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Abstract Book

Unravelling the structure-function relationships of the bi-functional enzyme FAD synthase (EC 2.7.7.2 / EC 3.6.1.18) to target LSMFLAD

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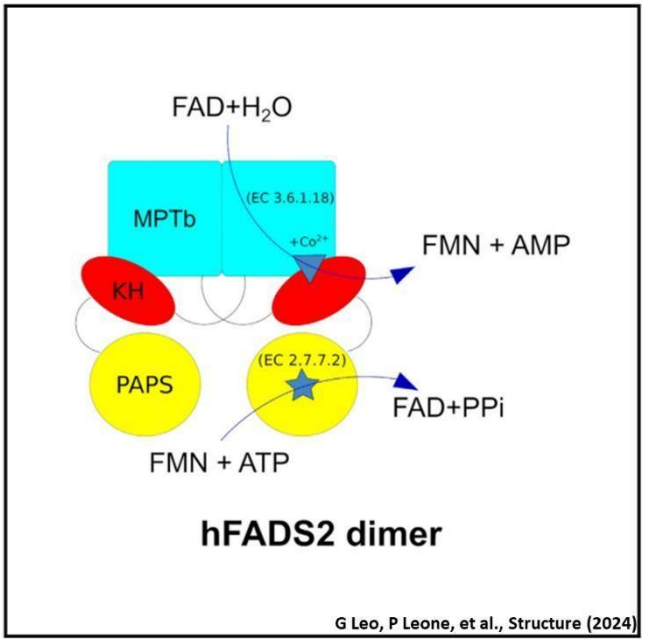
Human FAD synthase (FADS) is the last enzyme in the pathway converting riboflavin (Rf) into the redox cofactor FAD. Mutations in *FLAD1*, coding for FADS, are responsible for Rf-responsive and non-responsive forms of Multiple Acyl-CoA Dehydrogenase and combined respiratory chain deficiency (LSMFLAD, OMIM #255100) [1]. Over-expression of FADS has been recently associated to cancer [2]. The best characterized FADS, isoform 2, is a bi-functional enzyme. It consists of two major domains: a C-terminus PAPS reductase domain responsible for FAD synthesis and an N-terminus molybdopterin-binding resembling domain (MPTb), whose FAD hydrolytic activity strictly requires Co^{2+} [3]. Predictive models of the two principal domains of FADS's structure were previously described [4]. AlphaFold prediction of the entire protein highlighted the existence of a central inter-domain, with a Type I KH-like fold, whose role was still unknown. Here we report the crystal structure of the entire FADS2, revealing a dimeric organization. From a kinetical point of view, we report the functional characterization performed on complete and truncated constructs of FADS, demonstrating that the combination of the MPTb and KH domains is the minimal essential substructure required for the hydrolysis of FAD. These findings provide the molecular basis for elucidating the mechanism by which FADS ensures cellular flavin cofactor homeostasis, and for predicting the effects of mutations in LSMFLAD patients



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How *Pseudomonas aeruginosa* prevents H_2S toxicity

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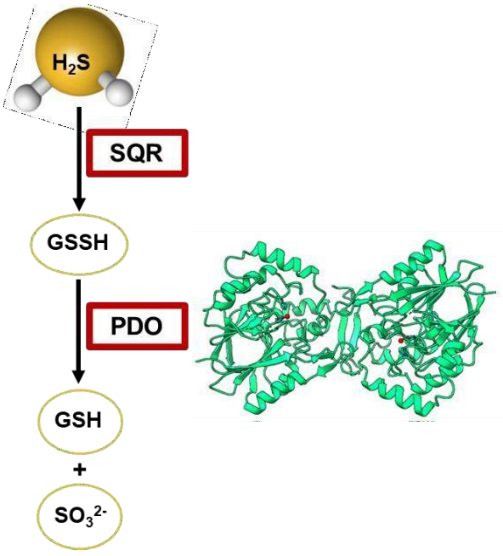
Pseudomonas aeruginosa (*Pa*) is a multidrug resistant pathogen responsible for life-threatening pneumonia in patients with cystic fibrosis [1]. Hydrogen sulfide (H_2S), while acting as a signaling molecule at low physiological concentrations, at higher levels is toxic. *Pa* was recently shown to be able to respire O_2 in sulfide-rich environments thanks to its *bd*-type Cyanide Insensitive Oxidase [2]. Like other bacteria, *Pa* codes for a multienzymatic unit putatively involed in H_2S detoxification to sulfite and (thio)sulfate, which comprises two isoforms of sulfide:quinone oxidoreductase (SQR) and a persulfide dioxygenase (PDO). Here we characterized these enzymes from *Pa* as recombinantly produced in *Escherichia coli*. The kinetic parameters of isolated *Pa*PDO for both O_2 and GSSH were determined by high-resolution respirometry. Then, combining this technique with nitric oxide (NO)-amperometry, the effect of NO on *Pa*PDO catalytic activity was assessed. Also, we will report on the biochemical properties of the isoform 2 of *Pa*SQR. Our findings suggest that *Pa*SQR and *Pa*PDO contribute to keep H_2S concentration low, thus possibly protecting *Pa* from H_2S poisoning.



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TMEM65 controls mitochondrial activity through respiratory complex I assembly

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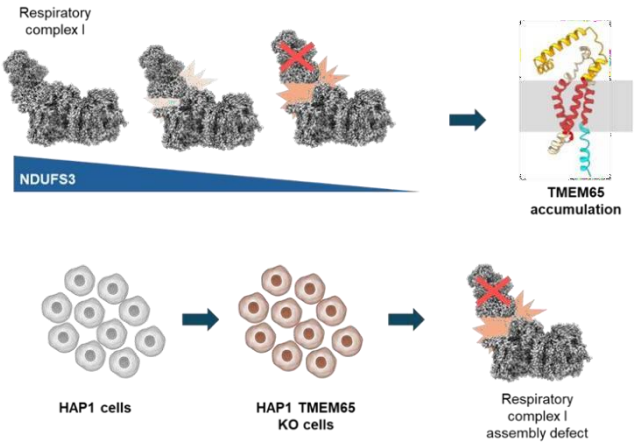
Transmembrane protein 65 (TMEM65) is a protein localized in the inner mitochondrial membrane. A previous work identified a homozygous loss-of-function mutation in *TMEM65* gene in a patient with a clinical presentation resembling a mitochondrial disorder [1]. Knock down of TMEM65 expression in human fibroblasts was shown to severely affect mitochondrial content and respiration [1], but the exact mechanism remains unknown.

Interestingly, by performing a quantitative proteomic screening, we observed an accumulation of TMEM65 protein in osteosarcoma cells lacking respiratory complex I (CI).

Thus, in order to get a deeper understanding of the function of this protein we obtained a TMEM65 KO human cell line and analysed the effects on mitochondrial function of the absence of the protein. Ablation of TMEM65 resulted in a mild reduction of mitochondrial OXPHOS capacity, associated with a reduction of fully assembled functional CI and abnormal accumulation of CI subassemblies. Moreover, TMEM65 KO cells showed impaired CI biogenesis kinetics, suggesting the presence of an assembly defect. Overexpression of full-length TMEM65 was able to partially rescue the assembly defect, confirming the role of the protein in promoting correct CI assembly.

Taken together, the data suggest TMEM65 plays a role in mitochondrial respiratory chain function by assisting CI assembly. Gaining deeper knowledge on the molecular function of this protein will help understanding the mitochondrial disorders caused by mutations in *TMEM65* gene.



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Investigating the functions of ATP synthase subunits e and g in *Drosophila melanogaster*

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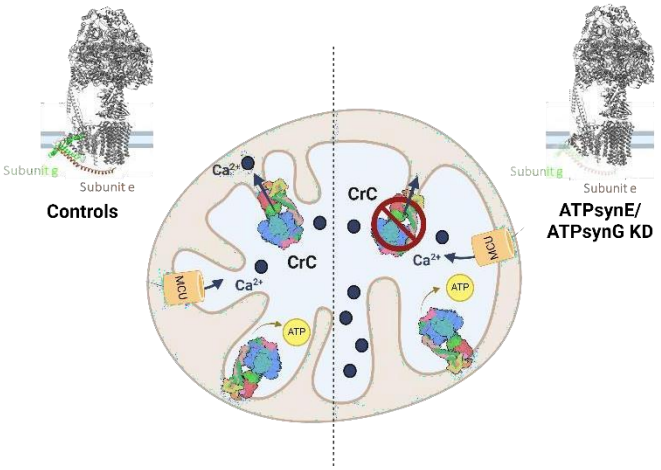
Differently from yeast and mammals, *Drosophila melanogaster* does not show a mitochondrial permeability transition (PT), a process caused by the opening of the so-called permeability transition pore (PTP). The PTP is defined as a Ca^{2+}-activated, high-conductance and unselective channel with a maximal conductance of 1.2 nS that allows ions and solutes up to 1.5 kDa to equilibrate across the inner membrane. PTP openings play a role in both Ca^{2+} homeostasis and cell death initiation. Conversely, *Drosophila* PTP appears to be specialized and operate uniquely as a selective Ca^{2+}-release channel (CrC), that does not induce mitochondrial swelling and cell death. ATP synthase was recently demonstrated to mediate the PT in mammals and yeast and to generate a peculiar 53 pS-channel in *Drosophila* that could represent the CrC. Genetic studies showed that the ablation of ATP synthase "accessory subunits" e and g dramatically affects PT occurrence in mammals and yeast, suggesting a primary role of these small proteins in PTP formation. To shed light on the roles of the two subunits in *Drosophila*, we generated knock-down (KD) lines for genes encoding either subunit e (*ATPsynE*) or g (*ATPsynG*) of ATP synthase. *In vivo* ubiquitous downregulation of each subunit causes a dramatic arrest in fly development at larval stage, impairs the dimerization and oligomerization states of ATP synthase, decreases mitochondrial respiration and *cristae* formation, yet the total amount of ATP is unaltered. Strikingly, the sensitivity to Ca^{2+} is decreased in both *ATPsynE* and *ATPsynG* KD mitochondria, which require higher matrix Ca^{2+} loads (1.5-fold and 3-fold, respectively) to induce the CrC. Altogether, our results confirm a key role of these two proteins in the formation of *Drosophila* channel and suggest that the phenotype of KD fliesis not entirely due to bioenergetic defects, but may also partially arise from a CrC-related Ca^{2+} dysregulation.



Unravealing the mechanism of channel formation by F-ATP synthase

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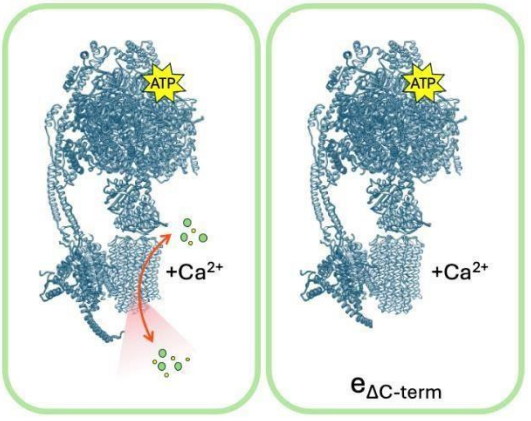
The Permeability Transition (PT) is a mitochondrial permeability increase to ions and solutes caused by the opening of a Ca^{2+} and voltage-dependent channel, the PT pore (PTP). The long-standing debate about the PTP molecular identity has been recently challenged by a substantial set of data reporting a key role of the ATP synthase. In presence of Ca^{2+}, the enzyme generates high conductance channels with biophysical properties matching those of the PTP. However, the mechanism of channel formation by the ATP synthase is still elusive. A central role is played by the dimerization subunits (e and g), which are required for the assembly of higher-order structures. In the absence of e and g subunits, the ATP synthase can no longer generate a channel, and a PT can eventually be mediated by the adenine nucleotide translocator (ANT), which represents an alternative permeation pathway. High-resolution structures of the ATP synthase revealed a physical connection between the e subunit C-terminus and the lipids filling the rotor (c-ring) and showed Ca^{2+}-dependent conformational changes that dramatically perturb this region. One hypothesis is that, in presence of Ca^{2+}, the e subunit C-terminus removes the lipids from the c-ring via a push-pull mechanism leading to the channel formation within the rotor. To test this, we generated HeLa cells expressing a truncated form of subunit e devoid of its C-terminus and evaluated PT occurrence. Our data show that in the mutant cells the PT is mediated by ANT, indicating that the e subunit C-terminus is an indispensable requirement for the channel formation by the ATP synthase.



Kinetic analysis of natural compounds on mitochondrial F_1F_O-ATPase activity

Antonia Cugliari[1], Cristina Algieri[1], Silvia Granata[2], Patrycja Anna Glogowski[1], Fabiana Trombetti[1], Micaela Fabbri[1], Salvatore Nesci[1]

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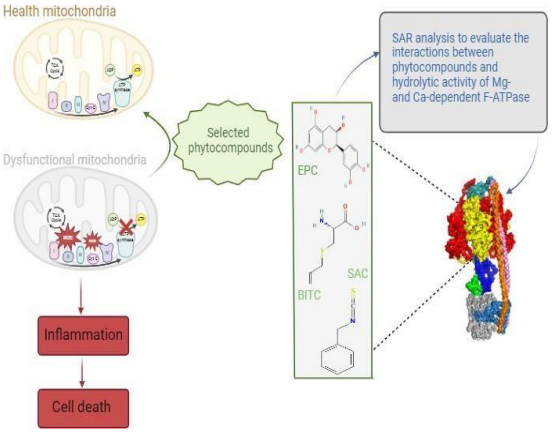
Several human diseases are characterised by mitochondrial dysfunction, a condition that causes the reduction of ATP production and induces excessive production of reactive oxygen species (ROS). To counteract this condition, in recent years particular attention has been focused on natural compounds, including phytocompounds, known for their antioxidant activity and ability to affect energy metabolism [1]. In this context, the effect of allyl-cysteine (SAC), epicatechin (EPC) and benzyl-isothiocyanate (BITC), selected for their antioxidant capabilities, was evaluated. A structure-activity relationship analysis was carried out to assess a possible interaction between the individual compounds and the hydrolytic activity of F_1F_O-ATPase from swine heart mitochondria by using enzyme kinetics studies. From these, organosulphide SAC was found to be a competitive activator with respect to the Mg^{2+} cofactor. The flavonoid EPC did not significantly modify the enzyme activity, while organosulphide BITC exerted a mixed competitive inhibitor with respect to cofactors on the hydrolysis of Ca^{2+}-dependent F_1F_O-ATPase andMg^{2+}-dependent F_1F_O-ATPase, which was more pronounced in the presence of dithiothreitol (DTT). In relation to the ATP substrate, BITC showed mixed competitive inhibition or competitive inhibition on Mg^{2+}- dependent F_1F_O-ATPase in the presence or absence of DTT, respectively. Otherwise, BITC showed a mixed competitive inhibition or uncompetitive inhibition on Ca^{2+}-dependent F_1F_O-ATPase in the presence or absence of DTT, respectively. This study aims to extend knowledge in the field of modulation of energy ATP transduction by phytocompounds considering the possible prevention or attenuation strategies for various pathological conditions.



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The binding of Cyclophilin D (CyPD) to F-ATP synthase is regulated by CyPD N-terminus cleavage.

Elisa Zamprogno[1], Gabriele Coluccino[1]; Camilla Bean[1]; Antonio Filippi[1]; Valentina Pia Muraca[1]; Clarissa Gissi[1]; Diana Canetti[2]; Andrea Urbani[3]; Francesco Ciscato[3], Marco Zancani[4]; Michela Carraro[3]; Paolo Bernardi[3]; Alessandra Corazza[1]; Giovanna Lippe[1]

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Cyclophilin D (CyPD) is a protein localized in mitochondria that plays an important role as regulator of the permeability transition pore (PTP). PTP is a Ca^{2+}-dependent, unselective channel involved in mediating fast Ca^{2+}release from mitochondria (short open times) and in generating a bioenergetic failure eventually leading to cell death (long open times). We had demonstrated that CyPD interacts with the OSCP subunit of ATP synthase, a site where CyPD binding inhibits catalysis and favors the transition of the enzyme complex to the PTP channel.

More recently, we identified two forms of CyPD in various cell lines and tissues. Mass spectrometry analyses showed that one form is ~1 kDa shorter than the other at the N-terminus, suggesting that CyPD could be cleaved by a mitochondrial protease. *In vitro* studies using human recombinant full-length CyPD (FL-CyPD) and a N-terminal truncated form (ΔN-CyPD) highlighted that the latter interacts with OSCP more avidly than FL-CyPD in a KCl buffer, which best resembles the physiological environment. Using a peptidase database and giving a bond input according to the MS data, we found out mitochondrial calpains as putative candidates, a finding that is consistent with the already described role of mitochondrial calpains in regulating PTP opening.

Overall, our data suggest that: i) the CyPD N-terminus regulates CyPD interaction with OSCP; ii) a N-terminally cleaved CyPD forms in cell, thus offering new insights into PTP regulation.

IF_1, the endogenous inhibitor of F_1F_o-ATPase, inhibits the ATP hydrolysis but not the ATP synthesis activity in cancer cells

Valentina Del Dotto, Silvia Grillini, Riccardo Righetti, Martina Grandi, Gianluca Sgarbi, Claudia Zanna, Valentina Giorgio, Giancarlo Solaini, Alessandra Baracca

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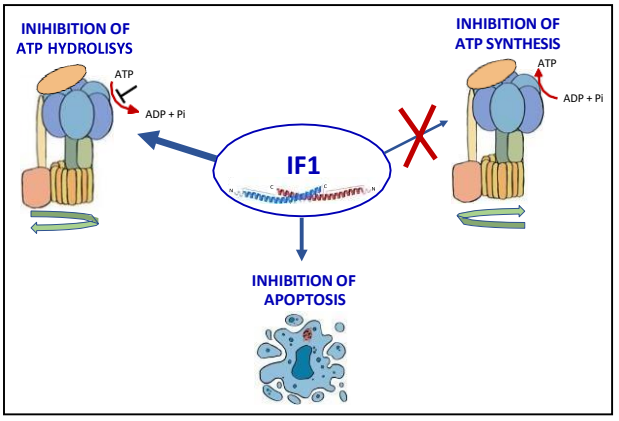
The pro-oncogenic role of the inhibitor protein IF_1 has been ascribed to several actions, including protection of cancer cells from severe hypoxia/anoxia and from both apoptosis and Ca^{2+} homeostasis dysregulation in normoxic conditions [1]. The present work concerns the possibility that IF_1 could express its pro-oncogenic action by inhibiting the ATP synthesis activity of F_1F_o-ATPase and oxidative phosphorylation (OXPHOS), thus challenging the reprogramming of energy metabolism under normoxia proposed by Cuezva and coworkers [2]. We prepared stably IF_1-silenced clones and compared their bioenergetics with that of three different cancer cell lines: 143B, HCT116 and HeLa. All functional parameters: respiration rate, ATP synthesis rate (OXPHOS) and mitochondrial membrane potential, were similar in IF_1-silenced and control cells, demonstrating that IF_1 does not inhibit the ATP synthesis in cancer cells. Furthermore, we investigated a possible PKA-mediated regulatory mechanism of IF_1 that should prevent its binding and action on the F_1F_o-ATPase in cancer cells [2]. We found that, regardless of the presence of IF_1, activation or inhibition of PKA similarly affected OXPHOS, but no effect on ATP synthase activity was observed [3], therefore supporting that IF_1 does not inhibit the ATP synthase in cancer cells when the enzyme works physiologically, synthesizing ATP.



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IF1 mutants recurrent in lung patients cause highly aggressive and death-resistant cancer phenotypes

Martina Grandi, Ida Schaldemose Nielsen, Valentina Del Dotto, Giancarlo Solaini, Alessandra Baracca, Valentina Giorgio

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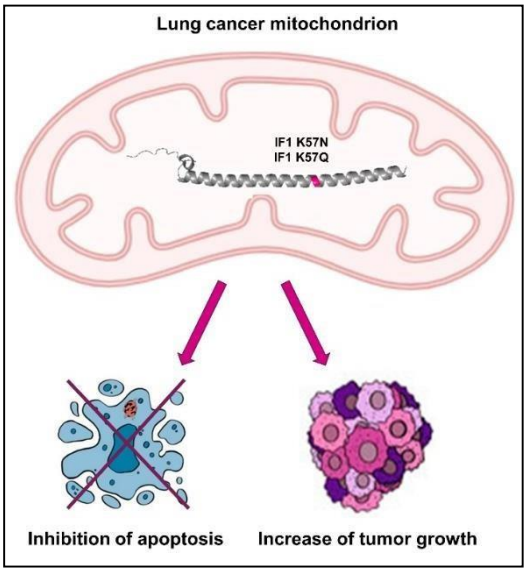
In many tumors, the mitochondrial inhibitor IF1 is upregulated and acts as a pro-oncogenic factor through different mechanisms [1, 2]. Its overexpression was shown to promote the IF1 interaction in cancer cells with the ATP synthase OSCP (oligomycin sensitivity conferring protein) subunit during oxidative phosphorylation [3].

We here show two IF1 mutant forms of the human mature protein, K57N and K57Q, that are recurrent in aggressive and therapy-resistant lung cancers (i.e. non-small cell lung cancer, NSCLC). The transient and stable re-insertion of the *ATP5IF1* gene in IF1 KO HeLa cells allowed us to overexpress the wild-type or mutant IF1 proteins and was instrumental for dissecting their effects.

IF1 mutants displayed an increased colony formation compared to wild-type IF1-expressing HeLa cells in soft agar. These IF1 mutants did not affect proliferation, but decreased the sensitivity to PTP opening. These changes are mediated by the interaction of IF1 with the OSCP subunit, as shown by immunoprecipitation in wild-type and mutant IF1-expressing cells.

Overall, our results suggest that the IF1 mutants found in lung patient biopsies might favor tumor growth by protecting cancer cells from PTP-dependent apoptosis.



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Von Willebrand Domain-containing Protein 8 (Vwa8) affects mitochondrial substrate preference and browning of subcutaneous adipose tissue

Aleksandra Marković[1], Camilla Bean[1], Hualin Fan[1], Habiba Hayat[2], Keisuke Takeda[1], Tomas Mracek[2], Lukas Alan[1,2] and Luca Scorrano[1]

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Background and aim: Obesity is turning into a worldwide disease, with most patients also affected by other comorbidities such as type 2 diabetes, hypertension, or cardiovascular disease. With mitochondria being a major site for fatty acid oxidation (FAO), they represent an important target for obesity treatment. Therefore, we focused our study on poorly characterized protein Vwa8, a putative AAA+ ATPase with a dynein conformation.

Material and methods: By combining 2-dimension blue native gel electrophoresis with proteomics and bioinformatics in heart tissue we identified a strong correlation between the key cristae biogenesis protein Opa1 and Vwa8. To study the role of the Vwa8 protein in mitochondrial physiology, we developed Vwa8 knock-out cell lines and Vwa8 knock-out C57BL/6N mice model.

Results: Vwa8 protein is located in mitochondrial intermembrane space. Deletion of Vwa8 leads to an increased mitochondrial respiration on FA but not on glucose or glutamine. The Vwa8 KO mice show decreased resting energy requirements as well as higher heat production, indicating a stronger preference for FAO. Moreover, the subcutaneous adipose tissue of Vwa8 KO mice shows increased markers of browning such as mitochondria content and lipid droplet multilocularity. The Vwa8 KO mice remained more insulin sensitive and with higher lean mass proportion upon a high-fat diet.

Conclusion: In conclusion, Vwa8 affects mitochondrial substrate preference, induces browning of subcutaneous adipose tissue, and represents a new target for obesity treatment.

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Respiratory complex I deficiency triggers OMA1-mediated integrated stress response and affects lipid homeostasis during glucose restriction

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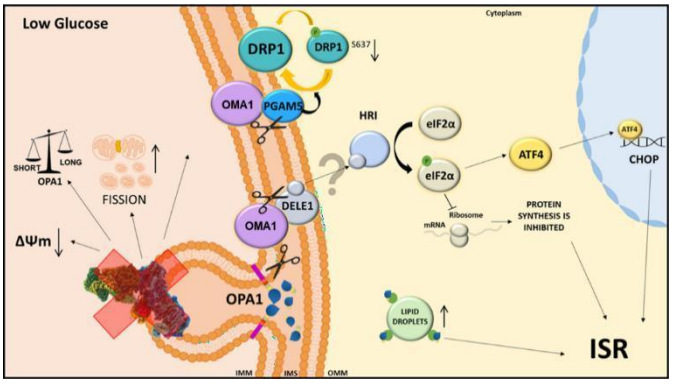
Respiratory complex I (CI) is a pivotal enzyme for cellular bioenergetics and its functional alterations trigger metabolic and molecular adaptive responses that help cancer cells survive despite the energetic deficit. Since such mechanisms are far from being completely understood, we exploited multiple cancer cell lines of different tissue origin lacking CI in which we knocked out the core subunit NDUFS3 (NDUFS3$^{-/-}$) to elucidate their response to energetic stress. During glucose restriction, NDUFS3$^{-/-}$ cells showed significant mitochondrial network fragmentation and depolarization, that in turns triggered the activation of the mitochondrial protease OMA1, as shown by OPA1 and PGAM5 cleavage and DRP1 dephosphorylation. These mitochondrial alterations were accompanied by a consistent activation of Integrated stress response (ISR) as proved by the phosphorylation of eIF2α, the block of protein synthesis, the nuclear re-localization of ATF4 and the increased expression of CHOP. This molecular mechanism is directed by OMA1 and suppressed when the protease expression is prevented by siRNA. We also found that glucose starvation triggered the accumulation of lipid droplets in close proximity to deranged mitochondria in CI-defective cells both *in vitro* and *in vivo*, accompanied by a profound alteration of lipid content, in particular we observed an increase in triacylglycerols and in cholesteryl esters amount. In this context lipid droplets could sequester misfolded proteins, excess lipids and prevent free calcium overload in the cytosol in order to alleviate endoplasmic reticulum stress. In conclusion, loss of CI and glucose restriction induce a striking mitochondrial distress with depolarization and network fragmentation and trigger OMA1-mediated ISR to possibly overcome the stress status. Further analyses are necessary to dissect the role of ISR in CI- defective cancer cells adaptation and survival.



Intermittent fasting rescues heart failure caused by cardiomyocyte ablation of the master mitochondria-shaping protein OPA1.

Martina Semenzato[1,2], Lorenza Iolanda Tsansizi[1,2], Marco Fantuz[1,2], Ludovica Zambello[1,2], Raffaele Cerutti[1,3] and Luca Scorrano[1,2]

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Levels of the inner mitochondrial membrane protein optic atrophy 1 (OPA1), a key regulator of mitochondrial morphology and ultrastructure, are reduced in different models of heart failure. Yet, the specific impact of OPA1 loss on heart structure and function remains unexplored.

We generated a mouse model of inducible Opa1 genetic ablation in cardiomyocytes (Opa1CKO) by crossing Opa1flox/flox with α-MHC MerCREMer transgenic mice. Four-weeks old Opa1CKO mice were fed with Tamoxifen for 4 weeks. We analyzed heart function by echocardiography, mitochondrial structure by histology and electron microscopy, function and signaling by functio nal and biochemical assays.

After the completion of the tamoxifen treatment Opa1 levels in cardiac tissues were reduced by 90%. One month later, in femal es and males Opa1CKO mice fractional shortening was severely decreased (FS: 40.2%±2.6 in WT vs.12.8%±0.9 in Opa1CKO) and cardiac mass was increased by 29% (heart weight/tibial length: 0.07%±0.01 WT vs. 0.09%±0.02 Opa1CKO), leading to premature death between 14 and 34 weeks of age. Histologically, Opa1CKO hearts displayed extensive fibrosis and reactivation of fetal genes indicative of hypertrophy before the alterations in FS and heart mass. As expected, Opa1CKO mitochondria were smaller with fewer and distorted cristae and their respiratory control ratio was reduced. Surprisingly, although RNAseq data displayed an upregulation of autophagy related genes in Opa1CKO mice, autophagic flux was reduced in Opa1CKO hearts upon starvation. A protocol of intermittent fasting not only restoring autophagy activation, but also improving heart contractility and reduced hypertrophy.

Our findings place dysregulated autophagy between primary mitochondrial dysfunction and cardiac hypertrophy and suggest that dietary interventions aimed at reactivating autophagy can improve cardiac hypertrophy.

Opa1 is required for melanocyte stem cell maintenance.

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Melanocytes produce melanin, which provides pigment to skin and protection against UV irradiation. In melanocyte biology, mouse models of hair graying processes can serve as useful systems to uncover mechanisms involved in aging and the maintenance of stem cell function. A recent genome wide association study helped to reveal a direct link between changes in mitochondrial function and melanin production, pointing to a role for these organelles in melanocyte function and dysfunction [1,2]. Despite being a crucial regulator of mitochondrial and cellular homeostasis [3, 4]. the roles of the large inner mitochondrial membrane dynamin related GTPase Opa1 in the graying processes and hair follicle genesis are yet to be defined. Here we show that Opa1 is essential for continuously maintaining adequate levels of differentiated melanocytes during the hair follicle cycle. Indeed, Opa1 ablation in mouse melanocytes reduced differentiated and melanocyte stem cells during hair cycles and resulted in early hair graying, indicating a critical role for Opa1 in melanocyte stem cell survival. We highlight the essential role of Opa1 in the melanocyte compartment and propose that Opa1 is a key player in melanocyte stem cells.

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ATAD3B knockdown decreases human embryonic stem cells proliferation rate without any influence on the cell's pluripotency, mitochondrial morphology, and volume density.

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ATPase family AAA domain-containing protein 3B (ATAD3) is a mitochondrial inner membrane protein specifically expressed in human embryonic stem cells (hESCs). Previous studies have shown that the differentiation of hESCs coincides with the loss of ATAD3B expression and the expression of the pluripotency marker OCT4. Our study aimed to investigate the role of ATAD3B in regulating hESCs' stemness and metabolism. To address this, we generated an ATAD3B knockdown stable cell line from H9 hESCs. Through qPCR analysis, we found no differences in the expression of pluripotency markers (*OCT4, NANOG, ZNF398, PRDM14*) between the knockdown and control cell lines. Similarly, there were no differences observed in the expression of OCT4 and NANOG proteins between the cell lines. Furthermore, differentiation of the cell lines into ectoderm, mesoderm, and endoderm did not reveal any influence of ATAD3B knockdown on the expression of specific differentiation markers (*PAX7, TBXT*, and *SOX17*, respectively). However, ATAD3B knockdown was found to decrease the proliferation rate of H9 cells, indicating an effect on cellular metabolism. Electron microscopy analysis showed no differences in mitochondrial morphology (mitochondrial area, aspect ratio, form factor), and western blot and qPCR analyses revealed no differences in the expression of OXPHOS genes (NDUFS1, NDUFA1, SDHA, UQCRC2, ATP5A, ND5, CYTB, ATP6). Additionally, qPCR analysis of glycolysis enzymes demonstrated a decrease in the expression of *LDHA* and *MCT4*, suggesting a potential impact of ATAD3B knockdown on lactate production and transport. In conclusion, our findings suggest that ATAD3B knockdown does not affect hESCs' pluripotency or mitochondrial volume density and morphology. However, it does decrease the proliferation rate, possibly affecting the cells' ability to utilize lactate.

Role of mitochondrial protease Lonp1 in the regulation of mitophagy

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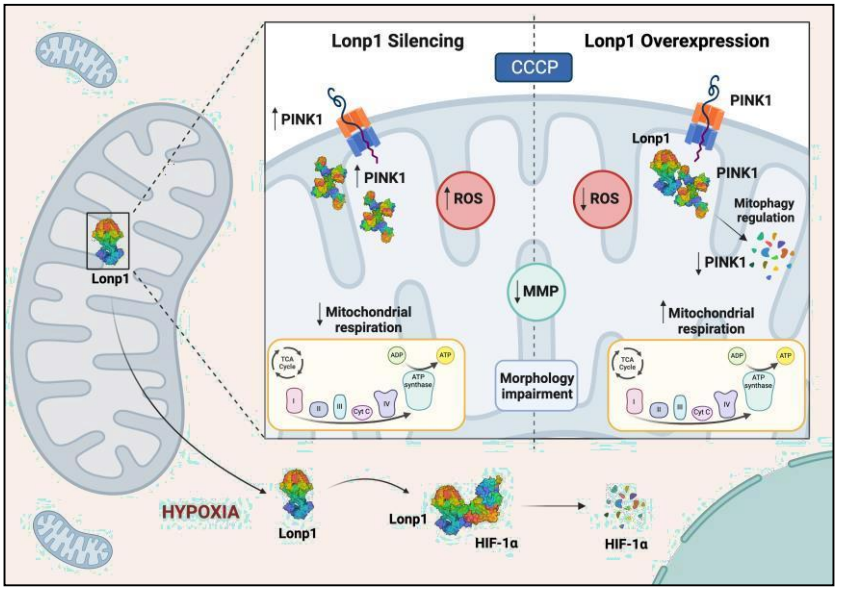
Lonp1 is a mitochondrial protease encoded by nuclear DNA essential for organelle homeostasis and cell response to stress. Lonp1 is induced by several stress stimuli, such as, heat shock, oxidative stress, and hypoxia, and could promote cancer cell survi val favouring cell resistance to stress. Several authors, including us, have previously shown that Lonp1 could play, directly or indirectly, a role in regulating mitophagy. Since HIF-1□enhances Lonp1 expression, we investigated the possible role of Lonp1 in regulating mitophagy in cancers and particularly under hypoxic conditions, often observed in solid tumours. Silencing of Lonp1 increases PINK1 levels and localization of PINK1 on mitochondrial membrane in the presence of CCCP, a known mitophagy inducer, while overexpression has the opposite effect; Lonp1 colocalizes with PINK1 and physically interacts with it. No changes were observed in other mitophagic pathways such as those mediated by ULK1 and FUNDC1. Hypoxia does not alter these effects in none of the conditions tested, excluding a possible role of Lonp1 in mitophagy-mediated adaptation to hypoxia. We interestingly observed that Lonp1 overexpression causes a reduction of HIF-1□ levels in hypoxic conditions; co-immunoprecipitation followed by proteolytic assay showed that Lonp1 can contribute to HIF-1□ degradation. The absence of Lonp1 and the induction of mitophagy by CCCP causes a complex alteration of mitochondrial dynamics proteins, increase in ROS levels and MMP reduction, and mitochondrial morphology impairment. Moreover, Lonp1 overexpression or silencing significantly modifies mitochondrial respiration. In conclusion, our study highlighted the involvement of Lonp1 in the regulation of mitophagy, also in a hypoxic environment. In particular, we showed modifications in mitophagic pathways activation, mitochondrial turnover and morphology, ROS production and mitochondrial respiration when Lonp1 is silenced or upregulated.



Metabolic control and fuelling of smouldering neuroinflammation

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There are currently no approved therapies to slow down the accumulation of neurological disability that occurs independently of relapses in multiple sclerosis (MS). International agencies are engaging to expedite the development of novel strategies capable of modifying disease progression, abrogating persistent CNS inflammation, and support degenerating axons in people with persistent inflammation of the, such as that occurring in progressive MS. Understanding why regeneration fails in the progressive MS bra in and developing new regenerative approaches is a key priority for the Pluchino Lab.

We aim to elucidate how the immune system, in particular its cells called myeloid cells, affects brain structure and function under normal healthy conditions and in disease.

Our objective is to find how myeloid cells communicate with the central nervous system and affect tissue healing and function al recovery by stimulating mechanisms of brain plasticity mechanisms such as the generation of new nerve cells and the reduction of scar formation.

Applying combination of state-of-the-art single cell analyses, and molecular approaches to study rodent and human disease models of inflammation and neurodegeneration, we aim to develop experimental molecular medicines, including those with stem cells and gene therapy vectors, which slow down the accumulation of irreversible disabilities and improve functional recovery after progressive multiple sclerosis.

By understanding the mechanisms of intercellular (neuro-immune) signalling, chronic diseases of the brain and spinal cord that include progressive MS may be treated more effectively, and significant neuroprotection and restoration of key functions may be achieved with new tailored molecular therapeutics.

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Disclosure

SP is founder, CSO and shareholder (>5%) of CITC Ltd and Chair of the Scientific Advisory Board at ReNeuron plc.

The mitochondrial aspartate/glutamate carrier does not transport GABA

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ɣ-aminobutyric acid (GABA) is a well-known inhibitory neurotransmitter in both invertebrate and vertebrate nervous systems. Its cellular metabolism involves well-defined compartments and its uptake from the cytosol into the mitochondrial matrix is required for degradation [1]. Previous studies carried out in *Drosophila melanogaster* suggested a putative role of the mitochondrial aspartate/glutamate carrier (AGC) in promoting GABA accumulation into mitochondria [2]. Here, we examined GABA transport catalyzed by human and *D. melanogaster* AGC proteins using a well-established method for assessing substrate specificity and kinetic parameters of mitochondrial carriers.

In this experimental system, the *D. melanogaster* spliced AGC isoforms (Aralar1-PA and Aralar1-PE) and the human AGC isoforms (AGC1/aralar1 and AGC2/citrin) are unable to transport GABA both in homo- and in hetero-exchange with either glutamate or aspartate, i.e. the canonical substrates of AGC. Moreover, GABA does not inhibit the exchange activities of the investigated AGCs. Our results demonstrate that AGC does not transport GABA and the molecular identity of the GABA transporter in human and *D. melanogaster* mitochondria remains unknown.

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Exploring the contribution of cysteine residues on VDAC3 biogenesis and maturation

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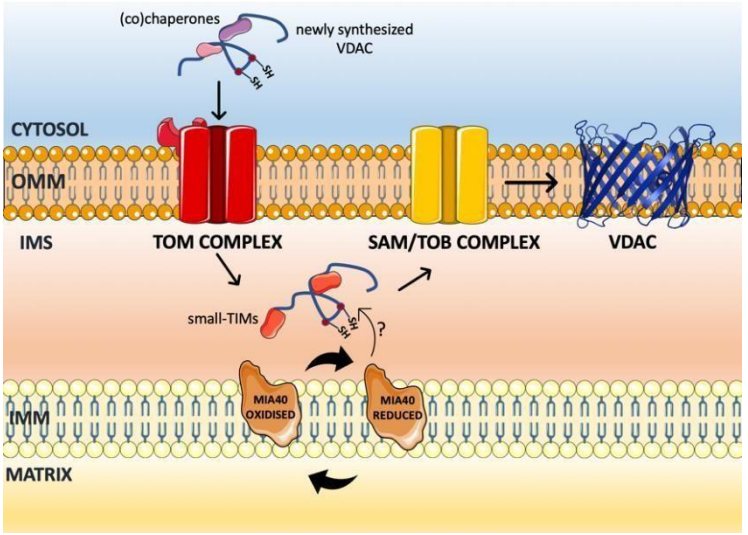
The Voltage-Dependent Anion-selective Channel (VDAC) family dominates as the primary pore-forming proteins in the outer mitochondrial membrane (OMM), regulating ion and metabolite flow within the mitochondrion. Among humans, VDAC3 boasts six cysteine residues with variable redox states, unlike VDAC1, which features only two cysteines. Recent findings from our group indicates the critical role of VDAC3's cysteine residues, exposed to the intermembrane space (IMS), in mitigating ROS-induced oxidative stress. Mass spectrometry analysis uncovered VDAC3's ability to form both intra- and intermolecular disulfide bridges, prompting further exploration of their potential biological functions. To that aim, we investigated the role of cysteines residues in the biogenesis of VDAC3 and VDAC1.

To monitor the biogenesis, in vitro import assays of radiolabeled ^{35}S-VDAC3 and ^{35}S-VDAC3-cysteineless were performed, while ^{35}S-VDAC1 and ^{35}S-VDAC1 cysteineless were used for comparison. The import kinetics of the radiolabeled proteins were investigated using mitochondria isolated from *S. Cerevisiae* cells. Since the Mia40 pathway facilitates disulfide bond formation of newly synthesized IMS proteins, we asked if the disulfide bridges of VDAC3 might be catalyzed by Mia40. Therefore, we compared import into control organelles to those isolated from *MIA40* mutant strain. Our results show that VDAC3 biogenesis is not affected by mutating Mia40, and surprisingly the ^{35}S-VDAC3 cysteineless variant is better imported into control mitochondria as compared to the native ^{35}S-VDAC3. Moreover, in vitro import assays followed by BN-PAGE highlighted that cysteineless VDAC3 forms mature complexes faster than the native protein. Overall, our results suggest that the cysteine residues of VDAC3 slow down its biogenesis and maturation.



The mitochondrial Aspartate-Glutamate carrier is a metabolic sensor orchestrating organelle morphology and cristae maintenance

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Mitochondria respond to cellular changes by undergoing fusion and fission events that control their shape, size, and number inside the cell. However, the molecular mechanisms that link cellular metabolism and mitochondrial dynamics are not fully elucidated. Through a MS-based complexomic screening[1] we identified the Ca^{2+}-sensitive mitochondrial aspartate-glutamate carrier (Agc1) as a potential interactor of Opa1, a mitochondrial shaping-protein essential for mitochondrial fusion and cristae maintenance[2]. Agc1 is part of the malate-aspartate shuttle (MAS) that upon dimerization efficiently delivers glycolysis-derived NADH to mitochondria. By profiling the dimerization status of Agc1 in response to individual carbon sources, we found that Agc1 dimerizes when cells are fed with high levels of glucose, suggesting that Agc1 dimerize when levels of glycolysis derived NADH are high. Agc1 ablation resulted in the expected decrease in mitochondrial NADH accumulation in high glucose media, membrane potential dissipation and decreased respiration in starvation. Reintroduction of dimerization-deficient and Ca^{2+}-insensitive mutants of Agc1 in Agc1-/- cells highlighted the functional Agc1 requirements to sustain mitochondrial respiration, membrane potential and cell viability. Starvation of Agc1-/- cells also resulted in the rapid disruption of cristae ultrastructure, accompanied by reduced Opa1 oligomerization, suggesting that Agc1 is required for starvation induced Opa1 oligomerization and cristae biogenesis. Our data reveal a previously unappreciated role for Agc1 in relaying changes in fuel availability to mitochondrial cristae via its dimerization and interaction with Opa1.

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Intracellular accumulation of amyloid- peptide impairs VDAC1 activity and mitochondrial functionality in Alzheimer's disease

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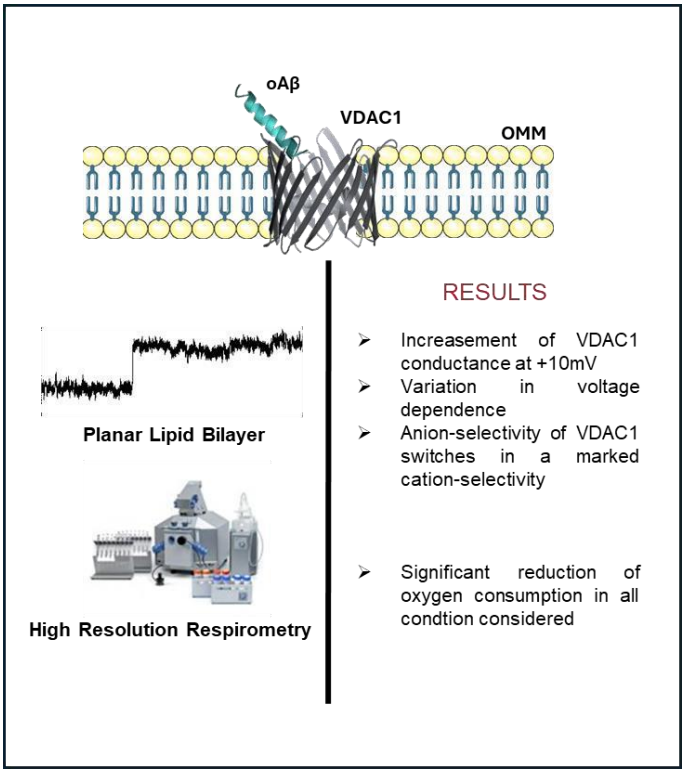
The Voltage-Dependent Anion-selective Channels (VDACs) are a family of pore-forming proteins located in the outer mitochondrial membrane (OMM) of eukaryotes, that allow the exchanges of ions and small metabolites, including ADP/ATP, between cytosol and mitochondria. In Alzheimer's disease, VDAC1, the main VDAC isoform, acts as the principal mitochondrial binding site for amyloid-β (Aβ) peptide, the major molecular hallmark of the disease. It has been established that the accumulation of Aβ peptide on the OMM surface increases the mitochondrial dysfunction and the oxidative stress. Having VDAC1 peculiar voltage-gating properties, that are relevant for the mitochondrial functions, here we analyze in detail the effect of $A\beta_{1-42}$ oligomers (oAβ) on VDAC1 electrophysiology at the Planar Lipid Bilayer. In physiological conditions, VDAC1 exhibits a conductance of ~3.5 nS in 1 M KCl, a voltage-dependent transition from open to closed state, and a selectivity for anions. In the presence of oAβ, however, we observed an increase of the VDAC1 conductance at the applied potential of +10 mV and a variation in the voltage-dependency. More interestingly, the anionic selectivity switched towards a marked selectivity for cations, as the possible results of a distortion of VDAC1 barrel structure embedded in biological membranes in the presence of oAβ. In addition, we evaluated the respiratory profile of permeabilized neuroblastoma cell line SH-SY5Y exposed to Aβ. Precisely, we found a significant reduction of the basal oxygen consumption and the maximal respiratory capacity, as well as of the oxygen flow linked to the ADP phosphorylation. In conclusion, our results reinforce our knowledgments about the VDAC1-Aβ molecular interplay, sheding a light on the future development of VDAC1-antagonist molecules able to counteract the mitochondrial accumulation of Aβ peptide.



The amino acid transporter LAT1 (SLC7A5) switches from antiporter to uniporter in presence of Cu-histidinate complex

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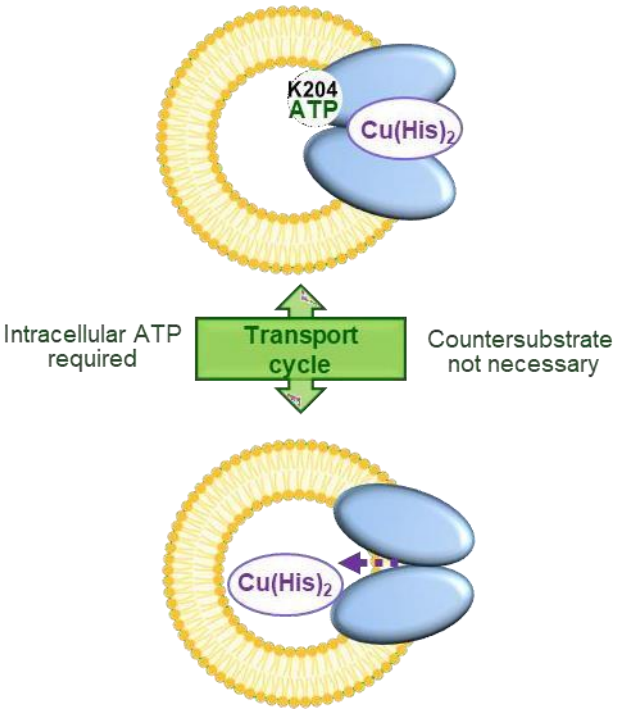
L-type amino acid transporter 1(LAT1) forms a functional heterodimer with the glycoprotein CD98 (SLC3A2). LAT1 is mainly expressed in critical body districts such as the blood-brain barrier (BBB) and placental barrier where it mediates the flux of essential amino acids, with an antiport mechanism. LAT1 over-expression has been described in virtually all human cancers. Thus, LAT1 is currently exploited as a pro-drug vehicle and as a target for anti-cancer therapies. Moreover, LAT1 is highly sensitive to inhibition by inorganic mercury, due to the interaction with cysteine residues. Considering that mercury is an environmental pollutant dangerous for brain and fetus development, we decided to test the interaction of LAT1 with several heavy metals and, surprisingly, we have found that while most heavy metals inhibit the transport, copper strongly activates LAT1 mediated histidine uptake in proteoliposomes. [1] Thus, we employed a combined approach of *in silico*, *in vitro*, and *ex vivo* technology to demonstrate that LAT1 can mediate the flux of Cu^{2+}-histidinate, with an unconventional uniport mechanism.



Given that LAT1 is expressed in the BBB and in cancer cells, the LAT1-mediated transport of $Cu(His)_2$ may have profound therapeutic implications: i) for the treatment of copper imbalance disorders, such as the rare Menkes Disease, ii) in the design of novel anti-cancer drugs, based on $Cu(His)_2$ scaffold, to overcome side effects of Pt-based therapies. [2]

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Riboflavin transporter 2 dimerization and stress responses in RTD2 (OMIM #614707)

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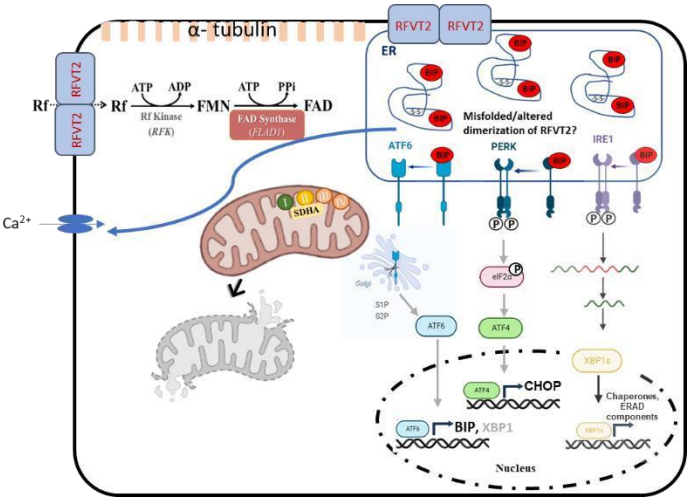
The vitamin B2, or Riboflavin (Rf), in its active forms, FMN and FAD, provides important functions in bioenergetics, ROS balance and so on. Rf transport is mediated by three members of the Solute Carrier family 52 (or *RFVT1-3*), which exhibit tissue specificity. *RFVT2* is ubiquitously expressed, but holds particular significance for brain [1]. Biallelic pathogenic variants in this gene are causative of a rare inherited neurodegenerative disorder, namely RTD2 (OMIM #614707), treatable with high doses of Rf (https://curertd.org/what-is-rtd/treatment/).

Despite the essential role of RFVT2 in neuronal economy, a comprehensive functional characterization of the RFVTs is far from reaching. We addressed this issue by producing WT and mutant recombinant proteins and reconstituting them in proteoliposome systems for transport assays [2].

Here we go ahead investigating a potential homodimerization of the protein, as suggested by [3]. The conclusive demonstration was achieved through the GPCA assay. Interestingly, certain mutants exhibit diminished dimerization capability.

The impact of RFVT2 mutation was also studied in patients' fibroblasts and iPSC-derived motor neurons. We unveiled altered mitochondrial morphology and Ca^{2+}-mediatedresponses as well as a reduced content of tubulin. Notably, in both cell models, UPR^{ER} sensor levels increased, presumably due to altered protein folding/dimerization. A chaperone-based therapy might be recommended in RTD2.



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3D structure of the ABC transporter MRP2 by Cryo-EM. Insights Post-translational modifications

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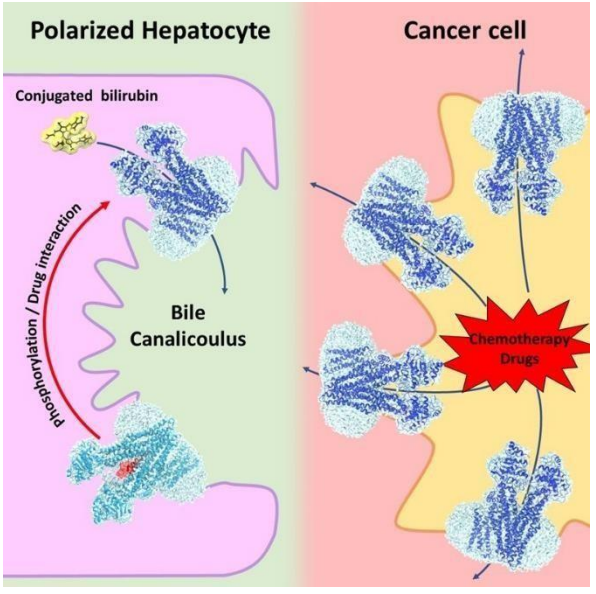
Multidrug Resistance-Associated Protein 2 (MRP2/ABCC2) is a transporter belonging to the ATP Binding Cassette (ABC) family. It plays a pivotal role in the cellular efflux of organic anions, including drugs and bilirubin glucuronides, with its dysfunction leading to jaundice and Dubin-Johnson syndrome. Moreover, MRP2 has been identified as the determinant in anticancer therapy resistance, mediating efflux of many anticancer drugs such as paclitaxel and cisplatin. We here present the structures of rat Mrp2 in its autoinhibited state and probenecid-bound obtained by cryo-EM at 3.21Å and 3.45 Å resolution respectively. The structure was solved by splitting the AlphaFold predicted model (AF-Q63120-F1) into 5 domains, each domain was individually fitted into the obtained EM-density map using UCSF ChimeraX. The model was subjected to iterative cycles of real-space refinement in phenix and manual rebuilding in COOT. The autoinhibited conformation reveals a unique regulatory domain arrangement within the transmembrane domain cavity, restricting transporter activity. Phosphorylation of S922 and S926 triggers a significant increase in transport activity, measured by in vitro phosphorylation studies, mass spectrometry, and functional assays in proteoliposomes using the fluorescent substrate 5(6)-Carboxy-2′,7′-dichlorofluorescein. The probenecid-bound structure uncovers two distinct drug-binding sites, providing a structural basis for the drug's modulatory effects on MRP2 activity. The proposed work underscores the critical role of structural modifications and kinase-mediated phosphorylation in determining MRP2's activity, offering potential avenues for therapeutic intervention in drug resistance and transport-related disorders.



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Role of MRP6 transporter in hepatic cells.

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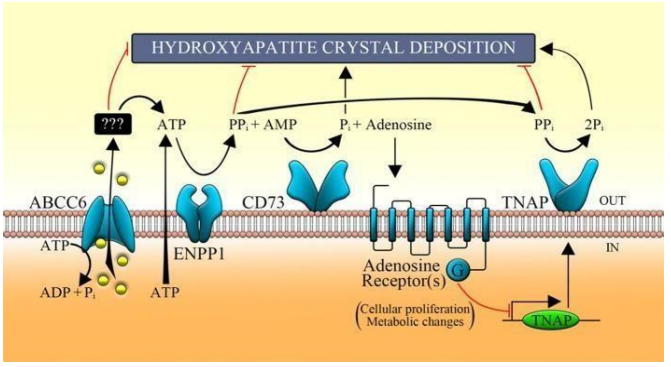
ABCC6 is an ATP-dependent transporter mainly expressed in the basolateral plasma membrane of hepatic and kidney cells, containing three transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). ABCC6 deficiency causes Pseudoxanthoma elasticum (PXE), a rare and multisystemic disorder characterized by calcification of elastic fibers in dermal, ocular and vascular tissues [1]. The exact nature of ABCC6 substrate is unknown, but it has been reported that ABCC6 facilitates the cellular efflux of ATP, which is rapidly converted by the ectonucleotidases ENPP1 and CD73 into inorganic pyrophosphate and adenosine, both inhibitors of calcification. PXE presents similarities with ACDC and GACI, disorders caused by mutations in CD73 and ENPP1 genes, respectively [2]. To shed light on ABCC6 functional role in hepatic cells, we investigated the effects of stable ABCC6 knockdown in HepG2 cells. Firstly, we demonstrated a direct relationship between reduced ABCC6 levels and the expression of genes involved in mineralization processes, showing an upregulation of genes promoting mineralization, such as TNAP, and a parallel downregulation of genes with anti-mineralization activity, such as CD73, Fetuin A and Osteopontin. So, we further characterized the cellular phenotype of ABCC6 knockdown HepG2 cells, demonstrating that these cells show typical features of replicative senescence, a cytoskeletal rearrangement and a reduction in cell motility. Although PXE does not affect the liver, our results demonstrated that ABCC6 deficiency causes profound changes in hepatic cells.



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ABCC6 transport activity alters the adhesion dynamics and aggressiveness of HepG2 cells

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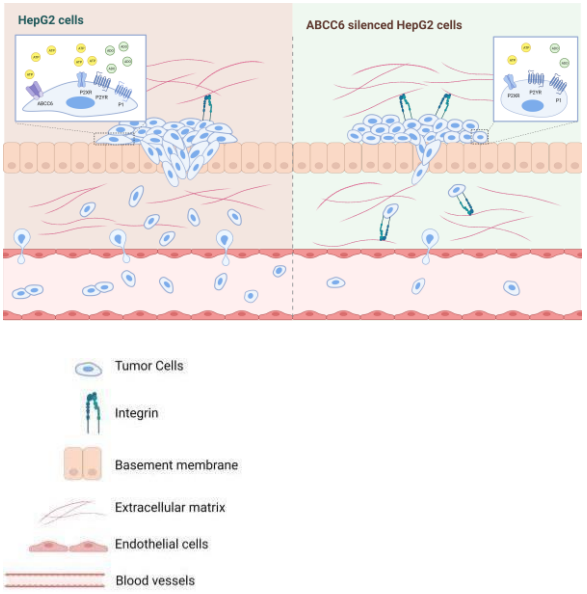
The human Abcc6 gene belongs to the multidrug resistance-associated protein (MRP) subfamily of ATP-binding cassette (ABC) transmembrane transporters and is mostly expressed in the liver and kidney. The ABCC6 transport along with the ectonucleosidases CD73 (ecto5′nucleotidase) and CD39 (ectonucleoside triphosphate diphosphohydrolase1) produce purine nucleosides involved in the Purinergic Signalling, a cell-cell communication system that affect different physiological and pathological cellular functions including cancer [1].

RNAseq results show that Abcc6 knockdown increases HepG2 cell adhesion to extracellular matrix(ECM)-mimicking hydrogels through the up-regulation of ITGA2 and ITGA6. The up-regulation of the epithelial marker E-cadherin and the down-regulation of mesenchymal markers Vimentin and N-cadherin suggest that ABCC6 knockdown cells have a less aggressive phenotype. Transwell invasion assays demonstrate that Abcc6 knockdown reduces HepG2 cells ability to infiltrate the ECM, thus reducing the secretion of MMP2 and MMP9 active forms [2]. Adding adenosine and ATP restores tumor cell invasion, demonstrating that Abcc6 affects tumor aggressiveness via the extracellular purinergic pathway. ABCC6 knockdown in HepG2 cells appears to induce a less aggressive phenotype, which may be beneficial for cancer treatment.



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Molecular basis for polyspecificity of the Organic Cation Transporter (Novel) OCTN1

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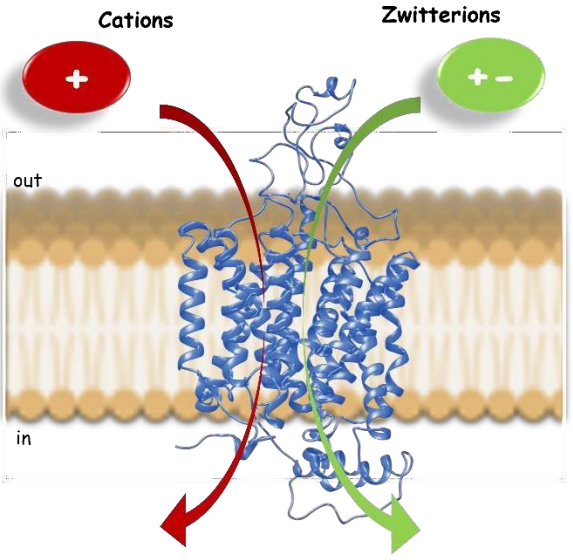
OCTN1 is a plasma membrane transporter encoded by the SLC22A4 gene. The most well described substrates of OCTN1 are some organic cations, such as the non-physiological substrate TEA (tetraethylammonium) and the neurotransmitter acetylcholine, but also zwitterions, such as carnitine and the mushroom metabolite ergothioneine. Moreover, OCTN1 is also recognized as drug transporter. The molecular bases of the described polyspecificity, as well as the actual physiological role of OCTN1, remains limited. Therefore, in this work, we deepened OCTN1-mediated uptake mechanisms of two model substrates: the cation TEA and the zwitterion carnitine. We employed a combined strategy of *in vitro* transport assays and *in silico* prediction of TEA and carnitine docking. Transport assays revealed that TEA and carnitine did not inhibit each other. Moreover, [^{14}C]-TEA uptake was strongly inhibited by external Na^+, whereas [^{3}H]-Carnitine uptake was stimulated by 75 mM of external Na^+. The homology model of OCTN1, built using the human OCT3 Cryo-EM structure as a template, was subjected to docking analysis and molecular dynamics. Computational data showed that TEA, Na^+, and carnitine interact with the residue E381 in the OCTN1 substrate binding site. Interestingly, in the presence of Na^+, carnitine can interact with the binding site via the R469, whereas TEA is displaced by Na^+. Taken together, experimental and computational evidences support the existence of two distinct transport pathways for cations and zwitterions. The results help to get further insights into the structure/function relationships, shedding new light on thecomprehension of OCTN1 role in physiological and pathologicalconditions, ranging from the non-neuronal cholinergic system, to senescence, inflammatory diseases, and cancer. Furthermore, the description of the molecular determinants for substrate binding can be exploited for drug design and drug screening [1].



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Overproduction and Purification of the SLC7a14 transporter in a functionally active form.

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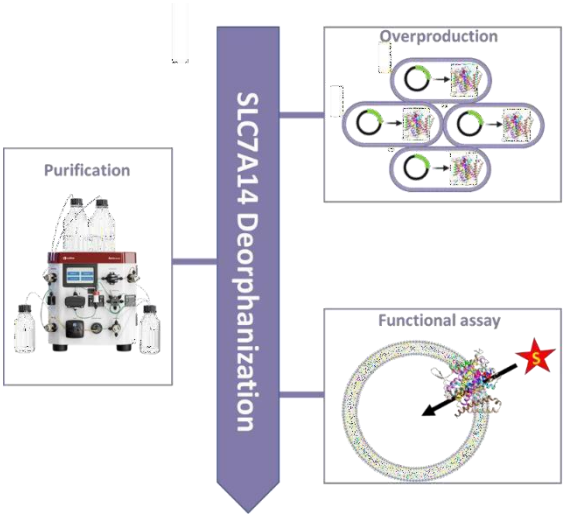
SLC7A14 is an uncharacterized membrane transporter that belongs to the CAT subgroup of the SLC7 family [1]. Like the other members of CAT, it is a probable cationic L-amino acid transporter with 14 predicted transmembrane domains and a cytoplasmatic loop, which is the longest of this subfamily [2-4]; consequently, SLC7A14 has a molecular mass of about 84.05 kDa which is the highest within the family. It is highly expressed in mammalian cochlear inner hair cells, retinal photoreceptors, subcortical regions of the brain, and at lower levels in human skin fibroblasts [5]. Mutations of SLC7A14 are associated with autosomal recessive retinitis pigmentosa and auditory neuropathy [2]. Despite its involvement in human pathologies, the function of SLC7A14 remains unclear. Therefore, we dedicated our efforts to identifying a strategy to produce the human recombinant SLC7A14 transporter in a functionally active state for assaying the activity in proteoliposomes. With this aim, the cDNA of SLC7A14 cloned into the bacterial expression vector pH6EX3 was used to transform the *E. coli* Rosetta strain. After investigating the expression conditions, the transporter's overexpression was achieved by incubating the bacterial culture with 0.4 mM IPTG for 6 hours. The recombinant protein was then purified by Ni-affinity chromatography and used for in-vitro transport assays in proteoliposomes using radioactive substrates. Our preliminary studies have shown that human recombinant SLC7A14 can catalyze transport of basic amino acids. In conclusion, we obtained the functional recombinant SLC7A14 that will allow us to shed light on the transporter's function!



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Generation and initial characterization of STACCATO, a family of Split-FAST Mitochondria-ER Contacts probes to measure contact sites of different widths.

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Endoplasmic reticulum (ER) physically contacts mitochondria via its specialized subdomain called mitochondria-associated membranes (MAMs) at sites of mitochondria-ER contacts (MERCs). Interaction between these organelles at the MERCs plays essential roles in lipid and calcium transfer and ultimately in the homeostasis of the two individual organelles. Since the width of the MERCs could be as narrow as 10 nm, which is below the diffraction limit, a handful of probes have been developed to evaluate MERCs formation and dynamics. However, the current probes are dim, or artificially induce tethering, or require complex imaging procedures that are not compatible with the common imaging setups available to most of the labs. To circumvent these problems, we are developing “STACCATO”, a new generation of probes to visualize MERCs that are based on split Fluorescence-Activating and absorption-Shifting Tag (FAST). STACCATO capitalizes on the reversible nature of splitFAST complementation so that the probe itself does not work as an artificial tether. We report the generation and the initial characterization of a series of STACCATO probes for MERCs of different width. In STACCATO, one half of the probe is anchored to the outer mitochondrial membrane and the second half to the surface of the endoplasmic reticulum via designer rigid linkers. STACCATO can be expressed in human and mouse cells, its moieties are targeted to the two organelles of interest and its fluorescence can be reconstituted by the perfusion with the fluorogens 4-hydroxybenzylidene-rhodanine (HBR, Lime) or 4-hydroxy-3-methylbenzylidene-rhodanine (HMBR, Coral). The backbone of STACCATO incorporates a rapamycin-induced dimerization domain, allowing maximization of its fluorescence of STACCATO increases when cells are pulsed with rapamycin. Thus, we report the initial characterization of a splitFAST-based probe to measure proximity between mitochondria and ER in microscopy and flow cytometry.

OPA1 processing regulates mitochondrial outer-inner membranes contacts and the TIM23 protein import complex

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Contacts between the mitochondrial inner (IM) and outer (OM) membranes participate in biogenesis of nascent proteins imported from the cytosol as well as in transduction of signals that need to reach the inner membrane from the extramitochondrial space. Our knowledge of the mediators of IM-OM contacts is scant, besides the roles for the interaction between TOM40 and TIM23 complexes and of the mitochondrial intermembrane space bridging (MIB) complex formed between the SAM and MICOS complexes. Here we show that processing of the mitochondria-shaping protein OPA1 controls the extent of contacts between OM and IM. Reintroduction of a mutant of OPA1 that is not processed into its short (S) form in an Opa1-/- cell line resulted in decreased OM-IM juxtaposition but not in cristae tightness, suggesting that OPA1 processing is required for IM-OM contacts. These ultrastructural changes were paralleled by defects in MICOS, MIB and the TIM23 import complex. This import pathway accounts for the import of most matrix proteins and is essential for mitochondrial biogenesis and respiration. Indeed, growth of cells lacking S-OPA1 was impaired. Our data indicate a role for OPA1 processing in the stability of the mitochondrial protein import complex TIM23, linking the core mitochondrial dynamics machinery to mitochondrial biogenesis.

The relationship between mitochondrial fission modes and mitochondrial DNA distribution

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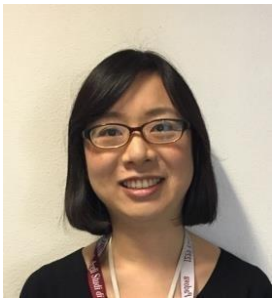
Mitochondrial fission that is essential for the distribution of mitochondrial DNA (mtDNA) among organelles can be further classified into “midzone” and “peripheral” fission. Daughter mitochondria originating from midzone division possess replicating mtDNA, whereas the mtDNA in the smaller daughter mitochondria from peripheral division is usually non-replicating. However, how the state of mtDNA replication is sensed to sort replicating/non-replicating mtDNAs to different daughter mitochondria remains unclear. My PhD project wishes to address these questions. We will first generate cell models characterized by a relative increase in either of the two distinct fission modes and expressing chimeras between the mtDNA polymerase POLG and the peripheral nucleoid factor TFAM with TurboID for identification of interactors. These different cell lines will be used to identify by proteomics the potential mtDNA sensing factors during the two modes of fission. After identifying the candidates, we will use orthogonal assays of candidate deletion by CrispR/Cas9 and siRNA silencing to confirm their function in replicating/non replicating mtDNA sorting. My project is expected not only to enhance our understanding of the connection between mitochondrial fission and mtDNA distribution, but also to identify new potential therapeutic targets for mitochondria-associated diseases.



Genetic and proteomic screening disclosed the molecular machinery of the mitochondria-endoplasmic reticulum contact sites

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Mitochondria contacts with endoplamic reticulum (ER) keeping proper distance at the mitochondria-ER contacts (MERCs) through poorly identified proteinaceous bridges. To provide a molecular MERCs atlas, we performed a genome wide shRNA screening coupled with ratiometric imaging analysis of a FRET ER-mitochondria proximity probe (FEMP) and iBAQ (intensity Based Absolute Quantitation) proteomics of mitochondria associated membrane (MAM) fraction. Automated imaging and plate- and genome-wise statistical analyses of the screening iterations yielded 107 gene candidates classified as tethers (i.e., genes whose ablation increases ER-mitochondria distance) including known mammalian tethers like Vamp and Mfn2; and 97 Spacers genes (i.e., genes whose ablation decreases ER-mitochondria distance). Gene candidates were enriched in calcium signaling, lipid biosynthesis and metabolism, processes known to concentrate in this interface. By crossing this list to the proteome of MERCs determined by iBAQ analysis we refined it to 25 spacers and 18 tethers. Among these potential tethers was interferon gamma induced GTPase (IGTP), a gene involved in autophagosome formation and immune response that indeed resided at MERCs as indicated also by biochemistry and superresolution microscopy. Orthogonal assays of mitochondria-ER juxtaposition confirmed that IGTP deletion increases the distance between the two organelles. Functionally, efficient transfer of ER Ca2+ to mitochondria also requires IGTP. Hence, IGTP satisfies the requirements to be considered a bona-fide MERCs regulator, highlighting the power of our screenings in the identification and functional annotation of MERCs components.

Gdap1l1

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Mitochondrial fusion and fission are essential processes to sustain mitochondrial bioenergetics and ultimately health of the cellular mitochondrial population. While the core components of these two processes are known, whether additional molecules participate in them is unclear. We therefore performed a high content imaging, RNAi genome wide screening to identify factors that once ablated promote elongation of mitochondria lacking the master mitochondrial fusion protein Opa1. Our screening identified a handful of potential novel regulators, including Gdap1l1, the paralogue of ganglioside-induced differentiation- associated protein 1 (Gdap1). Gdap1l1 displays 55% sequence identity with Gdap1 with which it shares conserved hydrophobic and he transmembrane domains. Gdap1 participates in mitochondrial fission through interacting by actin-depolymerization protein cofilin1 [1]. Mutation in Gdap1 cause Charcot-Marie-Tooth (CTM) type 4A and are associated with mitochondrial dysfunction, leading to the degeneration of peripheral but not central neurons which compensate by facilitating Gdap1l1 translocation from cytosol to mitochondria in responseto increased level of oxidized glutathione [3]. We will present results characterizing the effects of Gdap1l1 silencing and overexpression on mitochondrial morphology and mitochondria fission.

Von Williebrandt Associated 8 modulates mitochindria-sarco/endoplasmic reticulum contacts in b- adrenergic agonist induced cardiac hypertrophy

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The role of mitochondria-sarco/endoplasmic reticulum contact sites (MERCs) in cardiac hypertrophy progression remains ambiguous. We show that in an HL-1 cardiomyocyte hypertrophy model, MERCs increase alongside heightened mitochondrial Ca2+ uptake, fragmentation, and autophagy activation. Prolonged exposure to the β-adrenoceptor agonist isoproterenol induced Nppb expression and cell hypertrophy, concomitant with MERCs expansion, mitochondrial fragmentation, calcium overload, and autophagy activation. Through integrative analysis of transcriptomic data from isoproterenol-infused mouse hearts (GSE195466) and an unbiased MERCs proteomic catalog, we identified 111 differentially expressed candidate genes encoding MERCs proteins in hypertrophic hearts. Notably, the MERCs protein VWA8, a mitochondria/peroxisome AAA ATPase, was downregulated in hypertrophic hearts. In isoproterenol-treated HL-1 cardiomyocytes, VWA8 formed large punctuated structures associating with fragmented mitochondria, endoplasmic reticulum, peroxisomes, and autophagosomes. Silencing VWA8 enhanced mitochondria-endoplasmic reticulum proximity. Thus, gene expression rewiring in β-adrenergic agonist-induced cardiac hypertrophy orchestrates MERCs augmentation, altering mitochondrial morphology and function.

Chasing the nM: Lead Optimization of novel MYLS22-derived OPA1inhibitors

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The mitochondrial Dynamin-like protein Optic Atrophy 1 (OPA1) is a small GTPase involved in mitochondrial fusion, cristae remodeling, cytochrome c release and apoptosis. OPA1 upregulation has been increasingly identified as an exploitable vulnerability in cancer cells. From a previous screening of >10,000 drug-like molecules for inhibition of OPA1 GTPase activity, we identified MYLS22 as a promising hit. MYLS22 is not mitochondriotoxic but causes mitochondrial fragmentation and cristae remodeling, enhancing cytochrome c release following proapoptotic stimuli, recapitulating the effect of OPA1 downregulation in cells. Following a SAR analysis of MYLS22, multiple possible derivatives have been identified with increased water solubility. Three of these derivatives showed enhanced inhibitory effects on OPA1 GTPase activity *in vitro* and caused mitochondrial fragmentation in cells. To identify hits with further enhanced potency, solubility and overall availability of the leads, we generated a virtual, combinatorial library of 81,000 compounds. We docked these compounds to the GTPase domain of the currently available structure of OPA1 (PDB: 6JTG) and performed a virtual screening to discover second generation OPA1 inhibitors. We synthesized the best hits and analyzed their potency as OPA1 inhibitors in vitro and in cells. Our work identifies specific and potent OPA1 inhibitors with the potential to treat cancers where OPA1 is upregulated.

RAP1 inhibition restores mitochondria elongation and lysosomal distribution downstream of OPA1 deletion

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Changes in core components of the mitochondria-shaping machinery trigger retrograde signaling pathways that remodel gene expression and influence cellular signaling cascades, like angiogenesis that is transcriptionally controlled by Optic Atrophy 1 (Opa1) [1]. However, the nature and the cellular effects of these retrograde signals is largely unknown. Here we show that in endothelial cells the Ras-associated protein-1 (Rap1) signaling cascade activated when Opa1 is deleted regulates mitochondrial morphology. Unbiased RNA sequencing of ECs where we genetically ablated Opa1 revealed activation of the Rap1 small GTPase pathway. Indeed, Rap1 localized in proximity of mitochondria. Surprisingly, Rap1 downregulation in ECs lacking Opa1 restored mitochondrial morphology without affecting the core fission mediator Drp1. Conversely, Rap1 inhibition restored the normal distribution of lysosomes that is instead skewed towards the perinuclear region in cells lacking Opa1. Thus, by exploiting Opa1 deletion as a tool to investigate mitochondria-nucleus retrograde signals we unveiled a hitherto unappreciated role for Rap1 in mitochondrial dynamics, perhaps by modulating mitochondria-lysosome contact sites and lysosomal priming of mitochondrial fission.

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The interactomes of the mitochondrial cristae-shaping protein Opa1 vary depending on the oxidative metabolism of carbon sources

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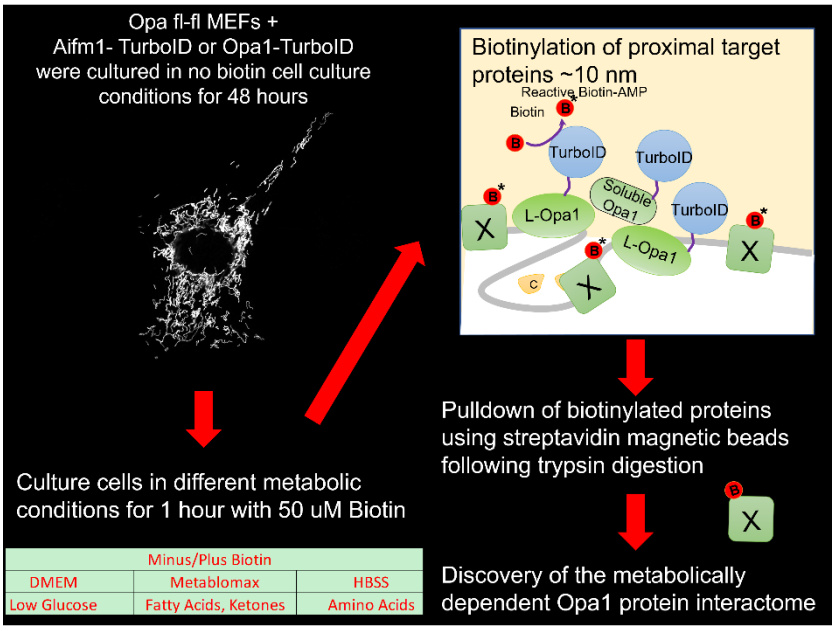
Mitochondrial cristae, the bioenergetic units of life, are dynamic compartments with distinct structures that contribute to respiratory efficiency, control of cytochrome c release and cellular proliferation. Optic Atrophy 1 (Opa1), a dynamin-related inner mitochondrial membrane (IMM) protein, is a central regulator of cristae shape that is retrieved in oligomeric complexes modulated by metabolic changes. Whether and how Opa1 connects fuel availability to cristae dynamics is unexplored. To address this question, we studied the Opa1 interactome upon changes in fuel availability. We generated an Opa1-TurboID (Opa1^{TID}) chimera that correctly localizes to the IMM, faces the intermembrane space, displays biotinylation activity, and lies in high molecular weight complexes of similar stoichiometry to Opa1. Once expressed in *Opa1*$^{-/-}$ cells, Opa1^{TID} restores mitochondrial ultrastructure and fusion, confirming it can vicariate untagged Opa1. We therefore used Opa1^{TID} to unbiasedly identify by label free proteomics the changes in the Opa1 interactome when *Opa1*$^{fl/fl}$ Murine Embryonic Fibroblasts stably expressing Opa1^{TID} were exposed to glucose, starvation, fatty acids, amino acids, or a complete cell culture medium mimicking human plasma. A pipeline of liquid chromatography-mass spectrometry detection of the biotinylated proteins identified 231 bona fide mitochondrial proteins as Opa1^{TID} interactors. A bioinformatic analysis indicated that unique proteins were significantly enriched in distinct metabolic conditions. This discovery of the carbon source-dependent Opa1^{TID} interactome indicates an axis between fuel availability and Opa1-mediated cristae dynamics and pinpoints metabolic enzymes that can relay individual fuel source to the cristae biogenesis machinery.



Unlocking sorafenib resistance: exploring the impact of mitochondria and metabolism in hepatocellular carcinoma

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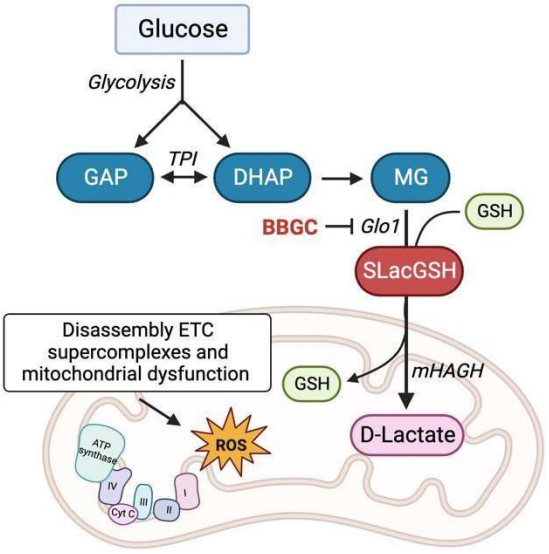
Hepatocellular carcinoma (HCC) is the most common type of primary liver malignancy worldwide often diagnosed in late stages with a median survival of less than a year. Current treatments, including the oral kinase inhibitor sorafenib (SOR), for advanced- stage HCC are unsatisfactory due to the onset of resistance. However, the biochemical and molecular mechanisms of SOR resistance remain unclear.

Here, we show that murine hepatoblasts with myc oncogene overexpression and p53-/- are resistant to SOR. We defined that SOR negatively affects mitochondrial function by acting as an uncoupling agent, disrupting electron transport chain (ETC) complexes, and altering ETC super-complex assembly. This causes reactive oxygen species (ROS) production within mitochondria. To counteract ROS production and continue to proliferate, SOR-resistant cells rewire their metabolism by activating the glyoxalase system to promote glutathione (GSH) shuttling into the mitochondria and the production of D-lactate.



Integrated data from transcriptomic and metabolomic profiles, along with metabolic tracing experiments, demonstrate that SOR-resistant cells increase glucose uptake for serine production. This serine is used in mitochondria by the one-carbon metabolism to generate enough NADPH for GSH regeneration and to promote purine biosynthesis.

Chemical and genetic inhibition of these key metabolic pathways engaged by SOR-resistant cells identify metabolic vulnerabilities that enhance sorafenib efficacy. Furthermore, analysis of SOR-resistant human hepatocyte cells (Huh7) and SOR-resistant patient samples revealed superimposable metabolic gene expression profiles identified in our SOR- resistant hepatoblasts, highlighting the role of these metabolic pathways in drug resistance.

Sapje zebrafish as a living platform to unveil the pathogenesis of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a severe X-linked disorder characterized by the absence of dystrophin, which leads to the progressive loss of muscle tissue. Several studies showed that mitochondrial dysfunction due to Ca^{2+} overload is the main cause of muscle fiber death triggered by the long-lasting opening of the permeability transition pore (PTP), a mitochondrial high-conductance channel sensitive to high Ca^{2+} levels. It has been demonstrated that PTP inhibition prevents mitochondrial dysfunction and muscle degeneration in both DMD patient cells and *sapje* myopathic zebrafish, a severe model of DMD [1, 2]. However, there is still a gap of knowledge about the early events linking the absence of the protein to Ca^{2+}-dependent muscle fiber death. We (i) generated *sapje* fluorescent biosensors to assess cell dynamics at the level of single muscle fibers in a living organism and (ii) performed RNA sequencing to identify differentially expressed genes at different stages of disease progression. RNAseq data from *sapje* zebrafish at 2 days post fertilization (dpf) showed a significant dysregulation of genes involved in Ca^{2+} homeostasis. At 5 dpf we observed dysregulation of genes involved in mitochondrial function, muscle contraction and differentiation. Significant dysregulation of Notch, Shh and Hif1α pathways, together with the impaired mitochondrial patterning and the severe macrophage infiltration, confirmed defects in muscle repair and differentiation. Treatment of *sapje* zebrafish with new triazole compounds that inhibit the PTP leads to a full rescue from dystrophic phenotype, showing a complete recovery of normal mitochondrial membrane potential and respiration, muscle structure, motor behavior, survival rate and signalling pathways activity. Identification of new druggable targets is key to perform new drug screenings and to develop novel compounds that can be used in combination with PTP inhibitors to slow down or arrest DMD progression.

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Mitochondrial and bioenergetic alterations of fibroblasts from ALS patients with A382T mutation of TDP-43

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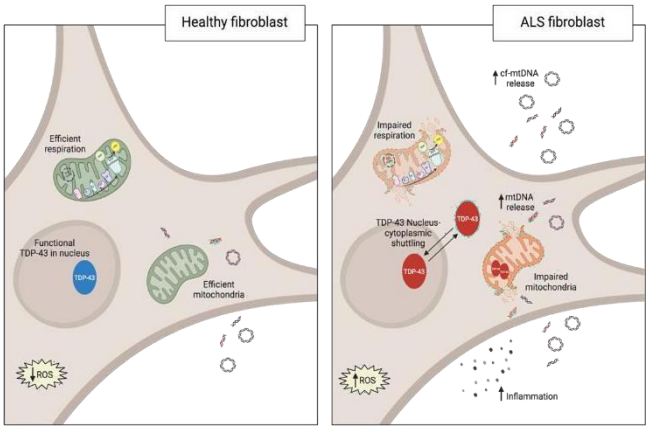
Amyotrophic lateral sclerosis (ALS) is a motoneuron disorder characterized by a progressive deterioration of both upper and lower motor neurons. TDP-43 is an RNA-binding protein encoded by TARDBP gene located on chromosome 1 whose mutations and pathological neuronal cytoplasmic ubiquitinated inclusions are associated with ALS with or without frontotemporal dementia (FTD). Alterations of mitochondrial (mt) morphology, metabolic activity, oxidative phosphorylation, and production of reactive oxygen species (ROS) was observed in ALS patients. Thus, we performed a morphological and functional analysis of mitochondria in human fibroblasts of ALS patient carrying A382T mutation in TARDBP gene. We set up a primary culture of fibroblasts from a skin biopsy of ALS patient and their characteristics were compared to those of fibroblasts from a healthy donor of matching sex and age. ALS fibroblasts were characterized by profound alterations of cell proteome, which impacts particularly the mitochondrial metabolic pathways and the endoplasmic reticulum. TDP-43 levels were similar to healthy fibroblasts, but a higher fraction localized in mitochondria. Mitochondrial network appeared fragmented, and the organelles smaller and more spheric. Mitochondrial respiration shows that basal respiration is lower in healthy fibroblasts than in ALS fibroblasts. Higher ROS production and a higher mitochondrial membrane potential were observed in ALS fibroblasts compared to control. MtDNA levels appeared normal, but a higher amount of mtDNA was present in the cytosol and in the supernatant, suggesting a pronounced mtDNA misplacement and extracellular release which can promote a proinflammatory response. This data confirms that mitochondria from ALS patients displays a wide range of functional and morphological alterations, but with a high degree of heterogeneity, indicating that the mechanistic links between mt impairment and ALS pathogenesis greatly vary on the basis of the genetic alterations.



Mitochondrial function analysis in bladder cancer cell line: targeting metabolism as a new therapeutic prospective

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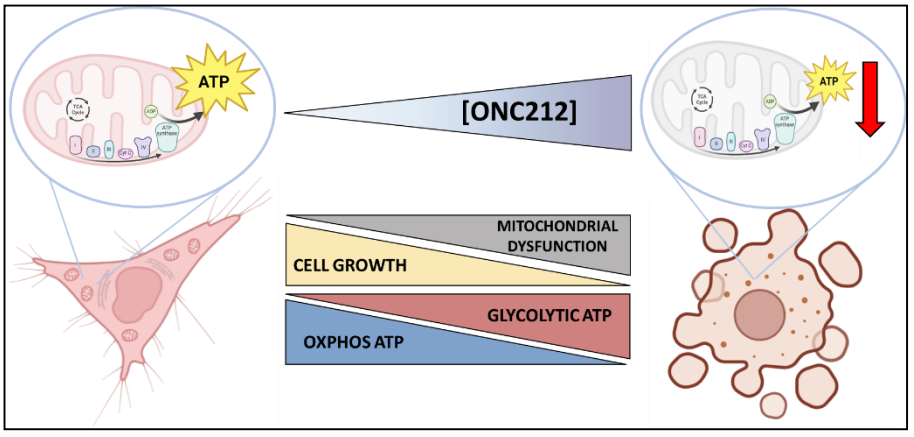
Bladder cancer (BC) refers to the malignant transformation of epithelial cells lining the inner surface of the bladder. BC-associated lesions can be classified as Non-Muscle Invasive and Muscle Invasive, with the latter characterized by an aggressive phenotype and poor prognosis[1]. As in most neoplasias, metabolic rewiring is one of the crucial step required for cancer progression and enhanced malignancy, and bladder cancer makes no exception, presenting with many modifications in bioenergetic pathways[2]. To better understand the molecular and metabolic alterations linked to tumour aggression, we adopted an in vitro model of the Low Grade phenotype BC cell line RT4, and the High Grade aggressive phenotype T24 cell line. Using a metabolic fluxes analyzer (SeaHorse technology) we characterized their metabolic phenotype, unveiling a more energetic profile in RT4 cell line as compared with T24 cell line. Moreover, confocal analysis with DCF and TMRE probes, revealed an increase in H_2O_2 content and a decrease in mitochondrial membrane potential in T24 as compared with RT4. Overall, our results highlighted a mitochondrial impairment in T24. Furthermore, western blot analysis revealed a significant reduction in OXPHOS proteins content, even if PCG-1α and SIRT1 were more expressed.

Interestingly, we found a differential expression of ClpP, a protease involved in mitochondrial quality control and protein homeostasis[3]. In this context, we used a pharmacological treatment with the ClpP activator ONC212, studying its effects on proliferation using real time monitoring of cell growth, as well as on mitochondrial respiration and on ATP production rates in both BC cell lines. As expected, we uncovered a chemo-resistant phenotype of T24 aggressive cell line, which is able to sustain cell growth despite the drug treatment, likely by enhancing ATP production through the glycolytic flux. The outcome of our study suggests that the impairment of mitochondrial function and metabolic switch toward glycolysis could account for tumour progression and chemoresistance in T24 High Grade BC cell line, although future studies are required to gain a better understanding of the underlying mechanisms involved.



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The metabolic setting of ovarian cancer cells modifies response to chemotherapy.

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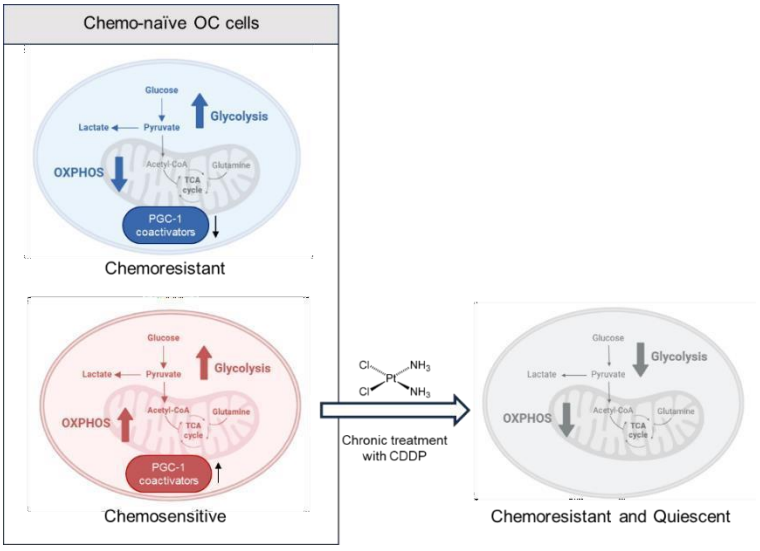
Epithelial ovarian cancer (EOC) is the most lethal and silent gynaecological malignancy. Despite initial response to conventional platinum- and taxane-based chemotherapy, 85% of treated patients develops chemoresistance and often relapses. Therefore, the understanding of the still elusive mechanisms leading to therapeutic resistance is a major challenge for the development of mo re effective treatments. In the last few years, metabolic reprogramming has emerged as crucial for tumour progression with growi ng evidence showing that EOC cells switch their metabolism between aerobic glycolysis and oxidative phosphorylation (OXPHOS) to sustain tumour proliferation, invasiveness, and chemoresistance. However, which metabolism is preferred by chemoresistant cells is still debated. Our analyses revealed that metabolically energetic chemo-naïve EOC cell lines, relying both on glycolysis and OXPHOS, were more responsive to platinum-based treatment, whereas purely glycolytic cells were chemoresistant. Indeed, we found higher abundance and activity of mitochondrial respiratory complexes in chemosensitive EOC cell lines. Such high-OXPHOS state correlated with the expression of PGC-1 family of transcriptional co-activators, the master regulators of mitochondrial biogenesis. In hypoxia, we observed an increase in chemoresistance that was prevented by HIF1α ablation. Importantly, the acquisition of cisplatin-resistance in two energetic cell lines induced a shift to a quiescent metabolism with the reduction of both glycolytic and OXPHOS function when compared to their syngeneic sensitive counterparts. Overall, our findings suggest that a high-OXPHOS condition is associated with chemosensitivity and upregulation of OXPHOS may be a possible therapeutic strategy to overcome chemoresistance.



Mitochondrial alteration and autophagy deregulation in Lipa$^{-/-}$ mice are rescued by a novel *in vivo* gene therapy treatment

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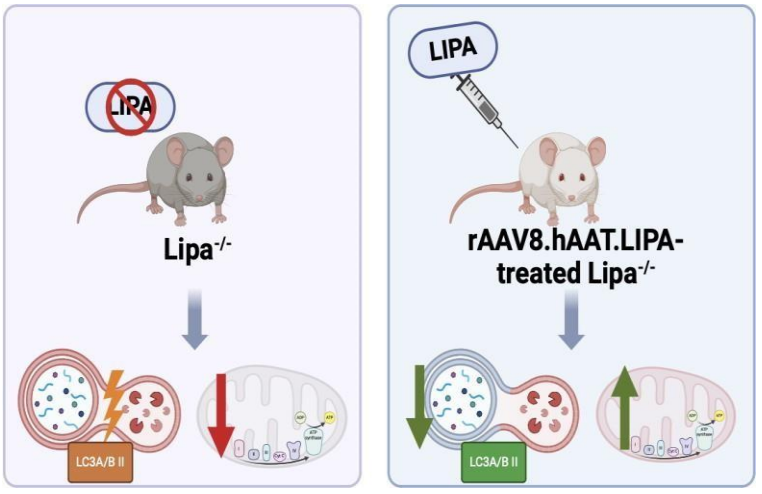
Lysosomal acid lipase deficiency (LAL-D) is an autosomal recessive disorder caused by mutations in the LIPA gene. Lysosomal acid lipase plays a central role in intracellular lipid metabolism since is the only lipase contained within lysosomes that hydrolyzes cholesterol esters and triglycerides into free cholesterol and fatty acids necessary for energy production, biosynthesis of cholesterol-derived compounds and for membrane formation [1]. Deficiency of LAL triggers fatty acids accumulation, leading to multi-organs failure, as observed in Wolman disease, a recessive autosomal genetic disorder [1].

In this study we investigated the mitochondrial function in WT, Lipa$^{-/-}$ and rAAV8.hAAT.LIPA-treated Lipa$^{-/-}$ mice models. Using Seahorse technology, we studied the mitochondrial oxidative function on isolated mitochondria from WT, Lipa-/- and Lipa-/- treated mouse frozen liver, at 12- and 34-week post-injection. The results obtained highlitghed that the OCR elicited by complex I substrate (NADH), was significantly reduced in Lipa-/- mitochondria and this deficiency was completely restored in mitochondria isolated from Lipa-/- treated mouse that showed respiratory values close to the WT. The spetrofotometric assessment the enzymatic activity of the individual OXPHOS complexes I-IV, confirmed the respirometric data. Moreover, preliminary western blot analisys on PCG1a, SIRT1 and LC3A/B II suggest impaired mitochondrial biogenesis and autophagy respectively, in Lipa-/- liver, fully restored following in vivo administration of recombinant AAV8 vector encoding for human LIPA transgene.

Altoghether, these findings provide valuable insights into the mechanisms underlying mitochondrial dysfunction in LAL-D in the pathogenesis of LAL-D-dependent diseases and offers a new therapeutic option for LAL-D patients.



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Proteomics strategies to disclose *BRCA1*-mutated breast cancer phenotype: functional and metabolic implications

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Germline mutations of the BRCA1 gene account for 5-10% of breast cancers and confer a risk of developing the disease 10- to 20-fold much higher than in non-carriers. By considering metabolic networks that could reconcile both genetic and non-genetic causal mechanisms in BRCA1-driven tumorigenesis, our studies are based on the hypothesis that BRCA1 haploinsufficiency might drive metabolic rewiring in breast cancer cells, acting as a push toward malignant transformation.

Cancer is a dynamic disease. As a consequence, tumour landscape might include a multivariate collection of cells exhibiting peculiar molecular signatures including metabolic features. In addition, it is well established that “committed cells” might acquire peculiar characteristics and adapt their metabolism to escape apoptosis and survive also in prohibitive conditions. Overall, tumour ce ll subpopulations might exhibit high spatial and temporal heterogeneity in their architecture.

In this study, by applying proteomic strategies on BRCA1 mutated breast cancer cells, we defined a novel localization for HIF-1α, hypothesizing that its translocation into mitochondria in response to BRCA1 mutated status might be critical for the acquisition of an OXPHOS phenotype sustaining proliferation and invasion. Our data establish a connection between BRCA1 and mitochondria pathways, capable of triggering metabolic changes which, in turn, sustain the high energetic and anabolic requirements of the malignant phenotype.

Establishment of a new cell model to identify drugs for Autosomal Dominant Optic Atrophy (ADOA)

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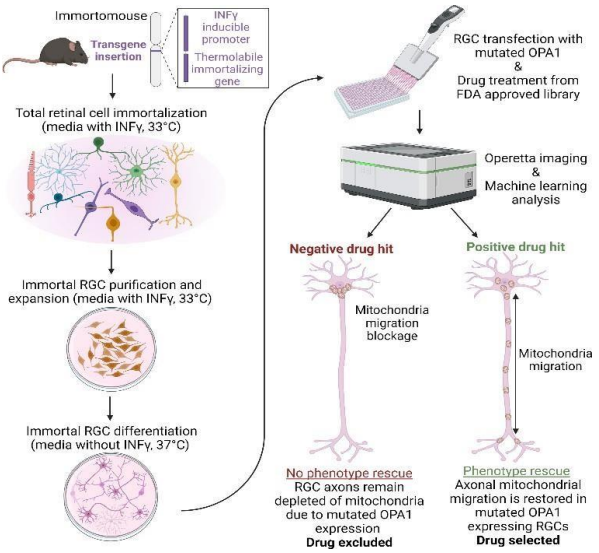
Autosomal dominant optic atrophy (ADOA) is a hereditary optic neuropathy characterized by the progressive bilateral loss of vision for which no treatment currently exists. Mutations in the nuclear encoded mitochondrial protein Optic Atrophy 1 (Opa1) are associated with ADOA which affect primarily Retinal Ganglion Cells (RGCs). Upon RGC death, the optic nerve composed of RGC axons degenerates resulting in blindness. The Scorrano lab has demonstrated that RGCs carrying mutated Opa1 display excess autophagy, accumulation of autophagosomes in axonal hillocks and mitochondrial depletion along axons, all associated with loss of vision in an ADOA mouse model. Remarkably, genetic inhibition of autophagy restored both axonal mitochondria distribution and vision in ADOA mice. We hence reasoned that pharmacological inhibition of pathways connecting ADOA mitochondria to autophagy hyperactivation could restore axonal mitochondrial distribution in ADOA RGCs, ultimately interrupting the pathogenetic cascade that leads to blindness. To this end, we seek to perform a high content imaging-based drug screening to identify compounds rescuing axonal mitochondrial content in ADOA RGCs. Such large-scale experiment however highlighted the technical bottleneck that is to work with primary mouse RGCs. Indeed, isolating primary RGC from mice cannot provide the sufficient amount of biological material that is necessary. To meet this demand, we have generated the first immortalized RGC line which will also facilitate our *in vitro* studies overall.



Microparticle sustained delivery of the calcineurin inhibitor FK506 to curtail autophagy and restore vision in an ADOA mouse model

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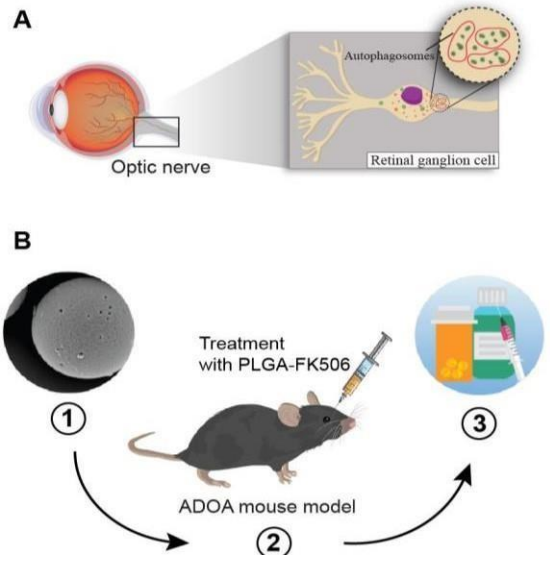
Autosomal dominant optic atrophy (ADOA) is the most common hereditary neuropathy that affects 1:35.000 people worldwide, and treatment is not available. Mutations in OPA1 in ADOA patients promote low levels of OPA1 protein, causing Retinal Ganglion Cells (RGCs) degeneration leading to blindness. Our group showed fragmented mitochondria, excluded from the axon but combined with autophagosomes in the axonal hillock of the ADOA mouse model. Moreover, genetic autophagy inhibition restores mitochondrial distribution in the RGC axon and curtails visual loss in the ADOA animal. Given that, this project aims to develop a pharmacological therapy through vitreal injection to curtail ADOA mouse visual loss. We developed PLGA microparticles loaded with FK506 (PLGA-FK506), an inhibitor of autophagy in RGCs. The ADOA mouse model (Opa1fl/fl::Grik4-Cre mice) was subjected to an PLGA-FK506 intravitreal treatment over 3 months. The visual acuity of mice was measured by optokinetic tests. The ADOA mouse model loses visual acuity after 3 months. However, PLGA-FK506 treatment prevented loss of visual acuity in ADOA animals, and it was maintained over 3 months.Therefore, the treatment with PLGA-FK506 prevented ADOA's vision loss for 3 months.



Specific OPA1 inhibitors that enhance apoptotic release of cytochrome c and cell death.

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The GTPase activity of the dynamin-related mitochondrial protein Optic Atrophy 1 (OPA1) controls cristae remodeling, cytochrome c release and apoptosis. OPA1 is hence being increasingly identified as a vulnerability of cancer cells. From a high throughput screening of 10,000 drug-like compounds for OPA1 GTPase inhibition, we identified MYLS22 as the most promising hit. This compound is not mitochondriotoxic but increases cytochrome c release in response to proapoptotic stimuli. MYLS22 causes mitochondrial fragmentation, and cristae remodeling and it sensitizes a panel of cancer cells to drug treatment. In vitro, MYLS22 binds recombinant OPA1 GTPase and did not inhibit recombinant Dynamin 1 GTPase activity. It does not display any additional effect over OPA1 deletion in cells, further substantiating its specificity. Structural activity relationship (SAR) analysis of the OPA1 inhibitor turned out into a series of MYLS22 derivatives with improved water solubility. Furthermore, three of these compounds exhibit significantly enhanced inhibitory effects on OPA1 GTPase activity *in vitro*, as well as mitochondrial fragmentation ability. In conclusion, MYLS22 and its derivatives are the first-in-kind specific OPA1 inhibitors that exert anti-cancer properties.

NADH aerobic oxidation pathway in mitochondria of Stroke Resistant and Stroke Prone Spontaneously Hypertensive Rats treated with high salt diet

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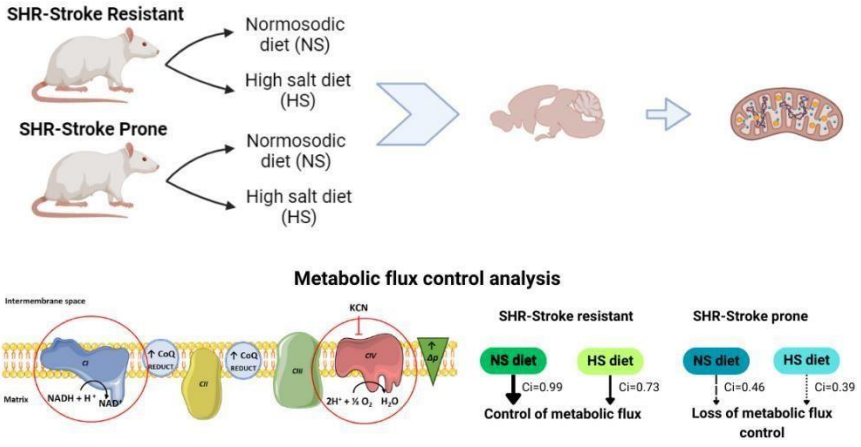
Stroke is one of the leading causes of death worldwide, and stroke survivors frequently experience cognitive impairment and physical disability. Since current treatments exhibit limited effects, it is important to discover new therapies. Mitochondrial dysfunction has been lately considered as a major contributor to cerebrovascular damage[1]. We aimed to understand the function of respiratory complexes organization in brain mitochondria of Spontaneously Hypertensive Rat (SHR) stroke-resistant (SR) and SHR stroke-prone (SP), fed with normosodic (NS) or high-salt (HS) diet. We carried out enzymatic kinetic studies to evaluate the kinetic metabolic control analysis for each group and condition. Our results on the flux of electrons from NADH to oxygen using flux control analysis on rats fed with NS diet showed that complex IV (CIV) exerted complete control on the NADH-driven respiratory rate in SHR-SR. These results were the opposite with respect to SHR-SP. On the other hand, HS diet decreased the control coefficient (Ci) in SHR-SR but, in the framework of metabolic control analysis, CIV still exerted complete control on the NADH-driven respiratory rate. In contrast, a very low Ci value was obtained in SHR-SP. To conclude, our research indicates a reduced control of the metabolic flow in SHR-SP, particularly when fed with HS diet which favours the stroke phenotype in this strain. On the other hand, the metabolic control was exerted by the terminal enzyme of the respiratory chain independently of the diet in SHR-SR. These findings improve our knowledge on the mitochondrial metabolism dysfunction underlying stroke susceptibility and they contribute to identify a more targeted treatment for stroke.



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Ketone bodies resume mitochondrial respiration in neuronal progenitor cells from induced Pluripotent Stem Cells of patients affected by AGC1 deficiency

Antonella Pignataro[a], Simona N. Barile[b], Maria C. Magnifico[a], Luigi Palmieri[a,b], Felix Distelmaier[c], Luigi Viggiano[a], Marica Antonicelli[a], Sabrina Petralla[d], Julia Hentschel[e], Vito Porcelli[a], Eleonora Poeta[d], Isabella Pisano[a], Giuseppe Fiermonte[a], Anna Maria Porcelli[d], Stewart A. Anderson[f], Luisa Iommarini[d], Gaia Tioli[d], Barbara Monti[d] and Francesco M. Lasorsa[a,b]

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AGC1 deficiency is a severe infantile encephalopathy (DEE39, OMIM# 612949) caused by mutations of *SLC25A12* gene encoding AGC1 the isoform 1 of the mitochondrial aspartate/glutamate carrier. AGC1 catalyses the Ca^{2+}-regulated and unidirectional exchange of mitochondrial aspartate with cytosolic glutamate plus a proton. AGC1 is a key component of the malate-aspartate shuttle transferring reducing equivalents of glycolysis-derived NADH into the mitochondria and plays a crucial role in the energy metabolism, in the balancing of cellular redox state and in the myelin synthesis in the central nervous system CNS [1]. AGC1 deficiency manifests in infants with brain atrophy, neuromuscular delay, hypotonia, epilepsy, and hypomyelination associated with reduction of brain N-acetyl-aspartate, the precursor of the myelin lipids in CNS. To gain further insights into the pathogenetic mechanisms of AGC1 deficiency, fibroblasts from two patients carrying different mutations in *SLC25A12* were reprogrammed into induced Pluripotent Stem Cells and differentiated into neuronal progenitor cells (NPs). NPs were obtained from a patient expressing the R353Q AGC1 mutant, which retained about 15% of WT AGC1 transport activity [2], and from a patient carrying two compound heterozygous mutations (c.225del; p(Glu76Serfs*17) and c.1747C>A; p. (=)) that completely prevented AGC1 expression due to altered splicing. All patient NPs revealed a proliferation deficit when the cells were grown in the absence of glutamine, along with an increased cell death, as compared to three unrelated healthy controls. As indirect measurement of the impaired malate-aspartate shuttle activity, the lactic acid released in the conditioned medium of patient NPs was found higher than in conditioned medium of control cells. Accordingly, NPs from both patients revealed a higher glycolytic activity associated with a dramatic reduction of mitochondrial oxygen consumption rates (OCR), when measured in the presence of glucose alone or in combination with other respiratory substrates, such as pyruvate or lactate. Furthermore, by examining the activity of the mitochondrial respiratory chain, in patient NPs we observed a significant reduction of complex I activity, whereas Complex II, II+III, IV and V activities were found similar to those measured in control NPs. Since the administration of a ketogenic diet improves the clinical outcomes in the patients with AGC1 deficiency, including resumed myelination [3], we evaluated the effect of the ketone bodies acetoacetate and beta-OH- butyrate on NPs mitochondrial respiration. OCR measurements revealed that ketone bodies significantly enhanced mitochondrial respiration in NPs with impaired AGC1, particularly in the absence of glucose and when combined with glutamine. Overall, our data suggest that NPs generated from AGC1 deficiency patients may benefit from the administration of alternative metabolites capable of generating acetyl-CoA in the mitochondria (ketone bodies) or TCA cycle intermediates (glutamine), bypassing the limited mitochondrial oxidation of pyruvate derived from glycolysis.

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Ganoderma adspersum ethanolic extracts: selective cytotoxicity on human renal tumor cell lines

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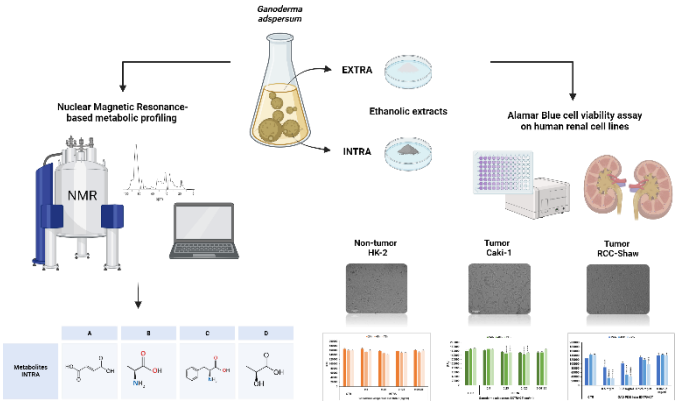
Despite their widespread use, chemotherapeutic agents in anticancer therapy often induce severe adverse effects and yield suboptimal outcomes, necessitating the pursuit of novel, more effective treatments. Natural products have emerged as promising source for new alternative drugs with more favorable safety profile. Fungal organisms, particularly *Ganoderma spp.*, are extensively investigated for their anticancer properties, prized for their safety, low toxicity, widespread availability, and cost-effectiveness as potential candidates for new cancer therapies. In this study, we aimed to explore the bioactivity of the limitedly investigated *Ganoderma adspersum* by evaluating the cytotoxic effects of ethanolic extracts obtained from both the fungal fermentation medium (extra) and the mycelium content (intra) on human renal tumor cell lines (Caki-1 and RCC-Shaw) as well as a non-tumor renal cell line (HK-2). Our findings revealed that the compounds extracted from the mycelium (intra) significantly impacted the viability of tumor renal cells, with a notable decrease of up to 65% in RCC-Shaw cell viability observed after 48 hours of treatment, while no significant effect on HK-2 cell proliferation was observed. Metabolomics analysis using NMR spectroscopy revealed substantial differences in the composition of the two tested extracts, notably the presence of succinic acid, alanine, xanthine, and phenylalanine derivatives in the bioactive extract. Further investigation is demanded to elucidate the potential therapeutic targets of these prioritized compounds in the context of cancer treatment, with a particular focus on their impact on the metabolic processes of tumor renal cells.



Combined *in silico/in vitro* approaches for identifying modulators of CRAT Enzyme activity

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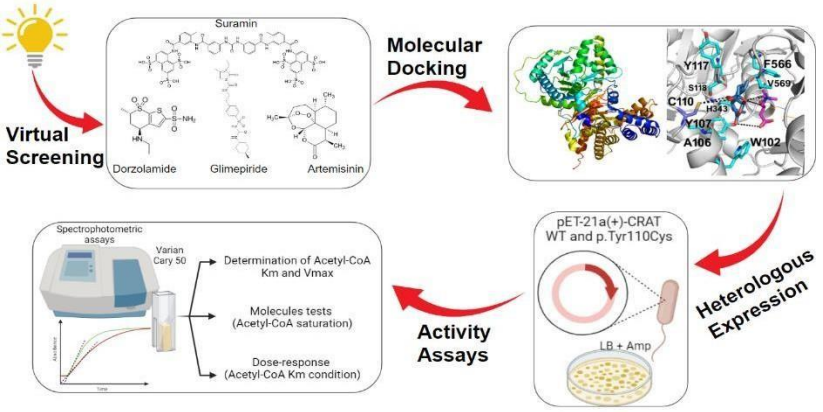
The impairment of the catalytic activity of Carnitine O--acyltransferase (CRAT) enzyme has recently been proposed as an additional cause for the onset of Leigh syndrome, or subacute necrotizing encephalomyelopathy. This syndrome represents one of the most severe pediatric disorders caused by mitochondrial dysfunction. The CRAT enzyme is responsible for the reversible transfer of acyl groups from acetyl--coenzyme A to carnitine. A missense mutation (p.Tyr110Cys, Y110C) was recently identified as the cause of severe impairment of the CRAT enzymatic function in a pediatric patient exhibiting Leigh--like syndrome symptoms [1]. The research here reported derived by an integrated approach combining *in silico* docking analysis and virtual screening of chemical libraries with subsequent *in vitro* validation in order to identify modulators of the activity of the wild type (WT) CRAT protein and the p.Tyr110Cys variant. The accuracy of the *in silico* analyses was validated through *in vitro* assays on the corresponding recombinant CRAT WT and p.Tyr110Cys proteins, and lysates from control fibroblasts and fibroblasts from a patient carrying the above cited mutation. Obtained results demonstrate the effectiveness of this combined approach in identifying novel compounds for modulating CRAT enzyme activity, offering valuable insights for potential therapeutic interventions targeting CRAT-related disorders.



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