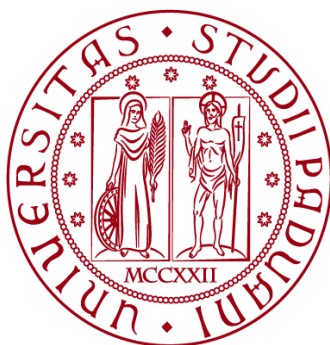


UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea in Biotecnologie



ELABORATO DI LAUREA

**Il ruolo di ACAD9 nel coordinamento tra
 β -ossidazione e fosforilazione ossidativa**

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Abstract

Mitochondria coordinate energy production by integrating fatty acid β -oxidation with the citric acid cycle and oxidative phosphorylation. Alterations in the enzymes involved can therefore simultaneously impair lipid metabolism, ATP production, and redox homeostasis. In this context, ACAD9 plays a unique role, since it acts both as an acyl-CoA dehydrogenase involved in the oxidation of unsaturated fatty acids and as an assembly factor for mitochondrial Complex I.

This thesis analyzes an experimental study that identifies ACAD9 as a regulator of ovarian cancer progression, a malignancy characterized by its ability to adapt to lipid-rich microenvironments. Through an in vivo CRISPR/Cas9 screen, cellular and animal models, gene silencing, metabolomic and lipidomic analyses, and mitochondrial functional assays, the authors show that high ACAD9 levels are associated with tumor growth, peritoneal metastasis, and poor prognosis.

Loss of ACAD9 reduces Complex I activity, oxygen consumption, and ATP production, leading to the accumulation of reactive oxygen species. In the presence of linoleic acid, lipid metabolism shifts from β -oxidation toward the synthesis of membrane phospholipids rich in polyunsaturated fatty acids. The resulting increase in lipid peroxidation, together with mitochondrial oxidative stress, therefore promotes ferroptosis.

ACAD9 thus emerges as a true metabolic gatekeeper, capable of coordinating mitochondrial respiration, lipid metabolism, and redox control in tumor cells.

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State of the Art

1.1) Mitochondrial bioenergetics

Mitochondria are cellular organelles responsible for the production of approximately 95% of the energy needed for biological processes in the form of ATP, which supplies energy for ion transport, biosynthesis reactions and growth and division of cells. ATP is an energy carrier, while the original energy comes from nutrients such as glucose and fatty acids. Mitochondria's structure includes an outer mitochondrial membrane, an intermembrane space, an inner mitochondrial membrane, cristae and the mitochondrial matrix. Different compartments are responsible for different phases of the ATP synthesis process. [1]

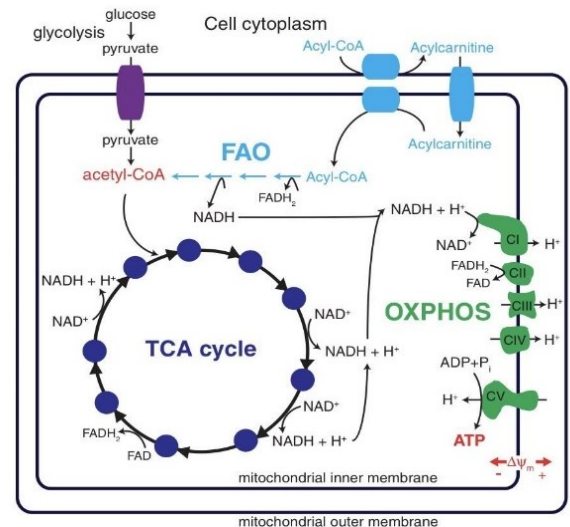
Within oxidative metabolism, mitochondria use three main pathways for energy production. The Krebs (TCA) cycle oxidizes acetyl-CoA into CO₂ and produces electron carriers such as NADH and FADH₂, which carry high-energy electrons to the respiratory chain. Fatty acid β -oxidation breaks down fatty acids to produce acetyl-CoA, which can enter the TCA cycle, alongside reducing equivalents that contribute electrons to the respiratory system. Ultimately, oxidative phosphorylation uses these electrons to generate a proton gradient across the inner mitochondrial membrane to power ATP synthesis. [1]

The oxidative phosphorylation system consists of five multi-subunit complexes located in the inner mitochondrial membrane. Complexes I, II, III and IV form the respiratory chain, whereas Complex V is ATP synthase. NADH transfers electrons to Complex I, while electrons derived from FAD dependent reactions enter through other pathways that converge on coenzyme Q. Coenzyme Q transfers electrons to Complex III, followed by cytochrome c which carries them to Complex IV. At Complex IV, molecular oxygen acts as the final electron acceptor and is reduced to water. As electrons move through the respiratory chain, Complexes I, III and IV pump protons into the intermembrane space, creating the electrochemical gradient used by Complex V to drive ATP production. [1]

Even though electron transfer through the respiratory chain is highly efficient, a small proportion of electrons may escape and react prematurely with oxygen, generating reactive oxygen species. Excessive ROS production causes oxidative stress and may damage proteins, nucleic acids and membrane lipids. [1]

As summarized in Figure 1, mitochondrial energy metabolism integrates different nutrient sources through the TCA cycle and fatty acid oxidation, which provide reducing equivalents to the OXPHOS system for ATP production.

Figure 1. Integration of mitochondrial energy metabolism. Fatty acid oxidation and glucose metabolism provide acetyl-CoA and reducing equivalents that connect the TCA cycle with oxidative phosphorylation and ATP production. [1]



1.2) Integration of metabolic pathways

Mitochondrial pathways don't work independently. They exchange substrates, products, electron carriers and intermediates, ultimately forming one connected network of metabolic reactions. Glucose, fatty acids and amino acids enter mitochondrial metabolism through different routes, but their degradation converges on common molecules such as acetyl-CoA, NADH and FADH₂. Acetyl-CoA enters the TCA cycle, while NADH and FADH₂ transfer electrons to the respiratory chain for ATP production. This relationship is bidirectional because the respiratory chain reoxidizes reduced cofactors, regenerating the oxidized forms required for continued activity of the TCA cycle and fatty acid oxidation. [1]

The coordination of mitochondrial pathways is not limited to the exchange of metabolites. Respiratory complexes can also organize into supercomplexes. In mammalian mitochondria, one of the best characterized is the respirasome, composed of Complex I, a Complex III dimer and one or more copies of Complex IV.

Supercomplexes have been proposed to contribute to the stability and organization of respiratory complexes and to facilitate substrate channeling by reducing the distance travelled by mobile electron carriers. Substrate channeling refers to the direct or localized transfer of intermediates between nearby proteins rather than their free diffusion. Their close organization may also reduce electron leakage and therefore limit superoxide and oxidative stress production. [1]

1.3) Link between respiration and lipid metabolism

Lipid metabolism and respiration are linked through both chemical and physical connections. FAO generates acetyl-CoA, NADH and electrons from FAD dependent reactions. Acetyl-CoA feeds the TCA cycle while the electrons eventually reach the respiratory chain. In addition to this metabolic connection, some FAO proteins are physically associated with respiratory chain proteins, which could allow nearby enzymes to coordinate reactions and transfer intermediates more directly. [1]

MTP and thiolase, two FAO related proteins, have been found associated with Complex I. In porcine liver mitochondria, ETF was identified in purified protein complexes containing Complex III, while in rat liver mitochondria several FAO proteins co-migrated with monomeric Complex I and the CI/CIII₂/CIV₁₋₃ respiratory supercomplex. In contrast, isovaleryl-CoA dehydrogenase, used as a non FAO control, did not co-migrate with these respiratory complexes, supporting the specificity of these associations. [1]

Functional evidence further supported this connection. Purified supercomplex fractions showed acyl-CoA dehydrogenase activity when substrates for VLCAD, LCAD, MCAD and SCAD were added. In addition, palmitoyl-CoA was metabolized in fractions containing OXPHOS supercomplexes in the presence of ETF, NAD and ATP without accumulation of FAO intermediates, suggesting that these assemblies may be capable of coordinated fatty acid metabolism. [1]

1.4) Fatty acid β -oxidation

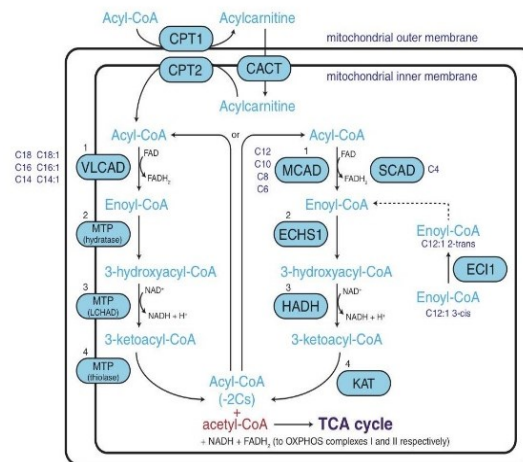
In order to use the energy stored in fatty acid chains, they need to be broken down through fatty acid β -oxidation. The process starts with the conversion of the fatty acid into fatty acyl-CoA, in which acyl-CoA synthetase adds CoA to activate it. Fatty acyl-CoA can either be used to make cholesterol, phospholipids and triacylglycerols or can be sent into the mitochondria to be broken down. [1]

Long chain fatty acyl-CoA uses the carnitine shuttle to enter the mitochondrial matrix. CPT1 transfers the fatty acyl group from CoA onto carnitine, CACT transports fatty acylcarnitine across the inner mitochondrial membrane and CPT2 transfers the fatty acyl group back onto CoA inside the mitochondria. [1]

The overall organization of mitochondrial fatty acid β -oxidation, including fatty acid transport and the enzymes acting on different chain lengths is summarized in Figure 2.

Figure 2) Mitochondrial fatty acid β -oxidation

Long chain fatty acids enter mitochondria through the Carnitine shuttle and undergo repeated cycles of β -oxidation producing acetyl-CoA and reducing equivalents while progressively shortening the fatty acid chain. [1]



1.4.1) Steps of β -oxidation

β -oxidation repeatedly shortens the fatty acyl-CoA by two carbons at a time, releasing acetyl-CoA. Each round consists of four reactions. The first is dehydrogenation by an acyl-CoA dehydrogenase, which converts fatty acyl-CoA into enoyl-CoA while FAD accepts the removed electrons and hydrogens. The electrons are then transferred through ETF and ETF-QO to coenzyme Q and ultimately enter the respiratory system through Complex III. [1,4]

The second reaction is hydration, where water is added and enoyl-CoA becomes 3-hydroxyacyl-CoA. This is followed by another dehydrogenation to form 3-ketoacyl-CoA. Finally, thiolysis releases one acetyl-CoA and leaves a fatty acyl-CoA two carbons shorter, which can enter another round of β -oxidation. For long chain fatty acids, mitochondrial trifunctional protein participates in these later reactions. The acetyl-CoA produced can then enter the TCA cycle. Unsaturated fatty acids also require enoyl-CoA delta isomerase 1 (EC1), which allows them to continue through β -oxidation. [1]

1.4.2) Role of acyl-CoA dehydrogenases

Acyl-CoA dehydrogenases catalyze the first dehydrogenation reaction of β -oxidation. Different members of this family act depending on the length of the fatty acid chain, including VLCAD, LCAD, MCAD and SCAD for very long, long, medium and short chains respectively. [1]

During this first reaction, FAD accepts the removed electrons and hydrogens. The electrons are then transferred through ETF and ETF-QO to coenzyme Q, linking the activity of acyl-CoA dehydrogenases directly to the mitochondrial respiratory system. [1,4]

1.5) Combined defects of oxidative phosphorylation and fatty acid oxidation

Mitochondrial diseases can involve OXPHOS defects caused by mutations affecting respiratory complex subunits, their assembly factors, mitochondrial DNA expression or other proteins involved in mitochondrial function.

Among these, isolated Complex I deficiency is the most common. FAO defects can similarly affect fatty acid transport, β -oxidation enzymes or proteins involved in electron transfer, reducing energy production and potentially causing the accumulation of toxic metabolic intermediates. [1]

Because FAO and OXPHOS are closely connected, a primary defect in one pathway can also cause a secondary defect in the other. Toxic intermediate accumulation and reduced cofactor sharing are possible mechanisms. However, the physical association between FAO and OXPHOS proteins provides another explanation, since the loss or dysfunction of an FAO protein may also affect the stability of associated respiratory complexes. [1]

LCHAD deficiency is one example, as patients can also show secondary Complex I deficiency. LCHAD is associated with Complex I and respiratory supercomplexes and also contributes to the formation of mature cardiolipin, meaning that its deficiency may affect OXPHOS through both protein interactions and altered cardiolipin metabolism. [1]

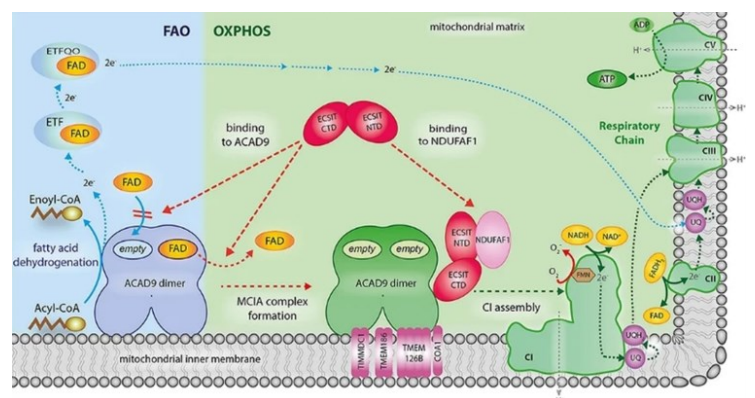
1.6) ACAD9: Structure and biological functions

ACAD9 is a member of the acyl-CoA dehydrogenase family and was first identified as a homologue of very long chain acyl-CoA dehydrogenase (VLCAD). It has a role in mitochondrial fatty acid oxidation and can partially compensate for the absence of VLCAD, as shown by the production of C12 and C14:1 carnitines in VLCAD deficiency. In addition to its role in fatty acid metabolism, ACAD9 also has a separate function as an assembly factor for mitochondrial Complex I. [1,3,5]

ACAD9 forms part of the Complex I assembly machinery together with NDUFAF1 and ECSIT, and knockdown of ACAD9 alters Complex I biogenesis, leading to isolated Complex I deficiency. This gives ACAD9 a direct role in both fatty acid oxidation and Complex I biogenesis. [1,3,5]

In patients with ACAD9 deficiency, a catalytically inactive form of ACAD9 was able to rescue Complex I biogenesis and activity, although in one patient it was less effective than the wild type protein. This suggests that the fatty acid dehydrogenase activity of ACAD9 is not required for its function in Complex I assembly. At the same time, mutations in ACAD9 can affect both fatty acid oxidation and oxidative phosphorylation. [1]

Figure 3) Proposed model of how deflavination of ACAD9 by ECSIT permits the coordinated regulation of the FAO and OXPHOS pathways [3]



Aim of the Article

The main aim of the study was to investigate the role of ACAD9 in ovarian cancer progression and understand how its metabolic functions help ovarian cancer cells survive in a lipid rich environment. ACAD9 was first identified through an in vivo genome wide CRISPR/Cas9 screen for genes involved in peritoneal metastasis. It became particularly interesting because of its dual role in fatty acid oxidation and mitochondrial Complex I assembly, meaning that it could represent a connection between lipid metabolism and mitochondrial respiration. [2]

The main question was therefore whether ACAD9 helps ovarian cancer cells maintain energy production and redox balance during tumor growth and metastasis. This becomes particularly important in the omentum, where ovarian cancer cells are exposed to an adipocyte and lipid rich environment. Fatty acids can be used as energy substrates, but polyunsaturated fatty acids can also become part of membrane phospholipids and increase their susceptibility to lipid peroxidation. At the same time, fatty acid oxidation supplies electrons to the respiratory chain, connecting lipid metabolism with mitochondrial energy production and oxidative stress. [2]

The study therefore focused particularly on linoleic acid metabolism and investigated whether ACAD9 allows ovarian cancer cells to maintain Complex I activity and oxidative phosphorylation while also controlling how linoleic acid is used. The main hypothesis was that reducing ACAD9 would impair mitochondrial energy production and increase oxidative stress while changing the metabolic fate of linoleic acid. The combination of these effects could then increase lipid peroxidation and promote ferroptotic cell death. [2]

Material and methods

Part of the experimental workflow included finding a gene important for ovarian cancer metastasis and manipulating that gene to see whether cancer behavior changes. The other part included establishing what ACAD9 does metabolically and testing mitochondrial functions, oxidative stress, lipid peroxidation and ferroptosis. [2]

In order to not rely on a single ovarian cancer cell line, different models were used in the experiment, including the human SK-OV-3, OVCAR-5 and OVCAR-8 cells, a mouse ovarian cancer model, ID8, and two non malignant cell models, HUVEC and GES-1. Non malignant cells were used to later compare if the reduction of ACAD9 has different effects between ovarian cancer cells and the non malignant cell models tested. 3T3-L1 adipocytes were also used because the study was interested in the lipid rich environment surrounding ovarian cancer. Adipocytes grow in medium and release substances into it. The medium is then collected and the ovarian cancer cells are exposed to it. [2]

Regarding the discovery of ACAD9, the process started with the GeCKO v2.0 library, which contains sgRNAs targeting a huge number of genes. Different SK-OV-3 cells carry sgRNAs targeting different genes, allowing the effect of disrupting many different genes to be studied across a population of cells. The cells were then injected into the ovaries of mice in an orthotopic model, since the cells were introduced into the organ corresponding to the tumor site.

The cells formed primary ovarian tumors and metastases at different sites. The sgRNAs were then collected from primary tumors, peritoneal metastases, omental metastases, intestinal metastases and liver metastases to be compared with each other. The comparison was performed using MAGeCK analysis to establish which gene targeting sgRNAs became enriched or depleted as the cancer progressed from the primary tumor to metastases. By comparing sgRNA abundance between the different sites, genes whose loss affected metastatic progression could be identified. ACAD9 then came out of this screening process. [2]

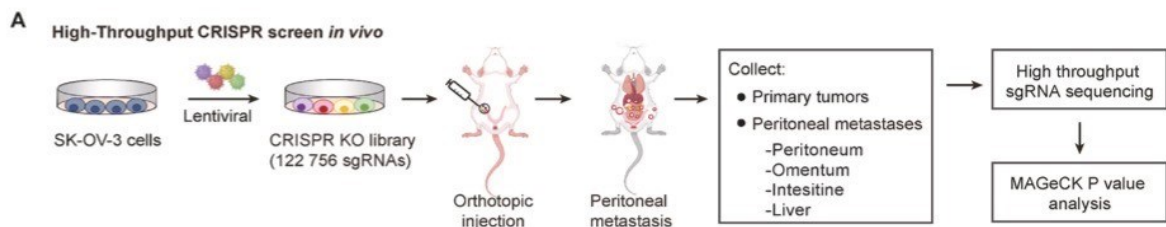


Figure 4) In vivo genome wide CRISPR/CAS9 screening workflow.

SK-OV-3 cells carrying the CRISPR knockout library were orthotopically injected into mice, followed by collection of primary tumors and metastases, sgRNA sequencing and MAGeCK analysis to identify genes involved in peritoneal metastatic progression. [2]

Once ACAD9 was found, shRNA mediated knockdown was used to reduce its expression in order to test its function. Two different ACAD9-targeting shRNAs were used to have two independent ways of reducing ACAD9. A negative control was also introduced. To check whether the knockdown actually worked, qRT-PCR at the RNA level and Western blotting at the protein level were performed. qRT-PCR was used to confirm the reduction of ACAD9 mRNA, while Western blotting confirmed the reduction at the protein level. In some ID8 experiments, the opposite was performed to test ACAD9 overexpression and see whether increasing ACAD9 produced the opposite phenotype. [2]

To test cancer cell behavior, several functional experiments were performed. A growth curve was drawn to follow the cells over time and see how their growth changes. A colony formation assay was also performed to check whether cells can continue proliferating long enough to form colonies. CCK-8 was then used to evaluate cell viability under different experimental conditions. [2]

The in vivo effects of ACAD9 were studied using orthotopic and intraperitoneal models. In the orthotopic model, tumor size and weight, ascites and the number of metastatic nodules were measured. An intraperitoneal model was also used to study metastasis more independently in case ACAD9 knockdown produced a smaller primary tumor, which could cause fewer metastases simply because there was less primary tumor to begin with. Instead of starting with an ovarian primary tumor, ID8 cells were injected directly into the peritoneal cavity, which allowed metastatic dissemination to be examined more independently from primary ovarian tumor growth. [2]

To check whether ACAD9 mattered clinically or not, actual human samples were used. There were six paired sets containing normal ovarian tissue, primary ovarian tumor and peritoneal metastasis. ACAD9 was then measured in two ways. The first was qRT-PCR with ACAD9 mRNA and the second was immunohistochemistry with ACAD9 protein in tissue sections. Public datasets such as TCGA, CSIOVDB and Kaplan-Meier Plotter were also used for expression, survival and co-expression analyses. [2]

After establishing ACAD9's effects on the behavior of cancer, the next step was to determine what changes inside the cell when ACAD9 is reduced. Control OVCAR-5 cells and ACAD9-silenced OVCAR-5 cells were compared using RNA sequencing to compare gene expression. Next, Gene Ontology, KEGG, Reactome and GSEA were used to understand which biological pathways were associated with those gene expression changes. [2]

Untargeted metabolomics was then performed to determine what small metabolites change when ACAD9 is silenced. LC-MS/MS was used for this purpose, including liquid chromatography to separate the compounds and then mass spectrometry to detect and analyze them, to ultimately compare metabolites associated with cellular energy, glucose metabolism, the TCA cycle and glutathione metabolism. [2]

With the focus on linoleic acid, it was also important to establish what happens to the cell's lipids when ACAD9 is missing. Using lipidomics with LC-MS/MS again, a comparison was performed between glycerolipids, glycerophospholipids, sphingolipids, saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids, especially after linoleic acid treatment.

Changes in cellular lipid composition could therefore be compared after reduction of ACAD9 to determine whether there was an association between them. [2]

Several assays were used to examine mitochondrial function from different angles. A Complex I activity assay was performed to directly measure its activity since ACAD9 is connected to Complex I. Seahorse analysis was also performed to measure the oxygen consumption rate, which gives information about mitochondrial respiration, including basal, ATP linked and maximal respiration. An ATP assay was also needed to measure the energetic consequence of ACAD9 loss. JC-1 was then used to evaluate mitochondrial membrane potential. [2]

Oxidative stress was measured through several measurements: DCFH-DA for general cellular ROS, GSH/GSSG to examine cellular redox status and MitoSOX to specifically examine mitochondrial superoxide. [2]

Lipid oxidation was then investigated through C11-BODIPY, a fluorescent measurement of lipid oxidation, and MDA, another measurement associated with lipid peroxidation. [2]

Finally, two different methods were used to establish whether the cells were actually undergoing ferroptosis. First, transmission electron microscopy was used to look at mitochondrial ultrastructure, so morphological changes could be observed under ACAD9-deficient and linoleic acid conditions. Second, ferrostatin-1 and troglitazone were used in rescue experiments to not only observe changes associated with ferroptosis but also interfere with the proposed cell death mechanism and determine whether cell viability could be restored. [2]

Results

4.1) ACAD9 regulates linoleic acid

Does ACAD9 control what ovarian cancer cells do with linoleic acid?

With ACAD9's presence, linoleic acid can contribute to β -oxidation and the production of ATP. Without enough ACAD9, the energetic benefit is lost and lipid composition shifts toward PUFA rich membrane lipids. This second pathway is inferred from ATP and lipidomics. [2]

ACAD9 had already been identified as important for ovarian cancer progression. But then ACAD9 expression in the ID8 mouse model was higher in primary orthotopic tumors and even higher in omental metastases. So ACAD9 expression increased as the cells moved into the in vivo tumor environment, particularly the omentum. Since the omental environment is rich in adipocytes and lipids, the adipocyte connection was tested more directly. ID8 cancer cells were exposed to conditioned medium from differentiated adipocytes and ACAD9 expression increased. These two related observations led to the investigation of whether ACAD9 is connected to the lipid rich environment. GSEA of ovarian cancer patient data also linked ACAD9 expression with fatty acid metabolic pathways, including linoleic acid metabolism. [2]

Before establishing linoleic acid as the main important lipid, several fatty acids such as palmitic acid, oleic acid, linoleic acid and eicosapentaenoic acid were compared in control versus ACAD9-silenced OVCAR-5 cells. If every fatty acid produced exactly the same ACAD9 dependent phenotype, then maybe ACAD9 loss simply makes cells generally unable to tolerate fatty acids. However, it was linoleic acid that stood out. In control cells, fatty acid supplementation generally gave a modest increase in proliferation and colony formation. When ACAD9 was silenced, linoleic acid produced the clearest negative effect. So ACAD9 deficiency doesn't just change fat metabolism generally, there's a particular relationship with linoleic acid, which was already relevant since ovarian cancer cells exposed to omental adipocytes had been reported to preferentially accumulate it. [2]

With the increase in the amount of linoleic acid, cell viability increased in control cells, while ACAD9-silenced cells had progressively lower viability. The same response was also observed in ID8 and OVCAR-8 cells, supporting the same results. Linoleic acid isn't simply good for or toxic to ovarian cancer; its effect depends on whether the cells have ACAD9. [2]

Since ACAD9 is connected to fatty acid oxidation, ATP was then measured to establish whether linoleic acid was contributing to energy production. Control cells saw an ATP increase with the addition of linoleic acid, while this increase was lost in ACAD9-silenced cells. So when ACAD9 is reduced, ovarian cancer cells lose the energetic benefit they normally obtain from linoleic acid. The results in control cells were consistent with linoleic acid being used for energy production through FAO. [2]

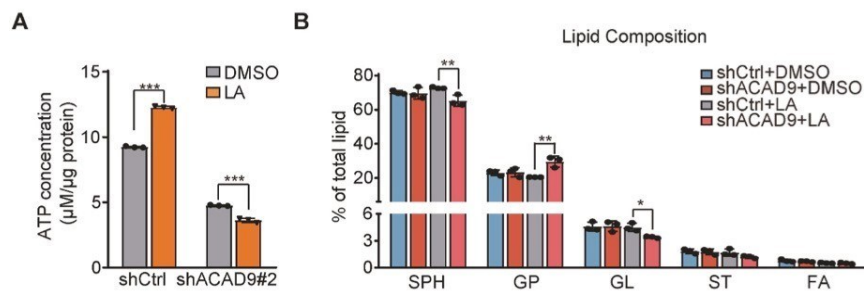


Figure 5) Effect of linoleic acid on ATP production following ACAD9 silencing.

ATP concentration was measured in control and ACAD9-silenced OVCAR-5 cells with or without linoleic acid treatment. Linoleic acid increased ATP levels in control cells, while this increase was lost following ACAD9 silencing. [2]

Lipidomics was then performed to determine what happens to cellular lipids when linoleic acid isn't contributing normally to energy production. Before linoleic acid treatment, there were no major overall lipidomic differences between control cells and ACAD9-silenced cells. So ACAD9 loss by itself does not automatically cause a massive change in lipid composition under these conditions. With the addition of linoleic acid, differences were observed, showing that the phenotype is strongly dependent on the lipid environment. [2]

In ACAD9-silenced cells exposed to linoleic acid, the levels of sphingolipids and glycerolipids were reduced, while glycerophospholipids increased. Triacylglycerols associated with fatty acid storage were also decreased, while phosphatidylglycerol belonging to the glycerophospholipid fraction increased. So under ACAD9 deficiency, more of the lipid pool is associated with membrane lipid synthesis rather than storage. Together with the fact that linoleic acid no longer increases ATP, these changes are where the proposed metabolic redirection stems from. [2]

In ACAD9-deficient cells treated with linoleic acid, saturated fatty acids decreased while polyunsaturated fatty acids increased. Looking at the number of double bonds, diunsaturated fatty acids increased particularly strongly. So ACAD9 deficiency causes mitochondrial dysfunction and an increase in ROS while also causing membrane lipids to become more PUFA rich. [2]

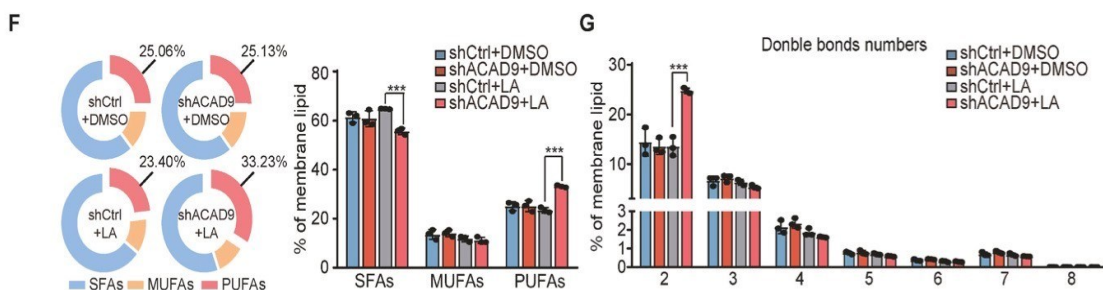


Figure 6) Effect of ACAD9 silencing on membrane fatty acid composition following linoleic acid treatment.

The relative proportions of saturated, monounsaturated and polyunsaturated fatty acids were analysed in membrane lipids of control and ACAD9-silenced OVCAR-5 cells. Under linoleic acid treatment, ACAD9 silencing was associated with reduced SFA and increased PUFA content. [2]

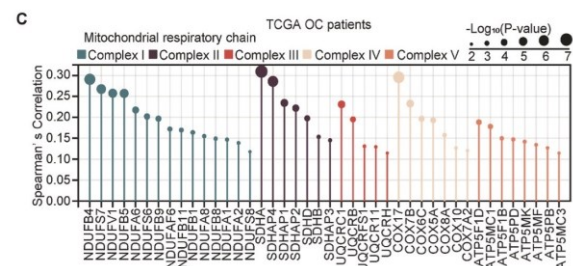
4.2) Crosstalk between lipid metabolism and redox signaling

What happens to the cell's energy and redox balance when ACAD9 is lost and how does that eventually interact with altered linoleic acid metabolism to cause ferroptosis?

Starting off with RNA sequencing in ACAD9-silenced OVCAR-5 cells, the goal was to determine which genes were being expressed more or less after ACAD9 was reduced. The results showed the downregulation of genes associated with respiratory chain assembly, electron transport and oxidative phosphorylation. So we're already getting the signal that ACAD9 loss is affecting the mitochondrial respiratory system broadly. Patient data analysis also showed a positive association between ACAD9 and electron transport chain genes. [2]

The positive correlation between ACAD9 expression and genes involved in the ETC in ovarian cancer patients is shown in Figure 7.

Figure 7) Correlation between ACAD9 expression and electron transport chain genes in ovarian cancer. Spearman correlation analysis of ACAD9 expression with genes involved in the electron transport chain in ovarian cancer patients from the TCGA dataset. [2]



Functional experiments were then performed to further show that mitochondrial respiration actually worked less efficiently. Complex I activity was measured directly and was reduced with ACAD9 silencing. In order to determine whether the whole mitochondrial respiratory phenotype changed, the oxygen consumption rate was measured looking at different aspects of respiration. Basal respiration, which is associated with the normal ongoing respiratory activity of the cells, was lower and ATP linked respiration was also reduced. Even when the mitochondria were pushed toward their maximum respiratory activity, ACAD9-deficient cells had less capacity. So ACAD9 loss doesn't merely alter the expression of mitochondrial genes or one isolated enzyme assay, it affects the actual respiratory performance of the cells. [2]

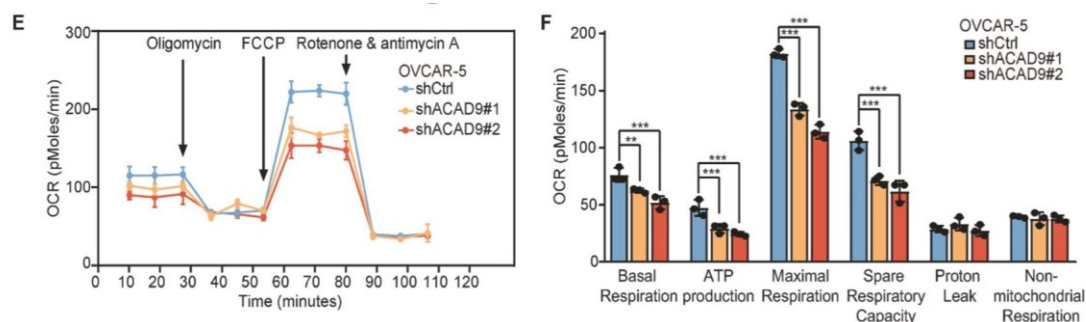


Figure 8) Effect of ACAD9 silencing on mitochondrial respiration.

Oxygen consumption rate measured by Seahorse extracellular flux analysis in control and ACAD9-silenced OVCAR-5 cells following sequential treatment with oligomycin, FCCP, rotenone and antimycin A. ACAD9 silencing reduced mitochondrial respiratory capacity. [2]

ATP was then measured to see whether the respiratory defect could actually produce an energy problem. In ACAD9-silenced cells ATP was lower, and metabolomics showed a decrease in ATP, dGTP and UTP levels, while ADP, AMP, UDP and TMP were increased. So there's a pattern pointing toward a lower energy cellular state. Part of this analysis was also repeated in ID8 orthotopic tumors, which resulted in decreased ATP levels and increases in AMP and ADP. So the energetic phenotype was also observed in the tumor model. [2]

So far, ACAD9-silenced cells showed reduced Complex I activity, reduced respiration and lower ATP levels. Next, the effect of this mitochondrial dysfunction on oxidative stress was observed. ROS was measured using DCFH-DA and a large increase was observed in ACAD9-silenced cells. This was detected using both fluorescence microscopy and flow cytometry. GSH/GSSG was also examined in cells and tumors, with the glutathione redox state being altered. [2]

ACAD9-silenced cells were also treated with CoQ10, which resulted in an improvement in cell viability. The rescue experiment gave additional support for the connection between ACAD9 loss, mitochondrial dysfunction and reduced cell viability, since an intervention connected to mitochondrial electron transport improved the phenotype. [2]

The cells also changed other metabolic pathways in response to compensate for failing mitochondrial energy production and rising oxidative stress. With integrated transcriptomics and metabolomics, changes were observed in glycolysis, the pentose phosphate pathway, TCA cycle related metabolism, amino acid metabolism and glutathione metabolism. [2]

In glycolysis, lower expression of PFKP, PFKL and PKM and lower pyruvate levels were observed. But at the same time, another glucose related pathway changed in the opposite direction. An increase in G6PD and ribulose-5-phosphate was observed in the pentose phosphate pathway, which was associated with increased use of this pathway. This change is connected with NADPH production and the response to oxidative stress. [2]

So with ACAD9 deficiency, oxidative stress increases and the cell shifts part of its metabolism toward pentose phosphate pathway related activity to support NADPH production and help deal with the oxidative stress. Changes were also observed in amino-acid and TCA cycle related metabolism. GOT1, GOT2 and GLUD1 were increased while the levels of aspartate and glutamate were decreased, suggesting that these amino acids are used more to support anaplerotic reactions that replenish TCA cycle intermediates. So if mitochondrial energy metabolism is struggling, the cells change amino acid metabolism to help support the TCA cycle. Another observation was an increase in isocitrate, malate, MDH1 and ACO1. These changes are connected with another possible way of supporting NADPH generation. [2]

There was another antioxidant related system change as well, with an increase in the expression of MAT2A, GCLC and SLC7A11, along with changes in S-adenosyl-homocysteine, homocysteine, cysteine and GSSG, as the cell tries to strengthen glutathione related antioxidant capacity. Despite all these compensatory mechanisms and responses, ATP levels remain lower and ROS remains elevated. So the compensation is not enough to completely restore metabolic balance. [2]

GSEA in linoleic acid treated ACAD9-deficient cells showed enrichment of pathways related to ferroptosis, oxidative stress induced cellular senescence and DNA damage responses. So pathway level data point toward a strong stress response and specifically toward ferroptosis. [2]

However, GSEA alone does not prove ferroptosis. So to collect several additional types of evidence, transmission electron microscopy was used to look at mitochondrial structure. Under ACAD9 silencing and linoleic acid treatment, mitochondrial volume decreased while membrane density increased and the cristae were almost completely lost. These morphological changes were considered consistent with ferroptotic stress. [2]

Another method was the use of JC-1 to evaluate mitochondrial membrane potential. Under these conditions, membrane depolarization occurred, providing additional evidence of mitochondrial dysfunction. [2]

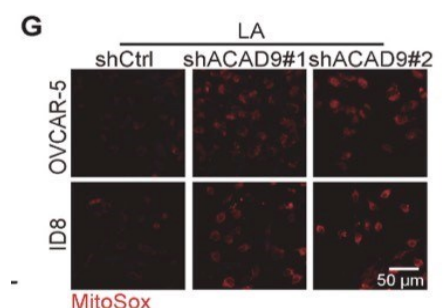
Next, lipid oxidation was looked at directly. C11-BODIPY oxidation increased strongly in ACAD9-deficient OVCAR-5 cells treated with linoleic acid and also in ACAD9-deficient ID8 cells under the same conditions. This was observed with both fluorescence microscopy and flow cytometry. MitoSOX then showed an increase in mitochondrial ROS, adding further evidence connecting the mitochondria with increased oxidative stress under linoleic acid conditions. [2]

Malondialdehyde levels were also measured and MDA levels increased, adding another measurement supporting increased lipid peroxidation rather than relying only on C11-BODIPY. [2]

Finally, ferrostatin-1 and troglitazone were used as ferroptosis inhibitors. In ACAD9-deficient cells treated with linoleic acid, the addition of these inhibitors restored cell viability. By interfering with the proposed cell death mechanism and reversing the viability phenotype, this provided additional functional evidence for the involvement of ferroptosis. [2]

Figure 9) Evidence of ferroptosis in ACAD9-deficient ovarian cancer cells under linoleic acid enriched conditions.

ACAD9 silencing increased C11-BODIPY oxidation and mitochondrial ROS following linoleic acid treatment, while treatment with ferrostatin-1 and troglitazone restored cell viability. [2]



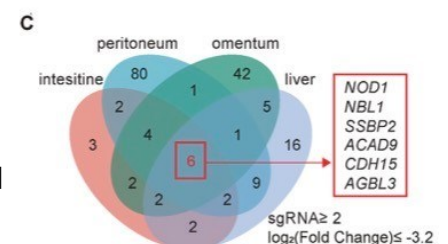
4.3) ACAD9 as a metabolic gatekeeper

How does ACAD9 act as a metabolic gatekeeper that supports ovarian cancer growth and metastatic progression?

The experiment didn't originally start with ACAD9. A genome wide CRISPR/Cas9 screen was performed, so there was a huge population of ovarian cancer cells with different genes targeted. These cells were allowed to form primary ovarian tumors and then metastases in the peritoneum, omentum, intestine and liver. The sgRNAs were then compared and ACAD9 targeting sgRNAs were negatively enriched in all four metastatic locations. If ACAD9-disrupted cells become much less represented when metastases are looked at later, that would suggest that cells lacking ACAD9 were at a disadvantage during metastatic progression. So ACAD9 became an interesting candidate. [2]

The comparison between the four metastatic sites identified six commonly negatively enriched genes, including ACAD9, as shown in Figure 10.

Figure 10) Common negatively enriched genes identified across ovarian cancer metastatic sites. Comparison of metastatic lesions from the peritoneum, omentum, intestine and liver identified six genes showing negative enrichment across all four sites, including ACAD9. [2]



Human patient data was then compared with these results. With higher ACAD9 expression, overall survival and progression free survival were worse. In tumor versus normal tissue comparison, ACAD9 expression was lower in normal tissues while the opposite was observed in ovarian tumors. Higher ACAD9 was also associated with a more advanced stage and higher grade of the disease. Tissues were then compared across progression from normal tissue to primary ovarian tumor and then to peritoneal metastasis, and ACAD9 mRNA and protein increased along with that progression. [2]

To test this association, ACAD9 was then manipulated directly using two independent shRNAs. Reducing ACAD9 resulted in decreased proliferation and colony formation across ovarian cancer models. [2]

In the orthotopic models, ACAD9 knockdown led to smaller primary tumors, lower tumor weight, less ascites and fewer peritoneal metastatic nodules. The problem was that if the control mouse had a much larger primary tumor and the ACAD9-knockdown mouse had a much smaller primary tumor, then naturally, the second mouse might produce fewer metastases simply because fewer tumor cells existed to spread. So the orthotopic model wasn't perfectly separating the effect on primary tumor growth from the effect on metastatic dissemination itself. To bypass this problem, ID8 cells were injected directly into the peritoneal cavity. Now the cells didn't first need to form the same kind of primary ovarian tumor before being tested in the peritoneal environment, and ACAD9 knockdown still reduced ascites and metastatic lesions. [2]

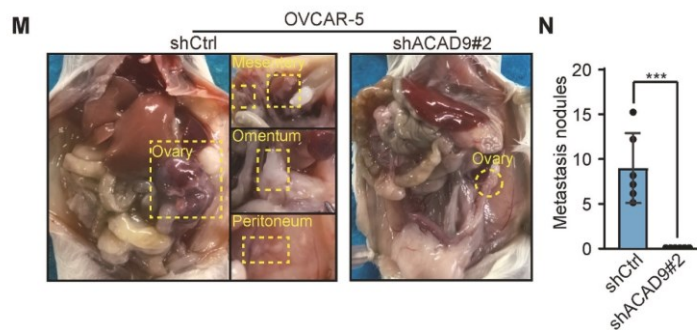


Figure 11) Effect of ACAD9 silencing on peritoneal metastasis in the orthotopic ovarian cancer model. Representative images of peritoneal metastatic lesions and quantification of metastatic nodules in control and ACAD9-silenced OVCAR-5 tumors, showing a reduction in metastatic burden following ACAD9 knockdown. [2]

ACAD9 controls two things simultaneously. The first side is mitochondrial respiration, energy and redox balance, and the second is linoleic acid metabolism and membrane lipid composition. Cancer cells need both sides to remain balanced.

Without enough ACAD9, ovarian cancer cells have an energy problem and an oxidative stress problem at the same time. They try to compensate for these changes through the metabolic responses described previously, but ATP remains low and ROS remains high. On the other hand, ACAD9 also influences how the cell handles the lipid rich environment. Under ACAD9 deficiency and linoleic acid enriched conditions, membrane lipid composition shifts toward a higher proportion of PUFAs. So ACAD9 deficiency creates increased oxidative pressure at the same time that more highly unsaturated membrane lipids are available for oxidation. [2]

These two effects together promote lipid peroxidation and ferroptosis. So ACAD9 acts like a metabolic checkpoint between energy metabolism, lipid handling and oxidative damage. [2]

However, these individual observations alone would not be enough to establish the involvement of ferroptosis, so several different types of evidence were used. GSEA first showed enrichment of ferroptosis related pathways. C11-BODIPY then showed increased lipid oxidation, while MitoSOX showed increased mitochondrial ROS and MDA provided another measurement supporting lipid peroxidation. Finally, ferrostatin-1 and troglitazone were used to interfere with the proposed ferroptotic mechanism. Both treatments restored viability in ACAD9-deficient cells exposed to linoleic acid. [2]

ACAD9 therefore gives ovarian cancer cells a metabolic advantage in the lipid rich environment, allowing them to use the available lipid resources while avoiding the combination of mitochondrial oxidative stress and excessive PUFA rich membrane accumulation. [2]

HUVEC and GES-1 were also tested as non malignant cells, and the same strong effects were not observed in proliferation, colony formation and the response to linoleic acid. So within the specific models tested, ovarian cancer cells appear more dependent on ACAD9. [2]

In the proposed model, with ACAD9 present, linoleic acid contributes more effectively to energy metabolism, while in ACAD9-deficient cells linoleic acid becomes more associated with membrane lipid remodeling. The combination of ATP data, lipidomics, PUFA changes, ROS, lipid peroxidation and ferroptosis rescue supports this metabolic redirection model. [2]

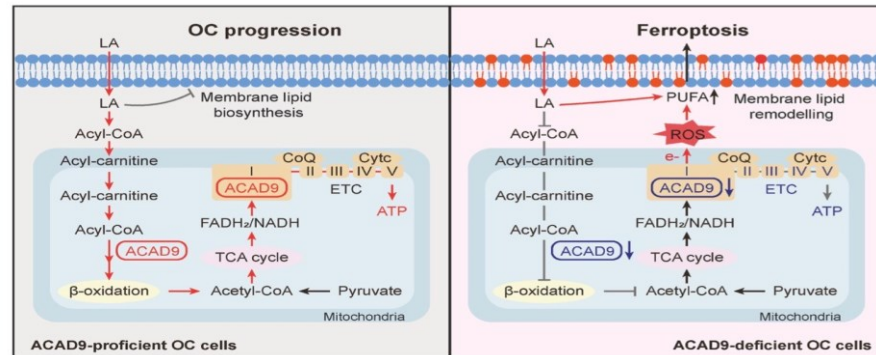


Figure 12) Proposed model of the metabolic role of ACAD9 in ovarian cancer.

In ACAD9-proficient cells, ACAD9 supports mitochondrial respiration and linoleic acid metabolism. ACAD9 deficiency is associated with impaired energy production and ROS accumulation together with PUFA rich membrane remodeling, promoting lipid peroxidation and ferroptosis. [2]

Conclusions

ACAD9 plays an important role in maintaining the metabolic balance of ovarian cancer cells by connecting mitochondrial respiration with linoleic acid metabolism. When ACAD9 is reduced, Complex I activity, mitochondrial respiration and ATP production decrease while ROS increase. At the same time, linoleic acid metabolism changes, with an increase in PUFA rich membrane lipids. So under linoleic acid enriched conditions, increased oxidative stress occurs together with more unsaturated membrane lipids, promoting lipid peroxidation and ferroptosis. This dual role is why ACAD9 can be considered a metabolic gatekeeper supporting ovarian cancer growth and peritoneal metastasis. [2]

However there are some limitations to consider. The CCK-8 assay depends on cellular metabolic activity, which may influence the interpretation of viability after ACAD9 silencing. More importantly, the proposed redirection of linoleic acid from β -oxidation toward membrane lipid synthesis was not directly followed through metabolic flux analysis. Approaches such as ^{13}C tracing would be needed to directly track its metabolic fate, together with further validation in primary ovarian cancer samples and patient derived models. [2]

ACAD9 could also represent a possible therapeutic target, although systemic inhibition may affect tissues with high oxidative requirements. Future approaches could therefore focus on ovarian cancer specific regulation of ACAD9 or tumor selective targeting strategies. [2]

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