figure 10: Suppression of TrxR2 expression sensitizes CAPAN-2 cells to cysteine deprivation. (A) Western blot analysis of TrxR2 protein expressions in Vec and shTrxR1 CAPAN-2 cells. (B) Relative cell viability of Vec or shTrxR2 cells after indicated doses of erastin treatments for 96 hours. (C) Relative cell survival indicated by the crystal violet staining in cells treated as (B).

We first conducted Western Blot analysis to ensure that TrxR2 knockdown was efficient. Again, sh#474 was able to lessen expression more than sh#475 (Fig. 10A). We then checked cell survival using ATP assays and found a significant change in resistivity to treatment in sh#475 (Fig. 10B). This was again mirrored by the crystal violet stain (Fig. 10C). These results suggested that TrxR2 likely plays a protective role in epithelial-type of PDAC against cysteine deprivation.

3 Chapter 3

3.1 Discussion and Conclusion

3.1.1 Sepp1's Role in Cysteine Dependence

Given our results with multiple cell lines, it was concluded that Sepp1 does not play a protective role in the cysteine deprivation pathway. This conclusion was the opposite of our preliminary data and led us to believe that those cells were unknowingly contaminated with mycoplasmas. Once we had treated our cells for mycoplasmas, their morphology and behavior went back to 'normal'. Once the cells were retested, we found very different results with all lines, regardless of whether they had a knockdown or knockout of Sepp1, showing us that erastin treatment did not affect them.

There was also no way to make a hard conclusion on the efficiency of the Sepp1 knockdown and knockout due to a poor primary antibody for Western Blot. If this study was to be continued, all cells would first be treated and checked for any ailments that may change results. A new antibody for Western Blot analyses would be used and RT-qPCR would be used to double check results found to ensure the knockout was efficient. We would also check the expression of other selenoproteins following Sepp1 downregulation to see if there was any effect in that manner.

3.1.2 The Role of TrxR in Cysteine Dependence

Following tests with both Capan2 and ASPC1 pancreatic cancer cells, it was concluded that TrxR1 did not play a large role in cysteine dependence. In both lines it was found

that after efficient knockdown there was little to no change in the knockdown versus vector lines after undergoing erastin treatments. Both shRNAs proved to have efficient knockdowns of their targets as seen from Western Blot analyses.

TrxR2 told us a different story compared to TrxR1, this could be because these isoforms are expressed in different areas of the cell. With TrxR2 being expressed in the mitochondria, it would have more exposure to ROS and would likely protect the cell from lipid peroxidation and ultimately ferroptosis more than TrxR1 would. Both shRNAs showed that their knockdown was efficient but shRNA #474 was the most effective when looking at the Western Blot. However, analyses of both the crystal violet and ATP assays show that cells with the shRNA #475 had the most cell death. There is a chance this is due to some off target hits of the shRNA. While off targets are not desired, both shRNAs are able to show that a lack of TrxR2 is detrimental to pancreatic cancer cells and their ability to protect themselves from cysteine deprivation.

If this study was to be continued, we would like to see if TrxR2 has a higher expression in PDAC cells versus TrxR1 before knockdown. This could also clue us in on how much more cells rely on TrxR2 compared to TrxR1. We would also want to look into the downstream effects of TrxR2 knockdown to see what other pathways could be affected.

4 Chapter 4

4.1 Materials and Methods

4.1.1 Cell Culture

All pancreatic cancer cell lines were obtained from ATCC. During culture, they were incubated at 37℃ with 95% humidity and 5% CO_2 concentration to mimic human body conditions. The cells were cultured in DMEM with fetal bovine serum (FBS) and 1% penicillin-streptomycin to allow for proper nutrient supplementation and fungal infection prevention.

4.1.2 Lentiviral Cell Infection

For shRNA knockdown, lentiviral infection was utilized. Viral particles were generated in transfected 293T cells using Lipofectamine 3000 (ThermoFisher Scientific). After 48 hours, the viral particles were collected from the cell media. The targeted cells were then infected for 48 hours with the virus and screened using puromycin (Cayman Chemicals). The shRNAs were purchased from Sigma. The empty vector PLKO was used for Sepp1 and TrxR knockdown.

4.1.3 CRISPR-Cas9 Knockout

For CRISPR-Cas9 knockout, lentiviral infection was utilized. Viral particles were generated in transfected 293T cells using Lipofectamine 3000 (ThermoFisher Scientific). After 48 hours, the viral particles were collected from the cell media. The targeted cells were then infected for 48 hours with the virus and screened using puromycin (Cayman

Chemicals). The gRNAs were purchased from VectorBuilder. The empty vector pLex was used for Sepp1 knockout. Cells were then seeded at 1 cell per well in 96 well plates. Cells were allowed to clonally expand before being transferred to 12 well plates for further expansion. After 70% confluency, cells were transferred again to 2 different 6 well plates for Western Blot analysis and cell survival assays.

4.1.4 Cell Viability

Cell viability was measured using crystal violet (CV) staining or by evaluating the ATP level using the Cell Titer-Glo Assay Kit from Promega. Crystal violet staining was done after cells were treated with Erastin for four days in 12 well plates. After four days, cell media was disposed of and 1mL of crystal violet stain was added overnight. For ATP assays, cells were treated with Erastin for four days in 96 well plates. After four days, cells were tested for ATP levels.

4.1.5 Western Blot Analysis

For western blot analysis, cells were first lysed to extract the protein using RIPA lysis buffer (Sigma) along with the protease and phosphatase inhibitor cocktail (ThermoFisher Scientific). BCA analysis was then done to determine protein concentrations, an equal amount of protein was then loaded for western blotting analysis. The signal was detected by the ECL Western Blotting Detection System (Amersham) and was visualized using LAS-4000 Lumino Image Analyzer.

4.1.6 RNA Extraction and RT-qPCR Analysis

RNA was extracted using the PureLink RNA kit (Invitrogen). The total RNA was then reverse transcribed into cDNA. The quantitative PCR was done using SYBR Green PCR mix (Applied Biosytems). The difference in mRNA expression was then normalized by the expression the Beta-Actin gene using the ΔΔCT method.

5 Works Cited

1. Chen, C., et al., *Gemcitabine resistance of pancreatic cancer cells is mediated by IGF1R dependent upregulation of CD44 expression and isoform switching.* Cell Death Dis, 2022. **13**(8): p. 682.
2. Yefei, R., et al., *DIAPH3 promotes pancreatic cancer progression by activating selenoprotein TrxR1-mediated antioxidant effects.* Journal of Cellular and Molecular Medicine (Online), 2021. **25**(4): p. 2163-2175.
3. Kleeff, J., et al., *Pancreatic cancer.* Nature Reviews Disease Primers, 2016. **2**(1): p. 16022.
4. McGuigan, A., et al., *Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes.* World J Gastroenterol, 2018. **24**(43): p. 4846-4861.
5. Nadine Abdel Hadi, G.R.-C., and Alice Carrier, *Targeting Redox Metabolism in Pancreatic Cancer.* International Journal of Molecular Sciences, 2021. **22**(4): p. 1534.
6. Maehara, S.-i., et al., *Selenoprotein P, as a predictor for evaluating gemcitabine resistance in human pancreatic cancer cells.* International Journal of Cancer, 2004. **112**(2): p. 184-189.
7. Motoo, Y., et al., *Metabolic Disorder, Inflammation, and Deregulated Molecular Pathways Converging in Pancreatic Cancer Development: Implications for New Therapeutic Strategies.* Cancers, 2011. **3**(1): p. 446-460.

8. Zhao, H., et al., *ROS/KRAS/AMPK Signaling Contributes to Gemcitabine-Induced Stem-like Cell Properties in Pancreatic Cancer.* Mol Ther Oncolytics, 2019. **14**: p. 299-312.

9. Kshattry, S., et al., *Enzyme-mediated depletion of l-cyst(e)ine synergizes with thioredoxin reductase inhibition for suppression of pancreatic tumor growth.* npj Precision Oncology, 2019. **3**(1): p. 16.

10. Zuzčák, M. and J. Trnka, *Cellular metabolism in pancreatic cancer as a tool for prognosis and treatment (Review).* Int J Oncol, 2022. **61**(2): p. 93.

11. Li, D., et al., *Pancreatic cancer.* The Lancet, 2004. **363**(9414): p. 1049-1057.

12. Yao, W., A. Maitra, and H. Ying, *Recent insights into the biology of pancreatic cancer.* eBioMedicine, 2020. **53**.

13. Ndombera, F.T., *Anti-cancer agents and reactive oxygen species modulators that target cancer cell metabolism.* Pure and Applied Chemistry. Chimie Pure et Appliquee, 2017. **89**(9): p. 1333-1348.

14. Gorrini, C., I.S. Harris, and T.W. Mak, *Modulation of oxidative stress as an anticancer strategy.* Nature Reviews Drug Discovery, 2013. **12**(12): p. 931-947.

15. Arfin, S., et al., *Oxidative Stress in Cancer Cell Metabolism.* Antioxidants, 2021. **10**(5): p. 642.

16. Su, L.-J., et al., *Reactive Oxygen Species-Induced Lipid Peroxidation in Apoptosis, Autophagy, and Ferroptosis.* Oxidative Medicine and Cellular Longevity, 2019. **2019**: p. 5080843.

17. Chen, G., et al., *Potential mechanism of ferroptosis in pancreatic cancer.* Oncol Lett, 2020. **19**(1): p. 579-587.

18. Xie, Y., et al., *Ferroptosis: process and function.* Cell Death & Differentiation, 2016. **23**(3): p. 369-379.

19. Lu, J. and A. Holmgren, *Selenoproteins*.* Journal of Biological Chemistry, 2009. **284**(2): p. 723-727.

20. Short, S.P. and C.S. Williams, *Selenoproteins in Tumorigenesis and Cancer Progression.* Advances in cancer research, 2017. **136**: p. 49-83.

21. Zou, Y., et al., *A GPX4-dependent cancer cell state underlies the clear-cell morphology and confers sensitivity to ferroptosis.* Nature communications, 2019. **10**(1): p. 1617.

22. Yang, Y., et al., *A Pan-Cancer Analysis of the Role of Selenoprotein P mRNA in Tumorigenesis.* International Journal of General Medicine, 2021. **14**: p. 7471-7485.

23. Kitabayashi, N., et al., *Role of selenoprotein P expression in the function of pancreatic β cells: Prevention of ferroptosis-like cell death and stress-induced nascent granule degradation.* Free Radical Biology and Medicine, 2022. **183**: p. 89-103.

24. Krasavin, M., et al., *Combining carbonic anhydrase and thioredoxin reductase inhibitory motifs within a single molecule dramatically increases its cytotoxicity.* Journal of Enzyme Inhibition and Medicinal Chemistry, 2020. **35**(1): p. 665-671.

25. Scalcon, V., A. Bindoli, and M.P. Rigobello, *Significance of the mitochondrial thioredoxin reductase in cancer cells: An update on role, targets and inhibitors.* Free Radical Biology and Medicine, 2018. **127**: p. 62-79.

26. Liu, C., et al., *Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications.* Journal of Controlled Release, 2017. **266**: p. 17-26.

27. Jiang, F. and J.A. Doudna, *CRISPR–Cas9 Structures and Mechanisms.* Annual Review of Biophysics, 2017. **46**(1): p. 505-529.

28. Mitra, S., et al., *Genome editing and cancer: How far has research moved forward on CRISPR/Cas9?* Biomedicine & Pharmacotherapy, 2022. **150**: p. 113011.

29. Kozovska, Z., et al., *CRISPR: History and perspectives to the future.* Biomed Pharmacother, 2021. **141**: p. 111917.

30. O'Geen, H., A.S. Yu, and D.J. Segal, *How specific is CRISPR/Cas9 really?* Current Opinion in Chemical Biology, 2015. **29**: p. 72-78.