ISTITUTO PASTEUR ITALIA
FONDAZIONE CENCI BOLOGNETTI

2022 Annual Report

ISTITUTO PASTEUR ITALIA – FONDAZIONE CENCI BOLOGNETTI
Viale Regina Elena 291 – 00161 – Rome
Tel. 0039 06 49255625
mail: pasteuritalia@istitutopasteuritalia.it
www.istitutopasteuritalia.it

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FOREWORD

The **Istituto Pasteur Italia - Fondazione Cenci Bolognetti**, the Italian member of the Institute Pasteur International Network (33 institutes worldwide), is a private *non-profit* foundation. It was established in 1964 thanks to the bequest and wishes of princess Beatrice Cenci Bolognetti to create a Center of Biomedical Research with the same mission and values of *Institut Pasteur* in Paris.

The **Istituto Pasteur Italia** research activity is committed to biomedicine, with a particular focus on infectious diseases, drug design, molecular medicine extended to innovative therapies (e.g. cancer immunotherapy; therapy of genetic diseases; regenerative medicine). The **funding of research projects** is possible thanks to the income from the donated real estates and to citizens' donations. In 2022 the Institute has invested a total of 560,126 Euros to fund **high level research projects in the affiliated laboratories of the Sapienza University of Rome, in different areas** (microbiology, virology, molecular genetics, molecular biology, cellular and molecular immunology as well as food-borne diseases), and also funded research projects on **Immunotherapies for Cancer and Infectious Diseases** carried on at **Laboratorio Pasteur Italia**, directed by Dr. John Hiscott. Moreover **the Instituto Pasteur Italia** supported young researchers with **fellowships** (i.e. and PhD courses to have experience abroad and to return in Italy).

The **Istituto Pasteur Italia** is also involved in a number of **collaborative projects with Institutes of the International Network of Pasteur** such as the *Actionnes Concertée International Pasteuriennes (ACIP)* and the *Programmes Transversaux de Recherche (PTR)*, funded by the Institut Pasteur of Paris.

The scientific excellence reached over 2022 is demonstrated by high quality publications in peer-reviewed scientific journals, for a cumulative impact factor: 1.290,403

Istituto Pasteur Italia hosted **Annual Meeting Pasteur Network "THE START OF A NEW CHAPTER"** in Rome, November 28-29, 2022.

The meeting of the representatives of all the Institutes present on the five continents was a great opportunity to discuss the goals achieved and the new development paths to be pursued together.

2022 was the **bicentenary of the birth of Louis Pasteur** and to celebrate the extreme relevance of his scientific heritage a conference was held on December 1st at the French Embassy – Palazzo Farnese.

The event entitled **"Louis Pasteur, a universal heritage"** was organized in partnership with the **Association française pour l'avancement des sciences** (AFAS) and **Scienzainrete**, the **French Embassy in Italy** and the **Institut français Italy** and dedicated to **school students**.

Last but not least, the Institute has been very active in promoting **educational** programs and scientific communication. The well–established dissemination project for secondary schools has been realized both in presence and through web platforms.

This Annual Report documents the results obtained during the year 2022 thanks to the enthusiasm and the effort of the Italian "Pasteur" community.

Luigi Frati
President

Angela Santoni
Scientific Director

RESEARCH PROJECTS

1. **LABORATORI PASTEUR ITALIA**

- **Immunotherapies for cancer and infectious diseases (PI: J. Hiscott)**

2. **SAPIENZA UNIVERSITY (IPI AFFILIATED LABORATORIES)**

- **“Anna Tramontano” Research Projects**
- **Under 45 Research Projects**

3. **COLLABORATIONS WITHIN THE INTERNATIONAL PASTEUR NETWORK**

- **Programmes Trasversaux des Recherch (PTR)**
- **Research Projects funded by IP Paris (ACIP)**

4. **SCIENTIFIC BOARD RESEARCH PROJECTS**

IMMUNOTHERAPIES FOR CANCER AND INFECTIOUS DISEASES

Director of Research: John Hiscott

IMMUNOTHERAPIES FOR CANCER AND INFECTIOUS DISEASES

John Hiscott

Istituto Pasteur Italia – Fondazione Cenci Bolognetti
john.hiscott@istitutopasteur.it

1. Prophylactic and therapeutic antiviral activity of VLPs-M8

SARS-CoV-2 pandemic has highlighted weaknesses in preparedness and response to global public health emergencies and emphasized the crucial need for timely and efficient interventions to control future outbreaks. The rapid development of SARS-CoV-2 vaccines is a clear demonstration of the extraordinary efforts made during the last years, with the release of several approved vaccines and others currently undergoing clinical trials. However, commercially available vaccines failed to induce a long-lasting protection against the virus due to the emergence of several variants of concern (VOCs). Therefore, a host-targeted therapy with a broad spectrum of activity represents an important therapeutic strategy.
Virus-Like Particles (VLPs) are nanostructures that share conformation and self-assembly properties with viruses but lack a viral genome and therefore the infectious capacity. In our study, we described the production of VLPs *in vitro* by co-expression of Vesicular Stomatitis Virus (VSV) glycoprotein (VSV-G) and HIV structural proteins (Gag), incorporating a strong sequence-optimized 5'ppp-RNA RIG-I agonist, termed M8 (*Palermo et al.,* Front Cell Infect Microbiol. 2022). Besides, we evaluated the ability of VLPs to release M8 into cells and to trigger a type I interferon antiviral response.

Treatment of target cells with VLPs-M8 generated an antiviral state that conferred resistance against multiple viruses, including VSV, Dengue virus, hCoV-229E and SARS-CoV-2 Spike pseudotyped VSV (VSV-Spike). Interestingly, administration of VLPs-M8 also elicited a therapeutic effect by inhibiting ongoing viral replication in previously infected cells. Finally, the presence of SARS-CoV-2 Spike glycoprotein on the VLP surface retargeted VLPs to A549 cells expressing the ACE2 receptor (A549-ACE2), thus selectively delivering M8 and blocking VSV infection in susceptible cells but not in wild-type A549.
These observations highlight the potential of VLPs-M8 as a therapeutic and prophylactic vaccine platform, directing specific innate and adaptive immune responses in target tissues, and boost immunogenicity while minimizing off-target effects.

2. Oncolytic virotherapy of pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is the most common lethal malignancy, with little improvement in patient outcomes over the past decades. Multiple challenges have hampered the efficacy of cancer therapies for PDAC, the most significant being the immunosuppressive pancreatic tumor microenvironment. PDAC cells adopt multiple mechanisms to evade and suppress antitumor immune responses, essentially establishing a non-immunogenic (or 'cold') tumor microenvironment (TME), with poor T-cell infiltration and low mutational burden (*Muscolini et al, CGFR 2020*). With the support of Associazione Italiana Ricerca sul Cancro (AIRC 2020 – 2024), we are investigating the implementation of oncolytic virotherapy for the treatment of pancreatic cancer. Oncolytic virotherapy represents an efficient immunotherapeutic strategy for cancer treatment. Oncolytic viruses (OVs) are native or genetically engineered viruses that promote antitumor responses through tumor-selective cell lysis and immune system activation. Because of their oncolytic and immune-stimulating properties, OVs are ideal candidates to counteract the pancreatic immunosuppressive TME and to design combination therapies that can be exploited in pre-clinical models to improve PDAC therapeutic options (*Tassone et al, CGFR 2020*).

A major hurdle to oncolytic virotherapy, especially when used as single treatment, is the resistance to OVs infection, due at least in part to the residual antiviral immunity of some cancer cell lines and primary tumors. The identification of host factors that determine the resistance to OVs by limiting viral entry, replication and oncolysis is crucial to extend the efficacy of OV-mediated therapy. To identify loss-of-function mutations that alter PDAC cell sensitivity to OV infection and to develop novel treatments for PDAC, we performed an unbiased genome-wide CRISPR-Cas9 knockout screen in a human PDAC cell line that emerged from our analyses as highly resistant to VSVΔM51. Cas9-expressing cells were transduced with a library of pooled single guide RNA (sgRNA)-expressing lentiviruses that target all human genes to obtain a population where each cell is knocked out for a single gene. Upon VSVΔM51 infection, due to the virus selective pressure there was an enrichment of resistant clones, while sensitive cells died. The variation in the relative abundance of each genome-integrated sgRNA was measured by NGS. NGS data analyses has uncovered more than 12 target genes that were missed by previous studies, including genes involved in transcriptional control, apoptosis regulation, oxidative metabolism, and tumor cell migration. The detailed protocol was described in Muscolini, Hiscott and Tassone, Methods Mol Biol., doi: 10.1007/978-1-0716-2788-4_25. To validate the top genes as host resistance candidates (HRC), single-knockout (KO) cell lines were generated in different PDAC models. Upon VSVΔM51 challenge, the number of KO-infected cells was at least 3-fold higher compared to control cells. The augmented sensitivity of KO cells to VSVΔM51 infection and replication was accompanied by a strong increase of cell death induction. Pharmacological inhibition of the HRC in resistant PDAC cells resulted in enhanced VSVΔM51 infectivity. Conversely, the overexpression of the HRC of interest in VSVΔM51-sensitive PDAC models was found to correlate with an augmented resistance to VSVΔM51 infection.

In vitro results confirmed a prominent role of the top HRC gene in PDAC intrinsic resistance to VSVΔM51 infection and replication. *In vivo* analyses and studies to elucidate the mechanisms responsible for the enhanced permissiveness to virotherapy in HRC-KO cells are ongoing. Our findings provide a resource amenable to the characterization of host factors involved in the resistance to different OVs in multiple tumor models and highlight the potential to understand and reverse host resistance to oncolytic virotherapy.

3. 4-OI induces metabolic reprogramming affecting Dengue virus replication

Dengue virus (DENV) is a mosquito-borne pathogen that annually threatens about half of the world's population with an estimated 100-400 million infections each year. Among the factors that influence dengue disease severity, the damage induced by oxidative stress has been correlated with inflammation and progression towards the severe forms of disease. Control of antioxidant enzyme expression is mediated in part through Nrf2, a global transcriptional regulator involved in the maintenance of redox homeostasis. Metabolic regulation is increasingly recognized as a powerful mechanism guiding the development of inflammatory and antiviral responses to viruses and microbial pathogens. The endogenous metabolite itaconate, a by-product of Krebs cycle metabolism, enables Nrf2 release and links metabolism to anti-oxidant response. In this study, we investigated the metabolic changes induced by the Nrf2 agonist 4-octyl-itaconate (4-OI) during DENV infection. We demonstrated that 4-OI increased DENV replication in a Nrf2-independent manner and impaired the activation of type I IFN response and inflammation. Moreover, we described that the proviral effect relied on the 4-OI – induced metabolic switch from glycolysis to fatty acid β-oxidation through the activation of the PDK4-CPT1 axis, thus identifying PDK4 as a key metabolic factor involved in modulation of viral replication.
These results have direct relevance to dengue immunopathogenesis, since disease severity has been directly associated clinically and experimentally with lipid metabolism disorders and chronic inflammation.

Publications

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Lab Members

Magdalini Alexandridi
Daniele Di Carlo
Michela Muscolini
Enrico Palermo

Collaborators

Nadine van Montfoort/Priscilla Kindermann – Leiden University Medical Center
David Olagnier/Soren Paludan – Aarhus University
Bernadette van den Hoogen- Erasmus University Medical Center, Rotterdam
Marisa Gariglio – University of Piemonte Orientale - Novara
Marco Sgarbanti – Istituto Superiore di Sanità
Mariateresa Fiorenza – Sapienza University
Guido Antonelli/ Ombretta Turriziani/Carolina Scagnolari – Sapienza University
Francesca Cutruzzolà/Serena Rinaldo – Sapienza University
Anna Teresa Palamara – Istituto Superiore di Sanita /Sapienza University
Peter Katsikis – Erasmus University Medical Center, Rotterdam

RESEARCH PROJETCS CARRIED OUT IN AFFILIATED LABORATORIES AT SAPIENZA UNIVERSITY OF ROME

“ANNA TRAMONTANO” RESEARCH PROJETCS – CALL 2020

PROJECTS LED BY UNDER 60-YEAR-OLD RESEARCHERS

FIRST YEAR REPORTS

ROLE AND MECHANISM OF ACTION OF PYRIDOXAL 5'-PHOSPHATE CARRIER PROTEINS IN VITAMIN B_6 METABOLISM

ROBERTO CONTESTABILE
RESEARCH AREA: Genetics and biology of microorganisms

Department of Biochemical Sciences
roberto.contestabile@uniroma1.it

Introduction - Pyridoxal 5'-phosphate (PLP), the catalytically active form of vitamin B_6, plays a crucial biological function as enzyme cofactor, and as such has key roles in many essential metabolic pathways. PLP is a very reactive molecule, therefore its cellular concentration in the free form must be tightly regulated to avoid toxic effects. At the same time, a sufficient amount of PLP must be supplied to satisfy cell requirements. Therefore, it is expected that the encounter between PLP and apoenzymes does not take place by simple diffusion and collision, but relies on a specific transport system. Experimental evidence suggests that PLP carrier proteins exist, which bind the cofactor protecting it from the environment and forming a PLP cellular reservoir, and transfer it to apoenzymes. While vitamin B_6 metabolism has been widely investigated with respect to reaction steps and enzyme catalysis, the mechanism of PLP delivery to apoenzymes is very poorly understood. The present proposal aims to investigate the role and mechanism of action of three putative PLP-binding proteins in bacteria: YggS (also called PLP-binding protein or PLP-BP), pyridoxine 5'-phosphate oxidase (PNPO) and pyridoxal kinase (PDXK). Although it has been proposed that all these proteins directly participate to PLP homeostasis and delivery in the cell, their actual involvement in these processes and the molecular mechanism of PLP transfer have never been clarified. Specifically, our project focuses on: i) the mechanism of PLP transfer from PLP carrier proteins to PLP-dependent apoenzymes; ii) the protein structural features involved in PLP binding and transfer capabilities; iii) *in vivo* studies on the actual role of PLP binding proteins in PLP homeostasis and delivery; these studies will mainly use *Escherichia coli* as bacterial model. Considering the importance of vitamin B_6 in bacterial physiology and virulence, the outcomes of our project may have a relevance for human health, indicating novel targets of antimicrobial intervention.

Main findings of the first year of the project

Studies on* E. coli *YggS - The physicochemical and PLP binding properties of recombinant *E. coli* YggS have been characterised in detail through spectroscopic, site-directed mutagenesis, crystallographic, mass spectrometry and limited proteolysis studies, and have been reported in Tramonti et al. 2022. Kinetic and equilibrium binding analyses showed a high affinity of YggS for PLP, indicated by a K_D of about 1 nM, and that PLP binding to apo-YggS is very fast, as the binding equilibrium is fully reached in the manual mixing time, which is not longer than 3 s. On the other hand, PLP

dissociates from YggS very slowly, as demonstrated by experiments in which holo-YggS was incubated with PLP phosphatase. These observations indicate that PLP binding is characterized by a large k_{on} and a small k_{off}, which is not really in favour of a role of PLP supplier for YggS. Another interesting finding is that PNP binds to apo-YggS with relatively high affinity (K_D is about 30 nM), suggesting that in particular conditions this vitamer may compete with PLP to bind to YggS. A fundamental role in PLP binding to YggS is played by the Lys36 residue, which establishes an aldimine linkage with the aldehyde group of this vitamer. Surprisingly we found that, when Lys36 is replaced by an Ala residue, other Lys residues are able to bind PLP covalently. The characterization of multiple lysine variants showed that K233, K234 and K38 bind PLP in the K36A variant. A number of lysine residues surrounding the active site (a "lysine cluster"), including also K65, K89, and K137, show a good degree of conservation. We believe that the covalent linkage of PLP by K38 and K233-K234, observed when K36 is replaced by an alanine residue, may only be an accidental consequence of the presence of these lysine residues around the active site. However, the lysine cluster as a whole may actually play an important structural and functional role in YggS. Our experiments showed that YggS undergoes a conformational change (or a marked increase of flexibility) when it binds PLP. We believe that this is a consequence of the energetic frustration due to the presence of K36 at the active site of apo-YggS, determining unfavourable electrostatic interactions of this residue with the lysine cluster, that is relieved when this residue binds PLP. We set up a novel *in vitro* discontinuous assay in which PLP transfer from holo-YggS to the apo-form of *E. coli* serine hydroxymethyltransferase (SHMT, a model PLP-dependent enzyme) could be followed. In this assay, the increase in SHMT activity is measured as PLP dissociates from holo-YggS and binds to apo-SHMT, forming the catalytically active holo-SHMT. A control experiment, in which YggS was replaced with a molar excess of free PLP gave 100% SHMT activity as reference value. Transfer experiments, carried out at 37 °C, used 20 µM holo-YggS and 20 µM apo-SHMT, in 50 mM NaHEPES buffer at pH 7.6. An exponential PLP transfer kinetics was observed, with a rate constant of 0.14 ± 0.02 min^{-1}, that reached the equilibrium in about 30 min (Fig. 1a). At equilibrium, approximately 15% of PLP initially bound to YggS was

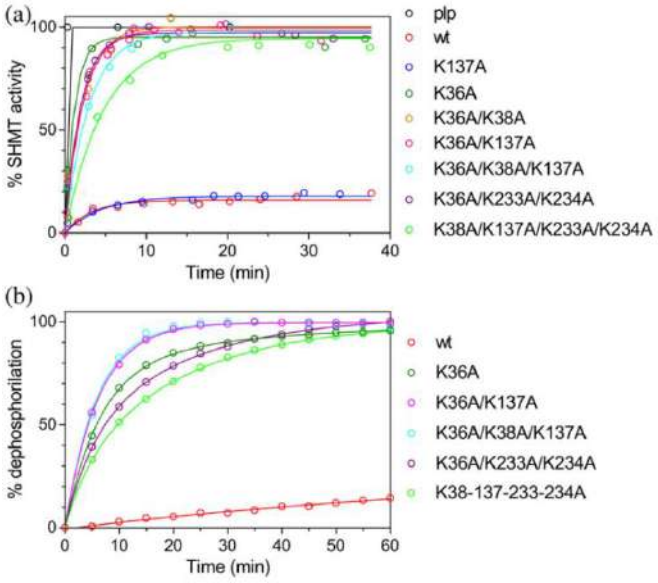

Fig. 1 PLP transfer from YggS to eSHMT. (a) The reactivation of apo-eSHMT in the presence of 100 µM PLP (black line) was used as the 100% activity reference. Transfer kinetics were measured in the presence of the indicated YggS variants. (b) The kinetics of the PLP hydrolysis was measured in order to compare k_{off} of PLP dissociation from WT and variant forms of holo-YggS. Data are represented as percentage of hydrolyzed PLP, with respect to total protein-bound PLP.

transferred to SHMT. PLP transfer was also studied using YggS lysine variants. While the single K137A variant showed the same transfer kinetics as that observed with WT YggS, variants that contained the K36A replacement (K36A, K36A/K38A, K36A/K137A, K36A/K38A/K137A, and K36A/K233A/K234A) showed a faster and much more extensive transfer of PLP to apo-SHMT (Fig. 1a). Interestingly, also the K38A/K137A/K233A/K234A variant showed PLP transfer properties similar to the latter variants. This is likely due to its reduced affinity for PLP when compared to the WT or the K137A variant. It is notable that the percentage of transferred PLP seems to correlate with the K_D value of PLP binding of the lysine variants. In order to assess whether the rate of PLP transfer depends on the apparent k_{off} value of PLP dissociation from holo-YggS, the kinetics of PLP hydrolysis of WT and variant forms by PLP phosphatase was measured. Experiments were carried out in which 20 μM holo-YggS samples were incubated with 0.5 μM PLP phosphatase, and the hydrolysis of PLP measured. In these conditions, the small fraction of free PLP contained in the solvent, in equilibrium with protein-bound PLP, is rapidly hydrolysed by PLP phosphatase, promoting PLP dissociation from the protein and its subsequent hydrolysis. The observed rate of PLP hydrolysis therefore depends on the k_{off} of PLP dissociation from the protein. Fig. 1b, in which PLP hydrolysis is reported as percentage of total protein-bound PLP, shows that PLP hydrolysis takes place much more rapidly in the case of the YggS variants containing the K36A mutation, indicating that the rate of PLP transfer correlates with the k_{off} of PLP dissociation from the protein. These PLP transfer experiments are not in favour of a role of YggS as a PLP donor. It should also be considered that the large concentrations of YggS and eSHMT used in our PLP transfer experiments (20 μM each) are undoubtedly far larger than the cellular concentrations of these proteins. Therefore, it is expected that PLP transfer kinetics would be much slower in the cellular conditions. A physiological role of YggS as a "PLP sponge" in the cell, which sequesters free PLP from the environment as this is produced and contributes to PLP homeostasis, would only be possible if YggS concentration in the cell were large enough to actually absorb large amounts of PLP; moreover, in order to redistribute PLP, YggS should also have a lower affinity for PLP than PLP-dependent apoenzymes. However, studies on the cellular concentration of *E. coli* proteins indicated that YggS is not an abundant protein, not even more abundant than SHMT for instance. In summary, YggS might not be suitable to play the proposed role of PLP carrier/distributor, and not even that of PLP reservoir in the cell. After all, a ΔyggS *E. coli* strain grows normally on a minimal medium containing only salts and glucose and this observation is not compatible with such hypothetic, fundamental functions. In order to further investigate any physiological roles played by the lysine residues *in vivo*, Δ*yggS E. coli* strains harbouring WT YggS and all various YggS lysine variant plasmids were constructed. Since the *ΔyggS* strain exhibits a concentration-dependent sensitivity phenotype to the pyridoxine analogue 4'-deoxypyridoxine (4dPN), assays were carried out to assess the ability of these YggS mutants to complement the 4dPN sensitivity phenotype of Δ*yggS*. Both K36A and K137A variant plasmids failed to complement the 4dPN sensitivity

phenotype, while mutating the K38, K233, or K234 residues did not affect complementation. These results show that PLP binding to YggS is essential for function, but also that the K137 residue, which is not involved in PLP binding, has an essential functional role. In conclusion, some features of YggS, such as the high affinity for PLP and the possible change in conformation induced by PLP binding, together with the striking results obtained in the *in vivo* experiments, point toward a role of YggS as a component of an unknown regulatory pathway. The concentration of free PLP in the cell is low since most of this cofactor is bound to proteins. Therefore, a signalling protein whose role was to sense PLP levels should have a high affinity for this cofactor. The binding of PLP to YggS, and the consequent changes in protein conformation or flexibility, may regulate its interactions with other cellular components involved in vitamin B_6 homeostasis. We also investigated the mechanism by which 4dPN disrupts vitamin B_6 homeostasis in *E. coli K12*. 4dPN is a long known B_6 antimetabolite but its mechanism of action was not totally clear. By exploring different conditions in which PLP metabolism is affected in the model organism *E. coli* K12, we showed that 4dPN cannot be used as a source of vitamin B_6 as previously claimed. In addition, we found that 4dPN sensitivity is likely the result of multiple modes of toxicity, including inhibition of PLP-dependent enzyme activity by 4'-deoxypyridoxine phosphate and inhibition of cumulative pyridoxine uptake. These toxicities are largely dependent on the phosphorylation of 4dPN by pyridoxal kinase. These results were recently published (Babor et al. 2022).

Studies on* E. coli *PNPO – In *Escherichia coli*, PLP formation is catalysed by PNPO, a homodimeric FMN-dependent enzyme that is responsible for the last step of PLP biosynthesis and is also involved in the PLP salvage pathway. We had previously observed that *E. coli* PNPO undergoes an allosteric feedback inhibition by PLP, caused by a strong allosteric coupling between PLP binding at the allosteric site and substrate binding at the active site. We have achieved the crystallographic identification of the PLP allosteric site, located at the interface between the enzyme subunits and mainly circumscribed by three arginine residues (Arg23, Arg24, and Arg215) that form an "arginine cage" and efficiently trap PLP (Fig. 2). The crystal structure of the PNPO–PLP complex, characterized by a marked structural asymmetry, presents only one PLP molecule bound at the allosteric site of one monomer and sheds light on the allosteric inhibition mechanism that makes the enzyme-substrate-PLP ternary complex catalytically incompetent. Site directed mutagenesis studies focused on the arginine cage validate the identity of the allosteric site and provide an effective means to modulate the allosteric properties of the enzyme, from the loosening of the allosteric coupling (in the R23L/R24L and R23L/R215L variants) to the complete loss of allosteric properties (in the R23L/R24L/R21L variant). These results were published in Barile et al. 2021 *J. Biol. Chem.* 296, 100795.

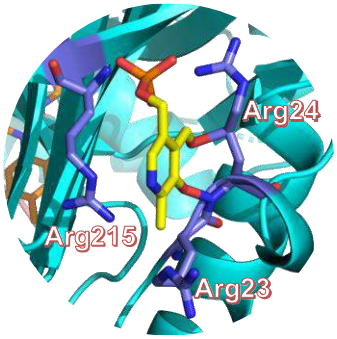

Fig. 2. "Arginine cage"

Publications

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Bunik, V., Aleshin, V., Nogues, I., Kähne, T., Parroni, A., Contestabile, R., di Salvo, M.L., Graf, A., Tramonti, A. ***Thiamine-dependent regulation of mammalian brain pyridoxal kinase in vitro and* in vivo** *J. Neurochem.*, 2022, 161(1), pp. 20–39. DOI: 10.1111/jnc.15576 - IF: 5.546

Research Group

Researchers
Angela Tramonti
Martino L. di Salvo
Alessia Parroni
Anna Barile

Ph.D. students
Claudio Graziani

Collaborations

Martin K. Safo, (Dep. of Medicinal Chemistry, VCU, Richmond, VA);
Valerie De Clergy-Lagard, (Dep. of Microbiology & Cell Science, University of Florida, Gainesville, FL).
Fiammetta Vernì (Dip. di Biologia e Biotecnologie, Sapienza Università di Roma).
Andrea Ilari (IBPM, CNR).

THE PERSONALIZED THERAPY OF CYSTIC FIBROSIS BY THERATYPING AND GENE TARGETING

MARCO LUCARELLI
RESEARCH AREA: Novel therapeutic interventions

Department of Experimental Medicine
marco.lucarelli@uniroma1.it

A new approach for precision medicine in Cystic Fibrosis (CF) is the so-called "theratyping". It is a methodology to identify, at cellular level, which pathogenic variants of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene respond to specific biochemical therapeutic modulators, allowing a quick clinical translation of their use.
By the "culture reprogramming condition" (CRC), we setup airway epithelial stem cells (AESC) from nasal epithelia of CF patients, to obtain patient-specific airway epithelial cells (CF-CRC-AESC) and corresponding airway organoids.
The possibility to obtain large amounts of CF-CRC-AESC from CF patients' upper airways, both undifferentiated (stem-like) and differentiated to mature airway epithelium, represents a powerful tool toward the introduction of more effective patient-specific biochemical and genetic therapeutic options, as well as to predict response to specific therapies for patients with rare pathogenic variants. The possibility to test cell response, virtually from each CF patient, to pharmacologic and genetic therapeutic approaches, may be of great translational impact, in the direction of theratyping and gene therapy.

Case series and general theratyping results

The patient-specific cellular system of nasal epithelial stem cells (CF-CRC-AESC) in use was previously completely setup by the applicant group (*Sette G., Lo Cicero S., Blaconà G., Pierandrei S., Bruno S.M., Salvati V., Castelli G., Falchi M., Fabrizzi B., Cimino G., De Maria R., Biffoni M., *Eramo A., *Lucarelli M. (*co-last authors) Theratyping cystic fibrosis in vitro in ALI-culture and organoid models generated from patient-derived nasal epithelial conditionally reprogrammed stem cells. European Respiratory Journal 2021; 58(6):2100908. IF:33.809*). During the first year of the present project, 35 long-term cultures, with 25 different genotypes, have been obtained and characterized by structural and functional tests. Overall, till now, 66 long-term cultures, with 47 different genotypes are available.
Although with quantitative differences, most genotypes tested (both rare and common) responded to Kaftrio (a combination of biochemical modulators already in clinical use for therapy: Elexacaftor + Tezacaftor (two correctors) + Ivacaftor (a potentiator), ETI) and to

a new modulator combination (Elexacaftor + Lumacaftor (another corrector) + Ivacaftor, ELI), while a minority, including genotypes with stop codons, did not respond.
Intriguing results were obtained by the theratyping of the L1077P/L1077P, L1077P/W1282X, W1282X/W1282X rare genotypes, which show all possible genetic combination of the L1077P and W1282X pathogenic variants. A synthesis of the results about the theratyping of L1077P variant is reported in the following section of this annual report. Complete results have been formalized through the following manuscript: *Lo Cicero S., Castelli G., Blaconà G. Bruno S.M., Sette G., Pigliucci R., Villella V.R., Esposito S., Zollo I., Spadaro F., De Maria R., Biffoni M., Cimino G., Amato F., *Lucarelli M., *Eramo A. (*co-last authors) L1077P CFTR pathogenic variant function rescue by Elexacaftor - Tezacaftor - Ivacaftor in cystic fibrosis patient-derived air liquid interface (ALI)-cultures and organoids: in-vitro guided personalized therapy of non-F508del patients. Re-submitted after revision to Respiratory Research* (2023), IF:7.162.
Also, the theratyping of the W57G/A234D rare genotype was performed, in collaboration with the group of Prof. Claudio Sorio (University of Verona) and Dr. Paola Melotti (AOU of Verona). In this case, also a comparison between respiratory and intestinal cellular model and organoids of CF was performed, which highlighted a good agreement. In addition, a good correlation between the theratyping results at cellular level and clinical treatment of corresponding patient could be revealed. These results were formalized through the following manuscript: *Kleinfelder K., Lotti V., Eramo A., Amato F., Farinazzo A., Dell'Orco D., Preato S., Conti J., Rodella L., Tomba F., Cerofolini A., Baldisseri E., Bertini M., Volpi S., Lo Cicero S., Castelli G., Villella V.R., Esposito S., Zollo I., Castaldo G., Laudanna C., Sorsher E.J., Hong J., Joshi D., Cutting G., Lucarelli M., Melotti P., Sorio C. In silico analysis and theratyping of an ultra-rare CFTR genotype (W57G/A234D) in primary human rectal and nasal epithelial cells. Re-submitted after revision to iScience (2023), IF:6.107.*
For some patients with genotypes involving F508del pathogenic variant and already in clinical treatment with Kaftrio, a comparison between the amelioration of clinical status after treatment and the enhancement of functional tests evidenced by theratyping could be attempted. Till now, all genotypes tested showed a good agreement between *in vivo* and *ex vivo* results. At the state-of-the-art, this kind of comparison, on both common and rare genotypes, is mandatory for the clinical validation of cellular models to be used for the personalized therapy of CF.

Theratyping of L1077P/L1077P, L1077P/W1282X, W1282X/W1282X rare genotypes

As an example of expected theratyping results, we report here the characterization of the L1077P pathogenic variant, performed comparing the L1077P/L1077P, L1077P/W1282X and W1282X/W1282X genotypes.
CFTR protein expression was assessed in differentiated CF-CRC-AESC of L1077P/L1077P and L1077P/W1282X genotypes, in untreated or pharmacologically treated cells. Basal CFTR protein levels were very low. CFTR band C (that of mature CFTR protein) increased dramatically following exposure to correctors included in Kaftrio (ET) or those included in the new combination (EL), in both genotypes, indicating strong correction of mutated CFTR. RNA analysis revealed dramatically reduced levels of W1282X CFTR mRNA, compatible with non-sense mediated decay (NMD) due to the premature terminator codon (PTC). These results suggested that Kaftrio correction (and that of the new combination of correctors) was restricted to, and dependent on, the L1077P variant. Functional studies confirmed a strong ability of Kaftrio (ETI) and the experimental drug combination (ELI) to rescue CFTR L1077P variant both in forskolin-induced swelling (FIS) assay of organoids and in short circuit recordings in Ussing Chamber assay, in both homozygous and compound heterozygous genotypes.
We also established CF-CRC-AESC for the W1282X homozygous genotype. Functional assays demonstrated that W1282X variant cannot be rescued by Kaftrio, as a consequence of heavily compromised CFTR protein expression. In this respect, undetectable levels of truncated protein in immunoblot are likely to result from two combined mechanisms. The most significant quantitative effect seems to be due to the degradation of mutated mRNA by the NMD, with consequent very reduced availability for protein translation. Nevertheless, a reduced amount of PTC mutated mRNA seems to escape degradation, as revealed by a very sensitive ddPCR expression assay. However, this reduced quote of W1282X mutated mRNA appears not to be properly translated/processed, with mutated CFTR protein degraded as a consequence of its abnormal conformation in untreated cells. Consequently, W1282X protein function cannot be recovered.
These results showed that Kaftrio, and the new combination of modulators, induced a strong rescue of L1077P pathogenic variant, even when present in single copy. Thus, in line with what already observed for F508del-bearing genotypes, our findings could validate the use of Kaftrio not only for L1077P homozygous genotypes, but also for genotypes that carry one copy of the responsive L1077P allele associated to any other variant on the second allele. This is particularly relevant also for genotypes including one variant that is not correctable or not expressed, as is the case of nonsense pathogenic variants, expanding the platea of non-F508del patients eligible for modulatory therapy.

CFTR and FOXI1 expression

In addition to the well-known role of CFTR in the pathogenesis of CF, its regulatory role in the respiratory epithelium differentiation is proposed but still to be demonstrated. As well, the Forkhead box I1 (FOXI1) gene seems to be highly expressed in a particular cell type called ionocyte, characterized from high levels of CFTR expression and chloride ion secretion, peculiar of the differentiated respiratory epithelium. The regulatory mechanism(s) involved in the induction of ionocyte properties is still to be elucidated. CFTR and FOXI1 regulatory role in differentiation, and their possible interaction and coordination, could add new insights into CF pathogenetic mechanism and therapy. With the aim of contributing to the clarification of this aspect, we studied CFTR and FOXI1 expression patterns (at mRNA level) in nasal brushing specimens, undifferentiated and differentiated CF-CRC-AESC, from several CF patients with different mutated CFTR genotypes. In particular, we analyzed 17 patient-specific brushing specimens and CF-CRC-AESC lines with 16 different genotypes. CFTR and FOXI1 gene expression was analyzed by both real-time PCR and digital droplet PCR (ddPCR), with excellent correlation between the two technical approaches (manuscript in preparation).

The expression pattern of CFTR gene resulted to be very similar in all the 17 patients and mainly linked to differentiative levels: highly expressed in nasal brushing specimens, no or very low expressed in undifferentiated CF-CRC-AESC and highly re-induced in differentiated CF-CRC-AESC. Interindividual quantitative differences in the CFTR expression were evidenced. These differences, only in some cases could be linked to the CFTR mutated genotype (for example the presence of splicing mutations or stop codon). In other frequent cases, different mechanism of expression control seems to be involved, enhanced in differentiated cellular models. In fact, expression levels often higher than that of brushing are reached after the re-induction of differentiation in CF-CRC-AESC cells.

In the same 17 patients, also FOXI1 gene expression resulted to be high in brushing specimens and undetectable or very low in undifferentiated CF-CRC-AESC. However, at least 2 different patterns of FOXI1 expression re-induction after differentiation were found: 11 CF-CRC-AESC lines showed no or very low re-induction of FOXI1 expression, while 6 showed from moderate to high re-induction. As a general conclusion, the expression of FOXI1 gene resulted to be generally lower than that of CFTR gene and inducible at a lower extent after the re-induction of differentiation, with the exception of some patient-specific cells.

These different patterns of re-induction after differentiation suggest a complex mechanism of CFTR and FOXI1 transcriptional control. These results reinforce the need of further studies aimed to clarify the mechanism(s) of CFTR and FOXI1 transcriptional control and their interaction, as well as their role in respiratory epithelium differentiation.

Amplificatory strategy

An emerging class of modulators are the so-called "amplifiers". They point to the increase of the expression of CFTR mRNA and, consequently, of the biosynthesis of the CFTR protein. This amplificatory strategy may both enhance the functionality of CFTR in mutated genotypes with residual function and provide a greater amount of mutated CFTR to be corrected or potentiated by other modulators in a synergistic way. An experimental DNA hypomethylating drug (3-deazaadenosine, 3-DZA) has been used as an amplificatory strategy aimed at increasing the CFTR transcription. It is a metabolic DNA hypomethylating drug already used by us in other contexts. In addition to its use as possible amplificatory drug, in this project it has been also evaluated as an epigenetic effector of DNA hypomethylation possibly enhancing respiratory epithelium differentiation. To this aim, in addition to CFTR, as differentiation and ionocyte marker we used also the FOXI1 gene. Our results showed that the expression of both genes, CFTR and FOXI1 measured at mRNA level by both real-time and ddPCR in differentiative conditions, can be highly enhanced by the hypomethylating treatment in both F508del/F508del and wild-type CFTR genotypes.
The DNA hypomethylation seems to be promising as amplificatory strategy for CFTR, as well as differentiation- and ionocyte-inducing strategy. It can represent a possible future new precision therapy for CF, possibly in combination with modulatory therapy.

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Research Group

Giampiero Ferraguti, Researcher
Andrea Fuso, Associate Professor
Sabina Maria Bruno, Research fellow
Giovanna Blaconà, Research fellow
Giancarlo Testino, Laboratory technician

Collaborations

Adriana Eramo, Researcher, Italian National Health Institute
Giuseppe Cimino, Director, Regional (Lazio) Reference Center for Cystic Fibrosis

EPI-DRUGS FOR TREATMENT OF PARASITIC DISEASES

ANTONELLO MAI
RESEARCH AREA: Novel therapeutic interventions

Department of Chemistry and Technologies of Drugs
antonello.mai@uniroma1.it

Neglected tropical diseases (NTDs) including trypanosomiasis, leishmaniasis, and schistosomiasis, represent a great problem in terms of morbidity and mortality worldwide every year. Limits of the current antiparasitic drugs are toxicity, no efficacy toward all of the forms of the parasites' life cycle, and/or induction of resistance. Histone-modifying enzymes play a crucial role in parasite growth and survival; on the other hand, infected cells share many features typical of cancer cells, such as escape from the immunity system and metabolic dysfunctions. Thus, the use of epigenetic drugs, useful/approved for cancer treatment, can represent a strategy for the treatment of NTDs too. We tested nine structurally unrelated HDAC inhibitors from our lab against *Trypanosoma cruzi*, *Leishmania* spp, and *Schistosoma mansoni*. Among them, a cinnamic hydroxamate emerged as the most potent against all of the tested parasites, but it was too toxic against host cells, hampering further studies. The retinoic 2′-aminoanilide was less potent than the previous compound in all parasitic assays, but as its toxicity is considerably lower, it could be a starting point for further development. In *T. cruzi*, a HDAC6/8-selective inhibitor pyrrolyl hydroxamate exhibited a single-digit micromolar inhibition of parasite growth combined with moderate toxicity. In *S. mansoni*, four close analogs of the cinnamic hydroxamate were tested in new transformed schistosomula (NTS) and adult worms displaying high death induction against both parasite forms, with two of them showing very low toxicity in human retinal pigment epithelial (RPE) cells, thus being promising compounds for further optimization (Di Bello et al., ACS Infect. Dis. 2022). Further research on HDAC inhibitors regarded the investigation on the molecular mechanism of the antifibrotic role of HDAC1 inhibition. Specifically, treatment with MS-275, a specific HDAC1-3 inhibitor, promoted the mesothelial-to-mesenchymal (MMT) transition reversal by induction of the expression of miR-769-5p that has bound to its promoter the transcription factor Wilms' tumor 1 (WT1), a master gene controlling mesothelial cells development (Bontempi et al., Cell Death Dis. 2022). In another work, we described the identification and characterization of 1,4-dihydropyridines acting as either pan Sirtuin activators or specific stimulators of Sirt3 or Sirt5, both in enzyme and cellular assays (Suenkel et al., J Med Chem 2022). In addition to research on HDAC/SIRT modulators, we reported design, synthesis and biological evaluation of both covalent and non-covalent LSD1 inhibitors, the first series belonging to tranylcypromine- (TCP-)based compounds, in which the TCP was decorated with (hetero)arylbenzoylamino substituents at the phenyl ring (Fioravanti et al., J Enzyme Inhib Med Chem. 2022), the latter obtained by chemical optimization of C2-, C4- and/or C7-substituted quinazolines acting as

reversible, highly active, and selective LSD1 inhibitors as well as dual LSD1/G9a inhibitors (Menna et al., Eur J Med Chem 2022). Finally, insights into the mechanism of action of an our DNMT inhibitor, MC3343, able to arrest Ewing sarcoma cell proliferation and to enhance tumor cell sensitivity to DNA damaging drugs by activating the DNA damage response, were also described (Cristalli et al., Front Endocrinol 2022).

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Research Group

Sergio Valente, **Dante Rotili**, **Rossella Fioravanti**, **Clemens Zwergel**, **Francesco Fiorentino**, **Alessia Raucci**, **Chiara Lambona**: Researchers,
Martina Menna, **Beatrice Noce**, **Emanuele Fabbrizi**, **Carola Castiello**: PhD students

Collaborations

Kathy Andrews, Griffith University, Australia
Artur Scherf, **Paola B. Arimondo**, Institute Pasteur, Paris
Raymond Pierce, Institute Pasteur, Lille
Marcelo Fantappie, University of Rio de Janeiro, Brazil
Gerald Spaeth, **Eric Prina**, Institute Pasteur, Paris
Marina de Moraes Mourão, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil.

SMALL MULTIFUNCTIONAL MEMBRANE-ACTIVE PEPTIDES TO TACKLE DRUG-RESISTANT RESPIRATORY AND WOUND INFECTIONS: ADVANCES IN DELIVERY SYSTEMS AND IN VIVO EFFICACY

MARIA LUISA MANGONI
RESEARCH AREA: Novel therapeutic interventions

Department of Biochemical Sciences "A. Rossi Fanelli"
marialuisa.mangoni@uniroma1.it

The plight of antimicrobial resistance continues to limit the availability of antibiotic treatment effective in combating resistant bacterial infections and the search for new antimicrobial compounds is of major importance. The World Health Organisation list of "priority bacterial pathogens" includes the Gram-negative *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and the Gram-positive *Staphylococcus aureus*. The ability of these microorganisms to persist in hostile conditions is primarily associated with their tendency to transform from a drug-tolerant planktonic to a more dangerous and antibiotic-resistant sessile life form, called biofilm. They can colonize host tissues which are most exposed to the external environment, such as the respiratory tract, especially in cystic fibrosis (CF) patients and the skin.

Nature represents one of the primary sources of bioactive compounds. Among these, antimicrobial peptides (AMPs) from amphibian skin, such as esculentins and temporins, are a promising class of alternative molecules to fight drug-resistant bacteria, being able to perturb the microbial cell membranes without damaging human cells. In addition, they have the potential to act as adjuvants of available antibiotics. Among these latter, carbapenems are currently the main agents to treat *A. baumannii*-associated infections. However, since these strains can easily develop resistance to carbapenems, colistin has become one of the last-resort drugs. Nevertheless, the appearance of colistin-resistant strains also makes treatment of the Acinetobacter infections very challenging. Interestingly, we discovered that when the frog skin derived AMP Esc(1-21) is used in combination with colistin, the mixture has a synergistic effect in inhibiting the growth and in killing colistin-resistant strains. When used at dosages below the minimal inhibitory concentration (MIC), the two drugs are also able to potentiate their membrane-perturbing effect. To the best of our knowledge, this is the first case showing synergism between AMPs and colistin against colistin-resistant *A. baumannii* clinical isolates, highlighting the potential clinical application of such combinational therapy.

We previously demonstrated that Esc(1-21) and its diastereomer Esc(1-21)-1c (Esc peptides) are valuable candidates for treatment of *P. aeruginosa* lung infection, especially in patients with CF. Furthermore, engineered poly(lactide-co-glycolide) (PLGA) nanoparticles (NPs) were found to be a promising pulmonary delivery system of AMPs. However, the "ad hoc" development of novel therapeutics requires consideration

of their stability, tolerability, and safety. We have now proved that Esc peptides as well as Esc-peptide-loaded PLGA NPs do not affect the integrity of the lung epithelium, nor change the global gene expression profile of lungs of mice compared to those of vehicle-treated animals. These findings contribute to emphasize PLGA NPs as suitable nanocarriers for pulmonary drug delivery-

Due to the relevance of biofilms in clinical infections, the activity of AMPs against the biofilm forms of microbes is essential. We discovered that when used at sub-MIC doses, the two AMPs Esc(1-21) and Esc(1-18) are able to reduce the formation of biofilm of *Escherichia coli* O157:H7 strain, as supported by both microbiological assays and scanning electron microscopy. Transcriptional analysis in *E. coli* O157:H7 showed that both AMPs induce the expression of several genes controlling the bacterial motility. The overexpression of the flagellar system highlighted in our study could be responsible for the inhibition of the cascade control for the synthesis of curli (Figure 1) with consequent inhibition of bacterial adhesion to surfaces and development of biofilm.

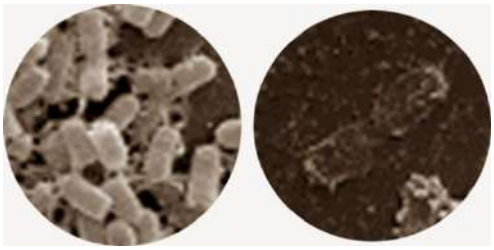

Figure 1.
Effects of Esc(1-21) at ½ MIC on biofilm formation of *E. coli* strain EDL933 after 24 h. Scanning electron microscopy (magnification 30,000×). Left image: control cells; right image: peptide-treated samples

The increasing resistance to antifungal drugs is also a widespread concern, and a selection of new compounds, active against different species of fungi, is demanded. Interestingly, we showed that the frog skin temporin G (TG) is active against (i) Candida species and Cryptococcus neoformans, with MIC_{50} between 4 μM and 64 μM after 24 h of incubation; (ii) dermatophytes with MIC_{80} ranging from 4 to 32 μM, and (iii) *Aspergillus* strains with MIC_{80} of 128 μM. The peptide reduced the metabolic activity of *Candida albicans* cells, with moderate membrane perturbation, and inhibited ~90% of yeast-mycelial switching, strongly preventing biofilm formation. Note that either *C. albicans* or *Aspergillus* spp have been associated with worse disease and lung function in CF.

To further understand the relationship between the physical-chemical properties of temporins and their antimicrobial activity/ selectivity, an analogue of temporin L, was designed by adding a norleucine residue at the N-terminus of the lead peptide sequence [dLeu9, dLys10]TL, previously developed. This modification promoted an increase of peptids's hydrophobicity and a β-type conformation in liposomes mimicking microbial membranes, making the peptide more active against both Gram-positive and Gram-

negative strains, without affecting the viability of mammalian cells. In addition, the increase of peptide hydrophobicity did not cause any loss of anti-inflammatory activity of the peptide in comparison with its lead compound. These results demonstrated that positive net charge, optimum hydrophobic-hydrophilic balance, and chain length remain the most important parameters to be addressed while designing small cationic AMPs.

In parallel, a de-novo peptide named KDEON WK-11, was designed building on previous work establishing effective residues and structures active in distinguished AMPs, such as lactoferrin. We assessed its antimicrobial activity against an array of bacterial strains and identified its highest activity against *P. aeruginosa* with a MIC value of 3.12 µM, lower than its counterparts developed with similar residues and chain lengths. We found out that KDEON WK-11 has a broad range of antimicrobial activity and specific capabilities to fight *P. aeruginosa* with low in vitro cytotoxicity and promising potential to express anti-lipopolysaccharide qualities, which could be exploited to expand its properties into an anti-sepsis agent.

Remarkably, our research work is also focussed on the antiviral activity of frog skin AMPs. Herpes simplex virus type-1 (HSV-1) and John Cunningham polyomavirus (JCPyV) are widely distributed DNA viruses causing asymptomatic infections, but also mild to very severe diseases, especially when these viruses reach the brain. Despite several drugs have been developed to inhibit HSV-1 replication in host cells, their prolonged usage can induce resistance. In contrast, there is no cure for JCPyV. Notably, we have discovered that TG, strongly affects HSV-1 replication by acting during the earliest stages of its life cycle and directly on the virion. Computational studies have revealed the ability of TG to interact with HSV-1 glycoprotein B (Figure 2).

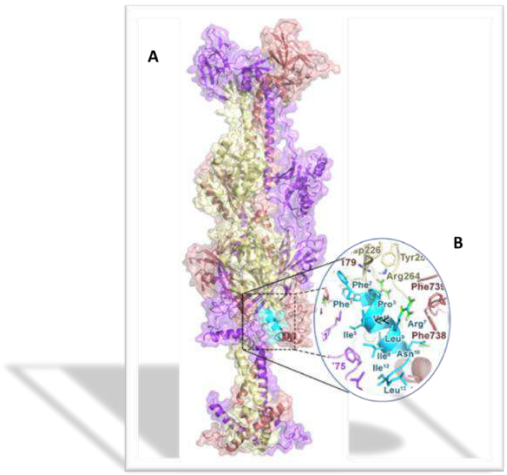

Figure 2.
(**A**) Model complex of the gB trimer and TG, as deduced by computational studies; gB is shown as a ribbon and in surface representations (the three monomers are in light orange, violet, and light yellow), while TG is shown as a blue ribbon.
(**B**) TG within the binding site of gB. Side chain of interacting residues are shown as sticks. The residue numbers of the peptide are also reported.

We also found that TG reduces JCPyV infection, probably affecting both the earliest phases of its life cycle and the viral particle, likely through an interaction with the viral capsid protein VP1. Altogether these results are promising for the development of short naturally occurring peptides as antiviral agents to counteract diseases related to HSV-1 and JCPyV and hopefully to other viral strains.
Importantly, significant inhibition activity against respiratory viruses (influenza and coronavirus including SARS-CoV2) has been discovered for lipidated temporins.

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Research Group

Bruno Casciaro, Assistant Professor (RTD-A)
Floriana Cappiello, Post-doc fellow
Maria Rosa Loffredo, Post-doc fellow
Elena Puglisi, PhD student

Collaborations

Maria Elena Marcocci, Sapienza University
Lorenzo Stella, Tor Vergata University
Francesca Ungaro, Paolo Grieco, University of Naples;
Loretta Ferrera, G. Gaslini, Genoa
Peter Di, University of Pittsburgh, USA
Francesca Ghirga, Sapienza University
Giammarco Raponi, Sapienza University

DISSECTING THE ROLE OF MAST CELL DURING THE TRANSITION FROM INTESTINAL INFLAMMATION TO COLON CANCER

ROSSELLA PAOLINI
RESEARCH AREA: Inflammation and immunity.
Cellular and molecular immunology

Department of Molecular Medicine
rossella.paolini@uniroma1.it

Mast cells (MCs) are tissue resident innate immune cells that act as sentinel of the surrounding environment thanks to the expression of a wide array of receptors. They are involved in physiological and pathological processes, such as infection and allergic response (1).
MCs are also frequently observed in tumors, suggesting their contribution in the transition from persistent inflammation to carcinoma. However, the exact role of MCs in tumorigenesis remains controversial: MC-derived mediators can either exert pro-tumorigenic functions, causing progression and spread of the tumor, or anti-tumorigenic functions, limiting tumor growth (2).
Particularly, there have been a considerable number of contradictory observations regarding the detrimental or protective roles of MCs in colorectal carcinoma (CRC) development, and the relationship between MCs, tumor progression and clinical outcome in patients with CRC have remained largely unclear (3). Consequently, a more comprehensive understanding of MC contribution in each stage of the disease is highly needed to unveil novel mechanisms underlying CRC pathogenesis.
To investigate the role of MCs during the transition from intestinal inflammation to CRC, we initially dissected MC phenotypes in a conventional mouse models obtained by an i.p. injection of azoxymethane (AOM) followed by administration of dextran sulfate sodium (DSS) in the drinking water for one week (4 cycles).
We characterized MC population within the tumor and in non-lesional colonic areas, by multicolour flow cytometry. After excluding CD3+/CD19+/CD11b+/NK1.1+/dead cells and positive gating for CD45+ cells, we observed an increase of c-kit/FcεRIsα double positive cells in colon of DSS-induced colitis mice respect to control mice (Figure 1A); moreover, we found a more pronounced accumulation in the tumor masses of AOM/DSS mice compared to inflamed colon and tumor-free surrounding tissue (Figure 1B). We then evaluated MC functionality by assessing the presence of several different cytokines by flow cytometric analysis upon intracellular staining. This analysis only revealed the presence of two MC pro-inflammatory cytokines, TNF-α and IL-6, with a much higher production of both cytokines in tumor-associated MCs compared to tumor-free tissue of AOM/DSS treated mice (Figure 1C).

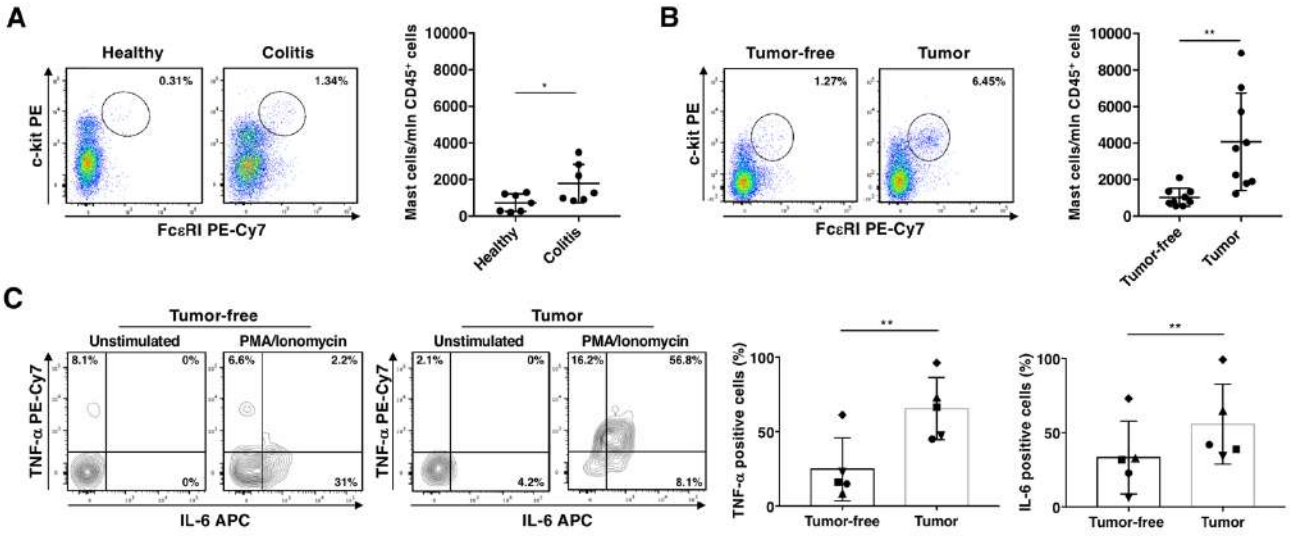


Figure 1. Evaluation of MC frequency and function in DSS and AOM/DSS mouse models. (A) Lamina propria cells were isolated by enzymatic digestion from colon of untreated mice (Healthy) and of DSS-induced colitis mice (Colitis). (B) Upon AOM administration and 4 cycles of DSS treatment, tumors/polyps (Tumor) were dissected out and tumor-infiltrating cells were isolated by enzymatic digestion. Cells isolated from the tumor-free colonic counterpart of AOM/DSS treated mice were used as control (Tumor-free). (A and B) MCs were identified as c-kit/FcεRI double positive cells gated on CD45+/CD19-/CD3-/NK1.1-/CD11b- cells. Representative dot plots (left panels) and number of MCs expressed as mean +/- SD of the mice analysed (right panels) are shown. (A) Unpaired Student' t test *$p<0.05$. (B) Paired Student' t test **$p<0.01$. (C) Cells were stimulated with PMA/Ionomycin for 2h and the expression of TNF-α and IL-6 was analyzed on c-kit+FcεRI+ MCs by flow cytometry. A representative dot plot is shown on the left. The right graphs show the percentage of cytokine-positive MCs upon PMA/Ionomycin stimulation. Each graph is representative of 2 independent experiments with 5 mice/group. Each symbol represents data obtained from an individual mouse and the same group of mice are indicated with the same symbol. Paired Student' t test **$p<0.01$.

To characterize the subtype of tumor-associated MCs, paraffin-embedded colon sections of AOM/DSS treated mice were stained with antibody against mMCP1, which is specifically expressed by mucosal MCs, or with antibody against mMCP4, a chymase observed into the secretory granules of connective tissue MCs and analysed by confocal microscopy. Tumor-associated MCs mainly express mMCP4 whereas reduced frequencies of mMCP4+ MCs and increased number of mMCP1+ MCs were found in the adjacent tumor-free tissue (Figure 1D). Similarly, MCs observed in colon sections from DSS-induced colitis mice were positive for mMCP4 (not shown).

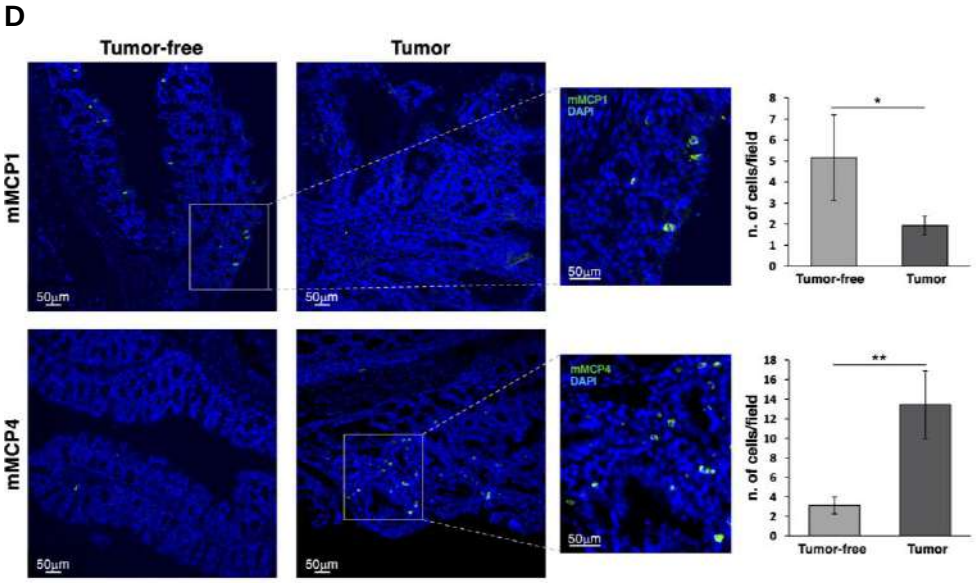


Figure 1D. Evaluation of MC frequency in AOM-DSS mouse model. Immunofluorescence of colon paraffin-embedded sections from AOM/DSS treated mice were stained with anti-MCP1 or anti-MCP4 Abs followed by Alexa Fluor 488 secondary Abs (green). Nuclei were counterstained with DAPI (blue). Images were acquired with 20x objective. The frequencies of MCs positive for mMCP1 and mMCP4 analysed in 20 fields randomly acquired from three independent experiments are shown (right panels) and classified as Tumor-free or Tumor based on normal or aberrant crypt architecture, respectively. Unpaired Student' t test *p<0.05; **p<0.01.

Altogether our findings demonstrate the accumulation of a connective tissue-like MC phenotype which through the production of IL-6 and TNF-α likely contribute to the establishment of a pro-inflammatory tumor microenvironment.

In the intestinal microenvironment, during CRC progression the c-kit ligand SCF and the alarmin IL-33 are both abnormally expressed and are considered biomarkers of poor prognosis for their pro-tumorigenic action (4-6). Thus, we first investigated the presence of SCF and IL-33 on colon tissue lysates obtained from DSS and AOM/DSS treated mice. Higher levels of SCF were observed on tumor lesions compared with tumor-free tissue (colon AOM/DSS) and inflamed colon tissue (colon DSS), while accumulation of IL-33 was detected on both inflamed and tumor lesions (data not shown).

IL-33 elicits a complex between its receptor and c-Kit supporting the finding that in the tumor microenvironment IL-33 and SCF can act synergistically to activate MC signaling (7).

To dissect the mechanism(s) by which tumor-derived SCF and IL-33 regulate MC phenotype and function, we generated primary bone-marrow derived mast cells (BMMCs), as previously described (8,9). Briefly, BM-derived cells were either cultured with IL-3 alone or in combination with SCF, and upon 4 weeks of culture the generation of pure BMMCs was monitored by flow cytometry evaluating the expression of FcεRI, c-kit and IL-33R.

To better reflect the phenotype of intestinal MCs, we have also generated primary cultures from isolated peritoneal committed MCs grown in the presence of IL-3 and SCF for 10 days (PDMCs).

BMMCs and PDMCs differentiated in the presence of SCF show a predominant connective-like phenotype characterized by high transcription levels of mMCP4, 5 and 6

in respect to BMMCs differentiated in the presence of IL-3 alone showing a prevalent mucosal phenotype.
Upon differentiation, SCF was unable to further affect protease content (not shown) while IL-33 stimulation was accompanied by dynamic changes in MC-protease expression with a selective increase of mMCP4 levels, leaving almost untouched the transcriptional levels of mMCP1 (Figure 2).

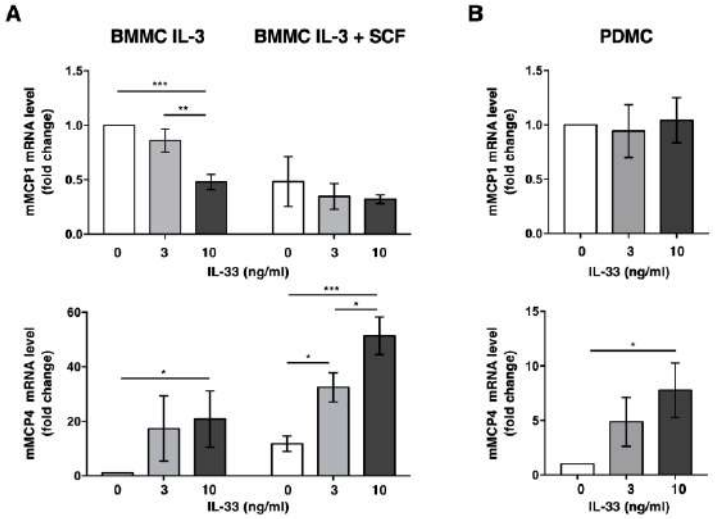


Figure 2. Dynamic changes in protease transcripts along BMMC and PDMC cultures upon IL-33 stimulation.
BMMCs (A) were differentiated from BM-precursors cultured in the presence of IL-3 alone (30 ng/ml) or in combination with SCF (25 ng/ml) while PDMC (B) were differentiated from isolated peritoneal committed MCs cultured in the presence of IL-3 (30 ng/ml) and SCF (25 ng/ml) for 10 days.
BMMCs and PDMC were stimulated with the indicated doses of IL-33 for 48h and the expression levels of mMCP1 and mMCP4 mRNA transcripts were evaluated by real-time PCR. (A) The amount of mRNA expressed in unstimulated BMMC differentiated with IL-3 alone was arbitrary set to 1. Means +/- SD of three independent experiments are shown as fold change. One-way ANOVA *p<0.05; **p<0.01; ***p<0.001. (B) The amount of mRNA expressed in unstimulated PDMC was arbitrary set to 1. Means +/- SD of three independent experiments are

Of note, after stimulation with SCF and IL-33 in combination, a superimposable production of IL-6 and TNF-α was observed in all primary MC cultures, demonstrating a synergistic effect of SCF and IL-33 in MC activation (Figure 3).

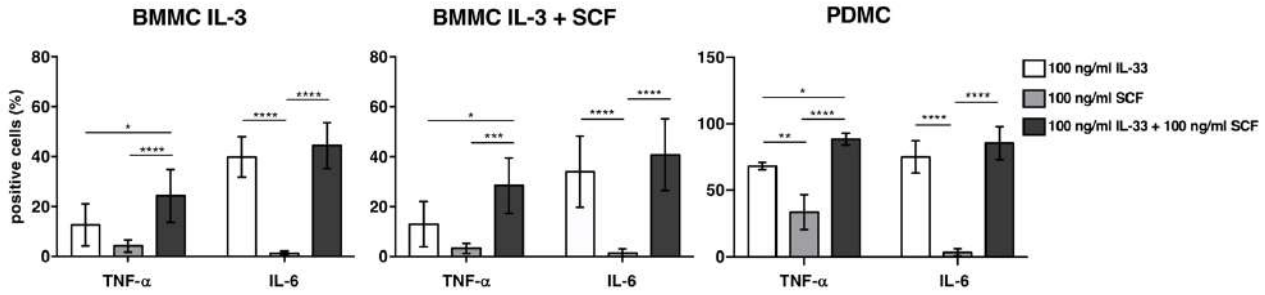
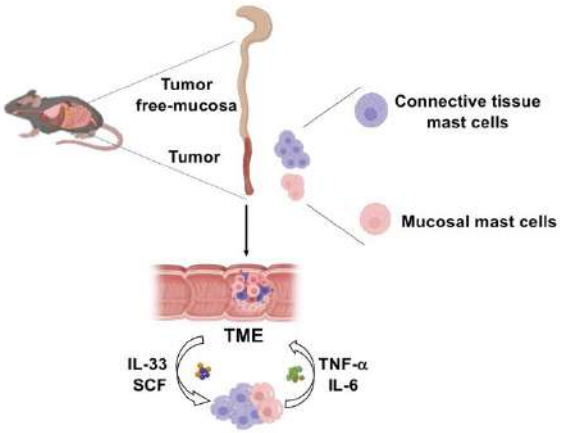


Figure 3. SCF and IL-33 stimulate cytokine production on primary MC cultures.
BMMC differentiated in IL-3 + SCF and PDMC primary cultures were starved overnight from SCF and then stimulated with 100 ng/ml of IL-33 or SCF alone or in combination for 6 h in the presence of Brefeldin A. TNF-□ and IL-6 expressions were analysed by flow cytometry on permeabilized cells. Means +/- SD of three independent experiments are shown as percentage of cells positive for the indicated cytokines.
One-way ANOVA *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Collectively, these results demonstrate the capability of IL-33 to induce a connective tissue-like MC phenotype and suggest a combined action of SCF and IL-33 in shaping MC plasticity in vivo, as depicted in our working model.



All these results are part of a manuscript entitled "SCF and IL-33 regulate mouse mast cell phenotypic and functional plasticity supporting a pro-inflammatory microenvironment" under consideration for publication in Cell Death & Disease.

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Research Group

Rosa Molfetta Researcher
Erisa Putro PhD student
Caterina Marangio PhD student

Collaborations

Giuseppe Sciumè, Researcher
Giuseppe Pietropaolo, Post-doctoral fellow
Enrico De Smaele, Researcher
Giovanni Bernardini, Researcher

TRIPLICATION OF miR802 IN DOWN SYNDROME: THE GENETIC LINK BETWEEN METABOLIC DEFECTS AND DEMENTIA

MARZIA PERLUIGI
RESEARCH AREA: Genetics, biology and pathophysiology of eukaryotes

Department of Biochemical Sciences
marzia.perluigi@uniroma1.it

Insulin resistance (IR) is a condition in which the body does not respond appropriately to circulating insulin. It is common in various metabolic disorders, such as diabetes, obesity, hypertension and coronary heart disease. IR occurs in a number of tissues, including liver, muscle, fat and brain (1). This latter is growing increasing attention as a key molecular aspect driving neurodegeneration in several age-associated disorders, including Alzheimer disease (AD). Accordingly, AD neurodegeneration is associated with energy imbalance, dysregulated lipid and carbohydrate metabolism, cytokine mediated inflammation, increased oxidative and other types of cellular stresses, cell death, and vascular degeneration. The finding that these abnormalities are also present in Type 2 diabetes mellitus (T2DM) and metabolic syndrome, supports the concept that insulin-resistance diseases are all inter-related, could have the same root causes, and may be treated by similar if not identical therapeutic strategies.
Indeed, AD should be regarded as a brain form of diabetes in which insulin resistance and deficiency develop either primarily in the brain, or due to systemic insulin resistance disease with secondary involvement of the brain. Nearly all pathologies in AD, including the typical Aß42 and phospho-tau containing, PHF-associated structural lesions, metabolic dysfunction, neuro-inflammation, cellular stress, synaptic disconnection with proliferation of dystrophic neurites (reflecting loss of neuronal plasticity), cell death, white matter atrophy and degeneration, and microvascular disease, could be attributed to impairments in insulin and IGF signalling.
Considering that Down Syndrome (DS) share many common features of AD neuropathology and that previous studies from our group and others have shown molecular markers of IR in DS post mortem brain that are associated with increased oxidative stress, decreased mitochondrial activity and energy metabolism, we made an attempt to identify putative Chr21 gene candidates that might play a causative role in the onset of IR in DS.
Previous studies from Bruning J and collaborators reported for the first time that the expression of miR-802 (encoded by Chr21) was increased in the liver of two obese mouse models and obese human subjects. The authors suggested that overexpression of miR-802 can cause IR and impairs glucose tolerance, whereas downregulated miR-802 expression in obese mice improved these metabolic parameters, suggesting that higher miR-802 expression results in an increased risk of obesity-associated IR. As well, inducible transgenic overexpression of miR-802 in mice caused impaired glucose

tolerance and attenuated insulin sensitivity, whereas reduction of miR-802 expression improved glucose tolerance and insulin action.
Based on these notions, we aim to investigate whether miR-802 over-expression represents a genetic alteration promoting brain-IR development and AD- onset both in DS and in the general population.
RESULTS. Preliminary results showed a significant increase of miR-802 expression levels in the frontal cortex of DS cases compared to control cases (figure 1) . Further, we measured the expression levels of miR-802 in the frontal cortex of Ts65Dn (a well-established model for DS), at 3 and 9 months, corresponding to young and adult age (Figure 1). We observed a significant increase of miR-802 expression levels along with inhibition of IRS1, a well-known marker of brain- IR, at 9 months of age in Ts65Dn mice compared to euploid mice, despite no significant changes were observed at 3 months of age.

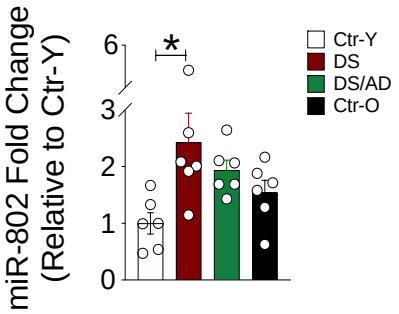


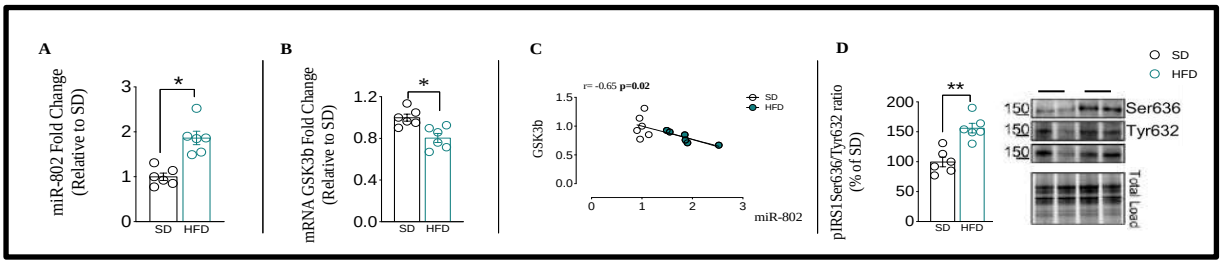
Figure 1. miR-802 expression levels in Frontal Cortex of DS cases compared with their age matched controls (Ctr young, Ctr-Y). (Data are presented as means ± SEM, **p < .01, (Student t-test n=4).

All together our data showed a strong link between brain-IR and miR-802 over-expression in both human and Tg mice. To gain insights into the mechanism(s) of miR-802-mediated development of brain-IR, we used a stringent bioinformatic approach to identify miR-802 target genes. Though bioinformatic tools were able to identify hundreds of targets for murin and human miR-802, we focused on selected Thus, it will be important to unravel the molecular events by which over-expression of miR-802 lead to IR and contributes to AD development both in DS and in the general population, where miR-802 over-expression has been observed. In detail, our analysis revealed two main genes involved in IS: Glycogen synthase kinase 3 ß (GSK3ß) and Phosphatase and tensin homolog (PTEN). Because PTEN negatively regulates the PI3K signaling downstream from IRS1 and GSK3ß regulates brain energy metabolism in response to insulin, we hypothesize that by blocking their transcription, could promote an impairment of IS. To prove this hypothesis, we measured miR-802 and GSK3ß mRNA levels in the brain of C57Bl/6j treated with high fat diet (HFD, 60% kcal from fat), known to promote brain-IR. Our preliminary results show that HFD-treated mice are characterized by a significant increase of miR-802 levels in the frontal cortex compared with mice treated with a chow diet (10% kcal from fat). Furthermore, higher miR-802 levels are significantly associated with reduced GSK3ß mRNA levels and IRS1 inhibition in the frontal cortex, thus supporting our hypothesis about a role for miR802 in brain-IR

development. Accordingly, a recent study reported that miR-802 over-expression downregulated insulin transcription and secretion and impaired glucose metabolism in pancreatic islets of HFD and obese.

Figure 2. (A) miR-802 expression levels in High fat diet (HFD) treated mice compared with standard diet (SD). (B) mRNA levels of GSK3beta in HFD-treated mice compared with standard diet (SD). (D) increased phosphorylation (ser36) of IRS1 in HFD treated mice compared with standard diet (SD)

(Data are presented as means ± SEM, **p <0 .01, (Student t-test n=6).



Publications.

Pagnotta S, Tramutola A, Barone E, Di Domenico F, Pittalà V, Salerno L, Folgiero V, Caforio M, Locatelli F, Petrini S, Butterfield DA, Perluigi M. **CAPE and its synthetic derivative VP961 restore BACH1/NRF2 axis in Down Syndrome.** *Free Radic Biol Med*. 2022 A;183:1-13

Research Group

Marzia Perluigi

Antonella Tramutola

Sara Pagnotta

PhD student
Lucrezia Romana Rolfi

Collaborations

Prof. DA Butterfield – The Alumni Association Endowed Professor of Biological
Chemistry, Director, Redox Metabolism Shared Resource Facility, Markey Cancer Center at
University of Kentucky (USA)
Prof. Elizabeth Head – Professor, Vice Chair for Research, Pathology & Laboratory
Medicine; Director, Experimental Pathology PhD Program University of California Irvine (USA)

ROLE OF MICROGLIA ON SYNAPTIC ADAPTATIONS IN THE NUCLEUS ACCUMBENS DURING ABSTINENCE FROM COCAINE: FROM MECHANISMS TO STRATEGIES FOR DRUG RELAPSE PREVENTION

DAVIDE RAGOZZINO
RESEARCH AREA: Novel therapeutic interventions

Department of Physiology and Pharmacology
Sapienza University or Rome
Piazzale Aldo Moro 5, 00185, Rome
davide.ragozzino@uniroma1.it

Most preclinical studies of cocaine substance use disorder have focused on neuronal mechanisms, but have barely considered microglial mechanisms, despite accumulating correlational evidence implicating microglia in the development and maintenance of drug addiction. This is an important gap in knowledge because studies on the interplay between microglia and synaptic plasticity in the context of craving may reveal novel targets for relapse prevention.

Cocaine-induced behavioral alterations are the result of modifications in synaptic connectivity and synaptic strength in NAc medium spiny neurons (MSNs). Two critical neuroadaptations observed after protracted cocaine withdrawal are changes in spine morphogenesis and synaptic calcium-permeable (CP-AMPAR) accumulation. The persistent accumulation of CP-AMPAR in NAc MSNs has been associated to the appearance of drug-induced behavioral adaptations, such as locomotor sensitization and incubated cue-induced cocaine seeking, contributing to the maintenance of addictive behaviors and increasing propensity of relapse.

The main objective of the proposal was to investigate the role of microglia, the resident innate immune cells of the central nervous system, as putative players for the maturation of silent synapses during cocaine withdrawal. Our central hypothesis was that microglia-neuron interactions dependent on fractalkine/CX3CR1 signalling contribute to the maturation and plasticity of silent glutamatergic synapses in the NAc, which embed critical memory traces that promote cue-induced cocaine craving.

Research results:

1) We found that microglia are not necessary for the maintenance and retrieval of drug-context associative memories. Both mice with microglia depletion and transgenic mice deficient of the fractalkine receptor (CX3CR1 knockout) acquired and maintained the memory of the Cocaine Conditioned Place Preference to a similar extent than control mice.

2) On the other hand, our results suggest that microglia contribute to the sensitization effects of drug-associated cues. Indeed, microglia depletion prevented the increase in locomotor activity displayed by cocaine control mice.

3) We confirmed our preliminary results showing that microglia contribute to the maintenance and growth of new immature spines induced by cocaine experience.

4) We extended these results with electrophysiological recordings confirming our hypothesis that microglia contribute to the maturation of synapses during withdrawal from cocaine. Indeed, microglia depletion prevented CP-AMPAR accumulation in the NAc.

These results have been presented in poster format (S03-297) at FENS Conference in Paris celebrated 9-13 July 2022 and at Research Activity Retreat of the Faculty of Pharmacy and Medicine of Sapienza University, February 2023.

We are currently preparing a manuscript to publish these results (see below).

Future directions: Based on the results reported above, we are currently investigating the specific role of fractalkine (CX3CL1)/CX3CR1 signaling on the electrophysiological properties of NAc excitatory synapses and incubation of craving after cocaine withdrawal.

Publications

Reverte I, Marchetti C, Pezza S, Ferruci L, D'Ottavio G, Pignataro A, Raspa M, Venniro M, Ramsey LA, Caprioli D, Ragozzino, D. **Microglia contribute to calcium-permeable AMPA receptor accumulation in the nucleus accumbens during withdrawal from cocaine** *(in preparation)*

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Research Group

PI: Davide Ragozzino, Full Professor, Dept. of Physiology and Pharmacology, Sapienza University of Rome, Rome, Italy
Daniele Caprioli, Associate Professor, Dept. of Physiology and Pharmacology, University of Rome, Rome, Italy
Silvia Marinelli, Group leader, European Brain Research Institute (EBRI), Rome, Italy
Ingrid Reverte, Researcher, Dept. of Physiology and Pharmacology, University of Rome, Rome, Italy
Laura Ferrucci, Post-doctoral fellow, Dept. of Physiology and Pharmacology, University of Rome, Rome, Italy
Ginevra D'Ottavio, PhD student, Dept. of Physiology and Pharmacology "V. Erspamer", University of Rome Sapienza

Collaborations

Marie-Eve Tremblay, Associate professor, University of Victoria | UVIC · Division of Medical Sciences, Victoria, British Columbia, Canada.

“ANNA TRAMONTANO” RESEARCH PROJETCS – CALL 2020

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JAGGED1, A NOVEL NON-CANONICAL PLAYER IN PANCREATIC CANCER. IDENTIFICATION OF NOVEL TARGET DRUGS

DIANA BELLAVIA

RESEARCH AREA: Genetics, biology and pathophysiology of eukaryotes

Department of Molecular Medicine
diana.bellavia@uniroma1.it

Jagged1 is a single-pass transmembrane protein, belonging to the Delta-Serrate-Ligand (DSL) family, which transactivates the Notch receptors through a cell-cell contact (1). Increased expression of Jagged1 occurs in several human malignancies and correlates with cancer progression, poor prognosis and recurrence (2-3-4-5). Recent works indicate that Jagged1 is processed by sequential proteolytic cleavages that involve ADAM-17/TACE and PS/γ-secretase complex, resulting in the release of Jagged1 intracellular domain (J1-ICD) that may play an important role in tumor development and carcinogenesis (6-7-8).

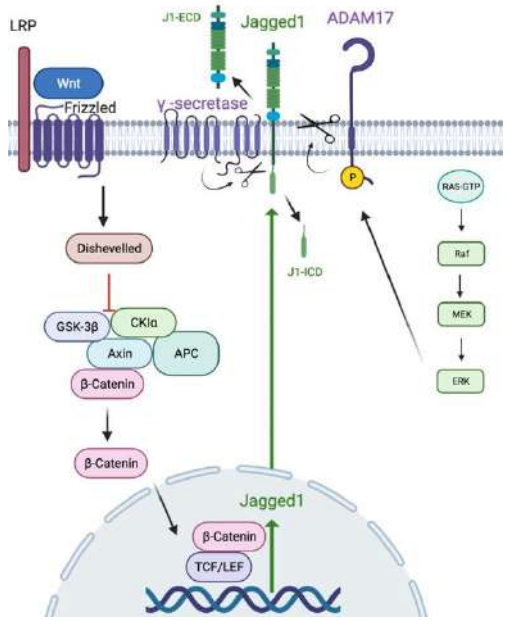

Figure 1. Schematic representation of J1-ICD processing/activation in CRC.

We previously reported novel findings about Jagged1 role in CRC tumors: β-catenin/TCF is responsible of a direct regulation of Jagged1 expression (9) and the oncogenic K-Ras controls ADAM17 activity *via* MEK/Erk/ADAM17 signaling axis, increasing the processing of J1-ICD (Figure1), which can translocate into the nucleus and favors the expression of genes correlated with proliferation, EMT, chemoresistance and decreases the apoptotic-related genes (10-11). Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant tumor with poor prognosis, and early diagnosis is difficult because of the lack of obvious symptoms and signs (12). PDAC is characterized by near-universal K-Ras constitutive activation (90% of cases) and frequent deregulation on different pathways, such as Wnt-βcatenin. K-Ras mutation is essential for the formation of pancreatic intra-epithelial neoplasias (PanINs) and strongly contributes to tumor progression. In addition, although β-catenin accumulation is not a universal characteristic of this disease, both nuclear and cytoplasmatic up-regulation of β-catenin are reported in many cases of PanINs and PDAC, and its increased levels correlate with PanINs grade and progression to PDAC (13-14-15). Interestingly, it is also demonstrated that Jagged1 is

overexpressed in pancreatic cancer, and it is associated with poor differentiation, tumor size, metastasis, invasion and high TNM stage in PDAC patients (16). Based on this observation, the aim of the project is to study the impact of Jagged1 and its intracellular fragment, J1-ICD, in PDAC, focusing on its role in sustaining the onset and the progression of the tumor.

We first assessed the relevance of Jagged1 in PDAC patients by *in silico* analysis and found that there is a positive correlation between K-Ras and Jagged1 in the same tumor samples from PDAC patients, and that patients with high Jagged1 expression levels have an unfavorable clinical outcome. We then monitored the Jagged1 protein expression *in vitro* in several PDAC cell lines with a K-Ras signature, such as KP4, PANC-1, HPAF-1, CAPAN-1 and PATU8902, and observed that the protein was upregulated in almost all of them, and target of a constitutive proteolytic processing, resulting in the release of J1-ICD fragment, localized into the nucleus of all PDAC cell lines. Furthermore, although the WT ductal pancreatic cell line, H6C7, also expresses Jagged1, the fragment, J1-ICD, is mainly localized to the cytoplasmic compartment, suggesting a different activity of the protein in a non-tumorigenic context. We also demonstrated that J1-ICD processing in PDAC is induced by K-Ras signaling and mediated by ADAM17/TACE, inhibiting the MEK/Erk/ADAM17 signaling axis by using U0126 (ERK inhibitor) and/or MK8353 (MEK inhibitor) and TAPI2 for ADAM17/TACE. Then, we performed J1-silencing in PDAC cell lines, resulting in a low proliferation rate, a decrease in EMT-related genes such as *Snail1* and *Snail2* and an increase in pro-apoptotic genes compared to scrambled sample. Starting from these observations, we generated PDAC cellular models: 1) J1-ICD overexpressing cell lines, by stable transfection (PDAC-Jag1-ICD) and 2) Jagged1-depleted cell lines by using Crispr-Cas9 technology (PDAC-Jag1-CRISP). We analysed both PDAC-Jag1-ICD and PDAC-Jag1-CRISP cell lines by evaluating proliferation, cell cycle, EMT and apoptosis. Using cell counting, MTT assay, qRT-PCR, Wound-Healing assays and FACS-Calibur analysis, we found that J1-ICD correlated with a high proliferation rate, an upregulation of EMT and anti-apoptotic related genes when compared to empty vector. On the other side, silencing of the full-length protein was associated with a decrease in proliferation, EMT-related genes, a lower recovery of the scratch, associated to an upregulation of pro-apoptotic genes and cell cycle arrest compared to the control sample.
The preliminary data reported here suggest a complex role of Jagged1 in PDAC initiation/progression. Our goal will be to unravel the canonical and non-canonical role of Jagged1 in PDAC onset and progression.

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Research Group

Maria Pelullo, PostDoc.
Sabrina Zema PostDoc.
Mariangela De Carolis, PhD student.

Collaborations

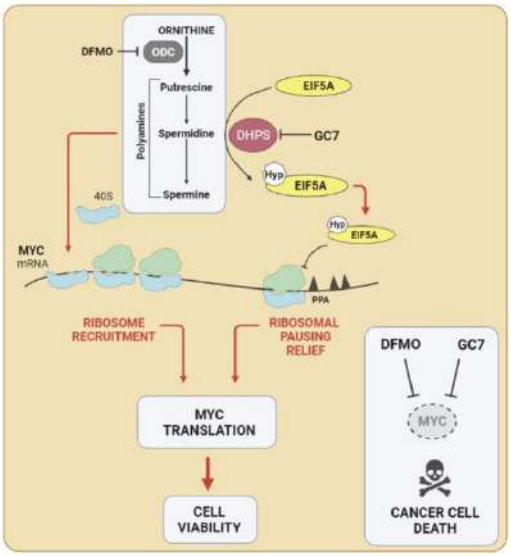
Prof. Giulia Franciosa, University of Copenhagen Novo Nordisk Foundation Center for Protein research
Prof. Bruno Botta, Dipartimento di Chimica e Tecnologia del Farmaco. Sapienza Università di Roma.

TARGETING REDOX STATE IN COLORECTAL CANCER

GIANLUCA CANETTIERI
RESEARCH AREA: Genetics, biology and pathophysiology of eukaryotes

Department of Molecular Medicine
gianluca.canettieri@uniroma1.it

Colorectal cancer (CRC) is a major cause of death from cancer worldwide. Despite the progresses made with early diagnosis and improvement of therapeutic protocols, the prognosis, specially at in the advanced stages, is still poor and requires a better understanding of the molecular determinants of CRC pathogenesis to find novel therapeutic opportunities.
Like other types of cancer, CRC is characterized by specific metabolic requirements, which may also represent potential actionable therapeutic vulnerabilities.
The main goal of this project is to identify specific metabolic and redox alterations in CRC and to identify novel pharmacological and nutritional strategies targeting metabolic deregulations.
During the year 2022 we have studied the contribution to colorectal tumorigenesis of polyamines, small polycations indispensable for cell survival, whose metabolism is tightly regulated by the enzyme Ornithine Decarboxylase (ODC). We previously found that elevated polyamines promote colorectal cancer in part by activating DHPS-mediated hypusination of the translation factor eIF5A, thereby inducing MYC biosynthesis. During this year we have discovered that combined inhibition of ODC (with DFMO) and eIF5A (with GC7) induces a synergistic antitumor response in CRC cells, leading to MYC suppression. We found that genes of the polyamine biosynthesis and hypusination pathways are significantly upregulated in colorectal cancer patients and that inhibition of ODC or DHPS alone limits CRC cell proliferation through a cytostatic mechanism, while combined ODC and DHPS/eIF5A blockade induces a synergistic inhibition, accompanied to apoptotic cell death in vitro and in mouse models of CRC and FAP. Mechanistically, we found that this dual treatment causes complete inhibition of MYC biosynthesis in a bimodal fashion, by preventing translational elongation and initiation. Together, these data illustrated a novel strategy for CRC treatment, based on the combined suppression of ODC and eIF5A, which holds promise for the treatment of CRC (Coni at al **Cancer letters 2023**).



We have also focused our attention on the alterations of glucose metabolism and the so-called Warburg effect, which consists in the ability of cancer cells to produce lactate through glycolysis, regardless to the presence oxygen in the extracellular environment. We have identified a novel selective and powerful class of Lactic Dehydrogenase A (LDHA) inhibitors capable of efficiently counteract the growth of CRC and other tumors (Di Magno et al **Eur J Med Chem 2022**) and to alter NADH homeostasis. We are currently characterizing novel derivatives of the same class of compounds and testing their efficacy in CRC cells as well as in cultured circulating tumor cells obtained from metastatic patients.
In parallel with these studies, we are investigating the redox alterations in response to biguanides (Di Magno et al., **Cancers 2022**) and are currently characterizing the mechanism of action and the effectiveness of metformin and phenformin administered in combination with different nutritional approaches (i.e. normal, high fat, ketogenic diets) (Di Magno et al., **in preparation**).

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Research Group

Researchers;

Sonia Coni, PhD
Laura Di Magno, PhD

Post Doc

Rosa Bordone, PhD

PhD students

Silvia Ripa
Lavinia Rutigliano
Devon Ivy
Tomoaki Tahara
Giorgia Della Rocca
Rodrigo D'Amico

Collaborations

Gregory Steinberg, McMaster University, Hamilton, CA
Natalia Del Galdo-Riobo, University of Leeds, UK
Robert Screaton, University of Toronto, CA
Paola Muti, University of Milano

CONJUGATIVE PLASMIDS SELECTABLE BY SHORT FRUCTOOLIGOSACCHARIDES AS VEHICLES OF GENETIC INFORMATION INTO MICROBIOTA

ALESSANDRA CARATTOLI
RESEARCH AREA: Microbiology

Department of Molecular Medicine
alessandra.carattoli@uniroma1.it

Plasmids are circular molecules of DNA that can replicate autonomously in bacterial cells. The
current genetic engineering technology is based on plasmids that contain an origin of replication and a resistance marker and can be introduced into bacterial recipient strain by transformation. We invented and patent in 2019 a new type of plasmid vectors (pFOS) that do not contain antibiotic resistance genes as selection markers but can be selected by a diet enriched with scFOS, short chain fructooligosaccharides. The scFOS are a group of linear fructose oligomers already available as prebiotics on the market. Metabolizing these sugars, a non-pathogenic *Escherichia coli* strain carrying the pFOS vector, is thought to have a selective advantage against other enterobacteria, transiently colonizing the gut. This project aims to test conjugation abilities of pFOS plasmids and a wider bacterial host range to measure conditions of selection and stability of the constructs, thinking that this plasmid may become a good genetic vector for future applications of microbiome genetic engineering.

This project is also based on knowledge of the plasmid content of well-known bacteria to be used as recipient in pFOS testing. The analysis of plasmids responsible of the spread of multi-drug resistance is a priority of the project to optimize the conjugation experiments and to correctly address the question of selection and stability of pFOS conjugants.

In the first semester we have completed the genome sequencing of several strains of *Klebsiella pneumoniae*, one of the greatest threats to public health. We studied *K. pneumoniae* isolates belonging to sequence type (ST) 37 that have been listed as a high-risk multidrug-resistant clone, typically linked to the dissemination of the 16S rRNA methylases RmtB and ArmA, this ST is poorly represented in both genomic databases and the scientific literature. In this period, we dissected an outbreak of ceftazidime–avibactam-resistant isolates belonging to ST37, comparing their genomes with others from different times and places belonging to the same ST. ST37 was the endemic clone in our hospital 10 years ago and nowadays it is striking back, with four KPC-producing isolates resistant to ceftazidime–avibactam (CZA). Ceftazidime-avibactam is a new combination of a cefalosporin (ceftazidime) with an inhibitor of class A carbapenemases,

like KPC. The new drug combination has been introduced in Italy in 2018, but used to treat KPC-producing *K. pneumoniae*, causing severe infections starting in January 2019. The ST37 isolates we studied were among the first *K. pneumoniae* reaching resistance to the new CZA combination. Isolates showing CZA-resistant phenotype were tested using CZA gradient test (Liofilchem). CZA-resistant isolates carrying bla_{KPC} were subjected to Whole genome sequencing by Illumina MiSeq (Illumina). Illumina reads were assembled using the SPAdes software. Genomes of strains of the contemporary ST37 clone were compared with thirty-nine genome sequences downloaded from the GenBank database to build a core genome alignment. Representative isolates of contemporary and historical ST37 isolates were also subjected to Illumina and Oxford Nanopore Technologies (ONT) sequencing in our laboratory. Illumina reads and ONT assemblies were integrated by the Unicycler tool version 0.4.8.0 using a bold bridging mode. This approach allowed the identification and complete assembly of many plasmids co-resident with the ST37 isolates. Synteny maps of the plasmids in ST37 isolates were created by blastn and visualized using the Circos tool. In particular, the plasmid named pKpQIL carrying the bla_{KPC} gene is responsible of the horizontal gene transfer of the bla_{KPC} gene in different *K. pneumoniae* strains. In the CZA-resistant ST37 strain, a novel variant of KPC has been discovered, named KPC-110 by the NCBI GenBank. In this variant an amino acid substitution occurring in the omega-loop of the protein caused an increment in the ceftazidimase activity of the enzyme and a reduction of the inhibition exerted by the Avibactam, resulting in CZA resistance. We endeavour to reconstruct the evolution of this clone and compare contemporary and historical isolates, showing the emergence of the new KPC variant in the pKpQIL plasmid. This strain represents a good example of plasmids circulating in this highly-resistant clones, whose stability could be perturbed using derivative of the pFOS plasmid.

Research Group

Dr. Gabriele Arcari Researcher;
Riccardo Polani PhD student
Valerio Capitani PhD student
Rita Trirocco PhD

Collaborations

Prof. Bianca Colonna , Dep. Charles Darwin Sapienza University of Rome
Prof. Gianni Prosseda , Dep. Charles Darwin, Sapienza University of Rome
Dr. Giammarco Raponi, Dep Public Health, Sapienza University of Rome

STUDYING THE MOLECULAR BASES THAT UNDERLIE THE CONSERVED FUNCTIONAL RELATIONSHIP BETWEEN HETEROCHROMATIN PROTEIN 1a (HP1a) AND NIJEMENGEN BREAKAGE SYNDROME 1 (NBS1) PROTEIN

GIOVANNI CENCI

RESEARCH AREA: Genetics, biology and pathophysiology of eukaryotes

Department of Biology and Biotechnologies
giovanni.cenci@uniroma1.it

We have recently reported an unanticipated and conserved functional relationship between HP1 and the MRN complex (Bosso et al., 2019). We have shown that *Drosophila* HP1a binds the MRN complex and that its levels are reduced upon the loss either of Rad50, Mre11 or Nbs. However, HP1a-encoding *Su(var)205* gene genetically interacts only with *nbs* in maintaining chromosome integrity. Interestingly, also loss of human NBS1 reduces HP1α levels. Molecular docking simulations and experimental data indicate that the pentapeptide-like motif PGPSL found in NBS1 binds the CSD of HP1α similarly to other HP1α interactors. Unexpectedly, the expression of hypomorphic NBS1 protein variants in Nijmegen Breakage Syndrome (NBS) patient cells causes the accumulation of HP1α and very likely delays its turnover. The NBS genetic disease shares several features with ataxia-telangiectasia (A-T), such as a high sensitivity to IR and predisposition to cancer (Digweed and Sperling, 2004). Interestingly, HP1α depletion in NBS cells decreases their hypersensitivity to IR. Overall, our data reveal that the NBS1-HP1 interaction preserves genome stability and that modulation of HP1α can affect NBS clinical features. However, the molecular mechanisms underlying the HP1-NBS evolutionarily conserved functional interaction remain still elusive. Our proposal is meant to fill this gap and our results will provide new insights on the implication of HP1a in cell metabolism and in the clinical features on Neijemegen Breakage Syndrome.

One proposed aim is investigating whether HP1a could be required for the targeting and retention of NBS on DSBs both in Drosophila and human cells (Task 1). We have generated Drosophila lines expressing an Nbs-GFP encoding transgene that allows tracking a real-time Nbs localization by time-lapse microscopy. We have already verified that following IR-induced DSBs, Nbs forms distinct foci *in vivo* in larval neuroblasts. In this first year of funding, we sought to understand whether this localization was affected by loss of HP1a (aim 1.1). We thus irradiated either wild-type or Hp1 mutant larval neuroblasts expressing Nbs-GFP and found no difference in both number or size of Nbs-GFP foci among wild-type and HP1-depleted cells after 10min, 30 min and 1hr Post Irradiation Time (PIR). This indicates that HP1a is not required for the formation of these IR-induced structures. In addition, we verified whether Nbs and HP1a localizations overlap on specific polytene regions in both wt and irradiated salivary glands by standard double IF. Immunostaining of polytene chromosomes with our

custom-made antibody revealed that Nbs localizes to many euchromatic regions along all chromosome arms; polytene chromosomes from *nbs1* mutants were not stained. Most Nbs signals on polytene chromosomes did not coincide with brightly fluorescent DAPI bands. About 45% of the Nbs signals corresponded to interbands that are not stained by DAPI, while the remaining 55% appeared to coincide with thin bands that were weakly stained by DAPI. In addition, we observed a diffuse Nbs staining of the chromocenter. To obtain additional insight into the Nbs localization pattern we co-immunostained polytene chromosomes for Nbs and HP1a. Examination of 20 Nbs/HP1a revealed that Nbs is enriched in ~110 clear-cut polytene bands. A total of 30% of these bands colocalized with HP1 signals. These findings indicate that Nbs is highly enriched in HP1- containing chromatin domains and demonstrate that Nbs localization is not restricted to a specific chromatin type. However, Nbs polytene signals did not change upon X-ray treatment, suggesting that unlike mitotic chromosomes, polytene chromosomes do not retain Nbs on chromatin breaks.

We have previously demonstrated that human fibroblasts derived from NBS patients who carried the NBS1 657del5 mutation which determines a high sensitivity to IR and predisposition to cancer (NBS1; OMIM #2512609) showed an approximate 3- to 5-fold increase of HP1α levels compared to corresponding controls (Bosso et al., 2019). However, no differences in levels of HP1α mRNAs were observed between MRC5 and NBS cells indicating that high HP1α levels are not due to increased transcription. As HP1α accumulation has been proven to cause deleterious effects on cell survival in different systems we speculated that part of the genome instability observed in NBS cells could arise because of the interaction with the p26-NBS1 and p70-NBS1 fragments that could enhance HP1α stability thus leading to its accumulation. To identify genetic and molecular factors that could modulate the genome instability of human NBS syndrome, we proposed to generate humanized flies expressing the human NBS1 657del5 mutation. To this end, we cloned *UAS HA-NBS1 WT -FLAG* and *UAS-HA-NBS1657del5-FLAG* expressing cDNAs in in pUAST-attB vectors under the UAS promoter, to generate Drosophila transgenic flies (Bestgene). All cDNAs have been inserted in the same genomic landing site (on the second chromosome) to achieve an equal expression avoiding position effects. Thus, by taking advantage of *Drosophila* UAS/GAL4 system, we induced the trans-activator GAL4, under control of ubiquitous enhancers (TubGal4 and or ActinGal4), to express each transgene during fly development. By WB analysis using anti-HA commercial antibodies, we verified that the expression of both human transgenes was very low. Consequently, we found that the NBS1 WT transgene was able to rescue neither lethality nor chromosome aberration phenotype of *nbs* mutants thus enabling us to use both lines for further studies. Although at the moment we cannot explain these negative results, we are generating new lines expressing human transgenes without the tags.

During this year of funding, we have also started a F1 screens to isolate deletions that affected the rough-like eye phenotype of *GMR_Gal4>NbsRNAi* flies (Task 6). To this aim, *GMR_Gal4>NbsRNAi* flies were crossed to flies bearing Deficiencies on chromosome 2 (99 lines) using the Bloomington Deficiency Kit (bdsc.indiana.edu). Interestingly we found that 10% and 15% of these lines enhanced or suppressed the eye phenotype, respectively, revealing potential genetic interaction among the genes uncovered by the deletions and *nbs*. By using small and molecularly defined deficiencies, we are currently narrowing down the Chromosome 2 regions that contain potential *nbs* genetic suppressors/enhancers to identify unprecedented genetic elements that interact with Nbs. In the meantime, we are in the process of carrying out the same screen on chromosome 3 and 1.

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*Co-corresponding

Research Group

Giovanni Cenci, Full Professor (Sapienza, University of Rome). PI
Elisabetta Bucciarelli, Researcher (CNR, IBPM, Rome)
Liliana Tullo, PhD Student (Sapienza, University of Rome)
Andrea D'Alessandro, PhD Student (Sapienza, University of Rome)

Collaborations

Alessandra Di Masi, Associate Professor (Roma Tre, University of Rome)
Valeria Naim, Researcher, NRS UMR9019, Université Paris-Saclay, Gustave Roussy, Villejuif, France

RNA-BASED MOLECULAR STRATEGIES TO ANTAGONIZE THE EPITHELIAL-TO-MESENCHYMAL-TRANSITION IN CANCER PROGRESSION

CARLA CICCHINI

RESEARCH AREA: Genetics, biology and pathophysiology of eukaryotes

Department of Molecular Medicine
carla.cicchini@uniroma1.it

The epithelial-to-mesenchymal transition (EMT) is a trans-differentiation process by which epithelial polarized cells can lose cell–cell contacts, acquiring motility and invasiveness properties. EMT plays a crucial role during embryonic development, tissue fibrosis and wound healing and, notably, it is determinant in epithelial tumor progression, favoring the metastasis process. Furthermore, it can confer the acquisition of a stem cell-like phenotype, drug resistance and immune-evasion. The EMT implies a series of gradual molecular and morphological changes and many states of partial EMT, where epithelial and mesenchymal markers coexist, can be encountered. Notably, EMT plasticity is underlined by the evidence that in some cases, such as in the secondary tumor sites, EMT can revert by means of an inverse mesenchymal-to-epithelial transition (MET).

At the basis of the EMT-MET dynamics, there is the interplay between several regulatory pathways and effector molecules. Particularly, "master" transcriptional factors are necessary and sufficient to induce and maintain the reprogramming of gene expression, triggering a profound modification of the chromatin state. A large body of evidence indicates that "master" transcriptional factors can enroll other factors, including co-activator or co-repressors, as well as chromatin modifiers recruiting them on specific regulatory sequences and, in turn, triggering or silencing specific gene expression programs. Notably, the formation of specific transcriptional complexes and their regulation can be cell- and context-dependent. In hepatocytes, we previously unveiled a molecular circuitry of direct and reciprocal transcriptional repression between Snail (i.e. master of EMT) and HNF4α (i.e. master of epithelial differentiation and MET), whose balance was found responsible for different cell physio-pathological outcomes (e.g. EMT *vs* MET; epithelial tumor progression *vs* tumor suppression) (Cicchini et al., JCP 2006; Santangelo et al., Hepatology 2011). This circuitry was further integrated at transcriptional and post-transcriptional levels by means of other co-regulators and different classes of non-coding RNAs (Garibaldi et al., Cell Death and Diff. 2012; Cicchini et al., BBA gene reg. mech. 2015; Battistelli et al. Oncogene 2017; Bisceglia et al., Front Pharmacol. 2019; Noce et al., Cell Death and Dis. 2019).

While master factors in the chromatin context can directly interact with transcriptional cofactors and chromatin modifiers, recent evidence points to the relevant role of long non-coding RNAs (lncRNAs) as bridges between some of them and epigenetic elements. This function would confer to lncRNAs an epistatic role with respect to the master activity itself. Notably, we highlighted that Snail acts as an organizer on epithelial

target genes of a molecular platform that includes HOTAIR, a lncRNA overexpressed in several epithelial cancers and strongly correlated to invasion. Specifically, HOTAIR is recruited by Snail to specific genomic sites and required for Snail repressive activity by acting as a scaffold for EZH2, responsible for the H3K27 trimethylation. In other words, HOTAIR is necessary to the Snail-mediated repression of epithelial genes in EMT and tumor progression (Battistelli et al., Oncogene 2017).

Building on this body of evidence, the design and the functional validation of mutant molecules as negative regulators of EMT can be conceived. Our recent published results described one HOTAIR deletion mutant form, named HOTAIR-sbid (for Snail binding domain), that includes the putative Snail-binding domain but is depleted of the EZH2-binding domain (Battistelli et al., Cancer Research 2021). It was functionally characterized as a dominant negative of the endogenous HOTAIR in regulating Snail activity: in both murine and human tumor cells, it impairs the ability of HOTAIR to bind Snail and, in turn, to trigger the EZH2-mediated repression of epithelial target genes. Notably, HOTAIR-sbid expression was proven to reduce cellular motility, invasiveness, anchorage-independent growth, and responsiveness to TGFß-induced EMT. This mutant represents the first of innovative tools we aim to validate for possible RNA-based approaches in counteracting epithelial tumor metastasis. Further effort is needed to characterize other HOTAIR mutants as able to interfere with its pleiotropic functions in EMT.

Current research is also focused on the study of the dynamic assembly of molecular platforms enrolled by master factors (involving ncRNAs and epigenetic modifiers) to drive specific cellular outcomes in different steps of cancer progression by modulating gene expression in a coordinated manner. Specifically, in the second half of 2022, we set up the experimental conditions to the large-scale identification of other partners of both HOTAIR and Snail, including other ncRNAs (e.g. miRNAs and circRNAs), in different EMT models.

Furthermore, in line with our previously evidence that the “master” factor HNF4α is a gatekeeper of the epithelial differentiation state (by both positively controlling epithelial genes and stably repressing the mesenchymal program, including HOTAIR transcription), as well as a MET inducer in invasive cancer cells (Santangelo et al., Hepatology 2011; Battistelli et al. Cell Death and Diff. 2019), our efforts are also focused on the characterization and the functional validation of other lncRNAs we identified as putative HNF4α transcriptional targets.

Overall, we believe that the knowledge acquired in gathering all this body of evidence could pave the way to the successive development of biotechnological tools for innovative ncRNA-based therapeutic strategies aimed at controlling epithelial tumor metastasis.

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Research Group

L. Amicone, Associate Professor
C. Cicchini (PI), Associate Professor
V. Consalvi, PhD student
A. Marchetti, Associate Professor
M. Ruggiero, Master degree student
M. Tripodi, Full Professor

RESTORING AN INFLAMED AND THERAPY-RESPONSIVE PHENOTYPE IN IMMUNE-COLD MYCN-DRIVEN TUMORS

GIUSEPPE GIANNINI

RESEARCH AREA: genetics, biology and pathophysiology of eukaryotes

Department of Molecular Medicine
giuseppe.giannini@uniroma1.it

MYCN deregulation is a cancer driver in neuronal and non-neuronal neoplasia of childhood and adulthood. MYCN-driven tumors are often aggressive and refractory to common and even multimodal therapies. Direct MYCN targeting is effective in preclinical models, but not yet achievable in patients. Most importantly, the mechanisms of MYCN-induced therapy resistance are largely not understood.

Accumulating evidence suggests that enduring anticancer effects (as opposed to short-lived mass destruction followed by cancer progression) are only achieved when the administered therapy (ie, chemo/radio therapy, target therapy or immune checkpoint blockade) drives the (re)activation of immune responses against the tumor. Unfortunately, MYCN-driven tumors are mostly characterized by a 'cold' phenotype, that is lack of tumor infiltrating leukocytes, low type-I interferon transcriptomics and reduced chemokine expression, which implies scant possibilities to reactivate the immune responses in these settings. Consistently, initial reports on the outcome of immune checkpoint blockade therapy in MYCN amplified (MNA) neuroblastoma were rather discouraging. Thus, we believe that unveiling the molecular mechanisms driving the MYCN-dependent cold phenotype may offer new perspectives for the treatment of MYCN-driven tumors.

Replication stress (RS) is a major cause of DNA damage, which may ultimately lead to cell death. However, the chronic exposure to RS due to the activation of specific oncogenic pathways in cancer cells is associated with an upgraded RS-response (RS-R), which fuels genomic instability and cancer progression. Exacerbation of RS to induce accumulation of intolerable levels of DNA damage is being pursued as a therapy approach at preclinical and clinical levels by several groups including ours. Building on the observation that MYCN induces RS, DNA damage and modulates the expression of a large set of DNA repair factors to restrain the deleterious effects of replication-born DNA damage, we and others showed that harnessing the RS-R might be a successful strategy to induce cell death in MYCN-driven tumors (Petroni M et al, 2016; Petroni M et al., 2018). Among the compounds in advanced clinical development that target the RS-R, we focused on CHK1 and PARP inhibitors (PARPi/CHK1i). Both PARP and CHK1 have important roles in securing DNA replication via replication fork stabilization and activation of cell cycle checkpoints. We reported that MYCN drives increased expression of PARPs and that PARP inhibitors (PARPi), such as olaparib or talazoparib, increase MYCN-dependent RS, drive premature entry in mitosis with damaged DNA, accumulation of micronuclei and anaphase bridges, leading to mitotic catastrophe and

cell death (Colicchia V et al, 2017). This could be exacerbated by the abrogation of the S phase checkpoint via CHK1 inhibition. Indeed, the combined treatment with PARP and the CHK1 inhibitors is far more effective in MNA compared to MYCN single copy cells (MNSC) in vitro and even suboptimal doses of CHK1 inhibitors in combination with PARP inhibitors cause accumulation of DNA damage and massive cell death in a MYCN-dependent manner, leading to therapeutic effects in multiple MYCN-driven preclinical animal models, with no major toxicities (Di Giulio S et al, 2021).

New insights indicate that, by causing replication stress, PARP inhibitors ignite the cytosolic DNA sensing (STING) pathway and promote innate immune responses, as part of their anticancer activity.

The STING pathway directs (innate)-immune and inflammatory responses upon pathogen infections. Indeed, cyclic GMP-AMP synthase (cGAS) senses cytosolic DNA derived from pathogens' infection and produces cyclic GMP-AMP dinucleotide (cGAMP). This in turn activates the stimulator of interferon genes (STING). STING recruits and activates the TANK-binding kinase 1 (TBK1), which phosphorylates interferon regulatory transcription factor 3 (IRF3), leading to its dimerization and nuclear translocation to promote transcription of IFN-I, induction of NF-kB activity and proinflammatory cytokines release. The resulting immune-stimulatory factors recruit effector immune cells, including dendritic (DC), natural killer (NK) and CD8+ T cells. Essential functions of the STING pathway in cancer immunity are also emerging. Indeed, cGAS may also sense the presence of RS-born self-DNA and micronuclei or mitochondrial DNA, all of which may be released in the cytosol of tumor cells spontaneously or upon DNA damaging treatments. Consistently, STING KO mice are prone to tumor formation. Intriguingly, PARPi may promote the STING pathway by releasing cytosolic RS-dependent damaged DNA suggesting that harnessing RS-Rs may promote anticancer immune responses and assist ICB therapy. STING pathway activation may then lead to proinflammatory modification of the microenvironment, increased tumor immunogenicity also via upregulation of MHC expression and antigen presentation and/or via the intrinsic induction of tumor immunogenic cell death. Consistent with this putative cancer suppressive role, STING pathway is repressed by multiple mechanisms in a variety of tumors, such as colon cancer and melanoma.

Promoting or enhancing STING activity may convert non-immunogenic cold tumors into inflamed and immunogenic ones, thereby facilitating tumor recognition and eradication by the immune system. STING/IFN-I pathway may up-regulate PD-L1 expression on cancer cells and in tumor microenvironment, further modulating immune responses. Consistently, therapeutic approaches focusing on STING reactivation eventually combined with ICB therapy are under investigation and the development of STING agonists for cancer therapy is an active and attractive area of research.

The role of the STING pathway in MYCN-driven tumors, and its potential connections with their cold phenotype has not yet been investigated. Moreover, although PARP inhibitors increase replication stress in MYCN-driven tumors, they appeared unable to activate the STING pathway. Rather, our data support the hypothesis that MYCN-

driven tumors enforce repression of STING activity to escape its cell-intrinsic and/or immune-mediated tumor suppressive effects, which would be otherwise activated by MYCN-dependent replication stress. Indeed, while the expression and the biochemical activation of the STING pathway could be easily revealed in non-MNA cells, it wasn't in MNA cells, suggesting this pathway might be repressed by MYCN.
By exploiting in vitro and in vivo MYCN-driven preclinical models, our grant proposal is meant to: a) provide significant proofs of principle for this hypothesis, by addressing the consequences of STING reactivation in MYCN-driven models; b) elucidate the epigenetic and non-epigenetic mechanisms of STING pathway repression by MYCN; c) provide evidence that restoring the STING pathway may convert cold MYCN-driven tumors into inflamed and therapy-responsive tumors.

Immunohistochemistry analysis of tumor sections from primary human NBs indicated that STING and cGAS proteins are substantially undetectable in neuroblastic tumor cells of aggressive MNA NBs, while they were detectable in endothelial cells and scant infiltrating immune cells. In silico analysis of RNASeq data from the R2 Genomics indicated that STING and cGAS are less expressed in MNA vs non-MNA primary tumor samples and that this correlation holds through also in stage IV tumors. RNAseq data from St. Jude database (https://platform.stjude.cloud/data/diseases/tumor) indicated that the expression levels of cGAS and STING are higher in primary advanced stage NBs compared with xenografts tumors (PDX), which comprises only neuroblasts tumor cells. Moreover, single cell RNAseq data from Dong_v3 GSE137804 datasets showed that STING and cGAS are highly expressed in CD4+ and CD8+ T cells, along with monocytes/macrophages, as compared to neuroblasts. Overall, we inferred that cGAS and STING expression is very poor in tumor neuroblasts, and that the scant expression found in RNAseq data most likely comes from the few inflammatory/immune cells infiltrating the tumor microenvironment.
To better address the status of the STING pathway in tumor neuroblasts we used a panel of NB cell lines, which demonstrated that STING is readily detectable in the majority of non-MNA cells and almost undetectable at protein and transcript level in all MNA cells. Other essential transducers of the pathway (ie, TBK1 or IRF3) were instead expressed in most cell lines. MYCN overexpression (MN-OE) repressed STING in stable and inducible model systems and led to the abrogation of type I IFN response upon stimulation with STING agonist, indicating it exerts a strong suppression on this pathway. Importantly, exogenous STING expression restores the response to STING agonists in MNA cells, consistent with the idea that the downstream elements of this pathway are present and active in MYCN-driven NBs. Moreover, STING and cGAS expression strongly inhibited colony formation in MYCN-driven cells, suggesting they are lethal in this context. To further address the functional consequences of STING pathway restoration in MNA NBs, we developed LAN1 cells with constitutive STING and inducible cGAS expression. Only one of these clones could be selected and it is characterized by a very low expression of both genes, consistent with the idea that

STING activity is deleterious in MNA cells. In fact, in these cells cGAS induction, in the absence of any STING agonist, induces a modest but reproducible increase in downstream target genes of the STING pathway such as ISG15 and CCL5, indicating that the reconstitution of cGAS/STING axis detects endogenous cytosolic DNA and activates a downstream response. These data support our hypothesis that MYCN-driven replication stress (with formation of micronuclei and release of cytosolic DNA) is not compatible with the expression/activity of the STING pathway.
In silico analysis on primary NB datasets indicated that STING promoter methylation is very high in MNA samples. Consistently, CHIP experiments showed that the chromatin of STING promoter is in a repressed state mainly in MNA cells, while cGAS is more broadly repressed, in line with our transcript expression data. Moreover, we found that MYCN binds to distal element of the STING promoter in MNA cells, and this binding is inversely correlated with H3K27Ac in a MYCN inducible systems. In silico analyses of RNASeq data evidenced that several epigenetic regulators are directly correlated with MYCN and inversely correlated with STING transcripts, in primary NBs. But in most cases their pharmacological inhibition with specific epidrugs failed to result in restoration of STING expression in MN-OE cells, as in the case of several EZH2 inhibitors. However, MNA and MN-OE cells showed increased expression of DNMT1/3 and the DNA methylation inhibitor 5-aza-2′-deoxycytidine (DAC) restored both cGAS and STING expression and activity. Similar data were obtained with the DNMT inhibitor GSK-3484862. Of relevance, treatment with DAC or GSK-3484862 not only restored the responsiveness to STING agonists in MN-OE cells, but also raised the expression of the IRF3 target ISG15, suggesting that they could restore the cystosolic DNA sensing pathway to activate a type I IFN response in these cells. Finally, we addressed whether DAC dependent restoration of the STING pathway could also restore the type I IFN response to our proposed PARPi/CHK1i combination therapy. Indeed, this is the case.
Overall, these in vitro data support the idea that reactivation of the STING pathway in MYCN-driven tumors is feasible and that it might be useful, further supporting the need for in vivo experiments in immunocompetent animal models.

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Research Group

Francesca Belardinilli, researcher**; Stefano Di Giulio and Alessio Balzerano,** post-doc fellows**; Debora Salerno,** Technician**; Maria Concetta Augusto, Damiana Battaglini, Emma Rullo,** PhD students.

Collaborations

Dr. Simone De Panfilis, Dr. Giovanna Peruzzi, Istituto Italiano di Tecnologia; **Dr. Marco Crescenzi**, Istituto Superiore di Sanità; **Dr. Paola Arimondo,** Institut Pasteur and CNRS, Paris, France; **Dr. Z. Liu and Dr. C.J. Thiele,** Pediatric Oncology Branch, National Cancer Institute Bethesda, MD, USA

INTERFERING WITH CHRONIC INFLAMMATORY RESPONSE AS THERAPEUTIC APPROACH TO TREAT MUSCOLAR DYSTROPHY

ANTONIO MUSARÒ

RESEARCH AREA: Genetics, biology and pathophysiology of eukaryotes

Department of SAIMLAL-Unit of Histology and Medical Embryology
antonio.musaro@uniroma1.it

Muscular dystrophy is a chronic disease that is largely refractory to different type of therapies aimed to rescue the dystrophin gene expression. Our working hypothesis is that the hostile dystrophic microenvironment might interfere with and limit the efficacy of the efficient rescue of the pathological phenotype. This is particularly challenging in the setting of chronic organ injury, where signals underlying homeostatic replacement of healthy tissue have been distorted, and thus the niche is not as receptive to transplanted cells and/or does not supply normal trophic signals to guarantee the survival of rescued dystrophin fibers. The therapeutic approach that has received most attention to date is the replacement of functional dystrophin by genetic, cell transplantation, or molecular interventions. Of note, the efficacy of stem cells transplantation, as reported in a recent clinical trial, was minimal, possibly due to the very low number of donor cells that engrafted the patients' muscles. Several critical variables, such as identification of the mechanisms controlling myogenic potential, homing of donor populations to all musculature, avoidance of the immune response, and lack of functional improvements in clinical trial are cooling the enthusiasm about stem cell therapy approach in DMD. In the context of gene therapy, one of the most promising approach aims at rescuing dystrophin by preventing the inclusion of specific exons in the mature dystrophin mRNA (exon skipping approach). Moreover, exon skipping provides a mutation-specific, and thus potentially personalized, therapeutic approach for patients with DMD. However, a defined dystrophin restoration levels seems to be required to slow down or prevent disease progression and improve overall muscle function. Our working hypothesis is that the hostile dystrophic niche might limit the efficacy of dystrophin replacement interventions. Among factors possibly explaining the establishment of hostile niche, the extent of chronic inflammatory response has been suggested to be linked to the severity of dystropathology.

While the evidence supporting a role of inflammation in muscle damage progression in DMD is rather convincing, the molecular mechanisms are still unclear.

The goal of the project is to characterize specific mediators of the pathogenesis of DMD and to pharmacologically target specific candidates involved in the switch from acute to chronic inflammatory response. We addressed whether a critical component of the immune system, namely the pro-inflammatory cytokines IL-6, plays a pivotal role in the pathogenesis of DMD.

IL-6 is a pleiotropic cytokine that can exert different and opposite effects (Figure 1). The muscle induced and transient expression of IL-6 can act in an autocrine or paracrine

manner, stimulating anabolic pathways associated with muscle growth, myogenesis, and with regulation of energy metabolism. In contrast, under pathologic conditions, the plasma levels of IL-6 significantly increase, promoting muscle wasting. How a single signaling molecule could be involved in diverse and opposite processes related to the metabolic response to exercise, inflammation, muscle regeneration (Figure 1) remained elusive for a long time. In this context, the purpose of our study is to shed light into the physiopathologic role of IL-6, dissecting the potential impact of IL-6 cytokine on muscle growth and maintenance. We demonstrated that skeletal muscle groups display a different vulnerability in response to the chronically elevated levels of IL-6 in the bloodstream, with fast-twitch glycolytic fibers that resulted more vulnerable than slow-twitch oxidative fibers. The chronic increased plasma levels of IL-6 affect skeletal muscle homeostasis, promotes alteration in skeletal muscle growth, functional performance, and metabolism.

Thus, we aim to interfere with cytokine activity. At first, we aimed to evaluate whether blockade IL-6 activity would not interfere with the delicate post-natal developmental stage and would not present critical drawbacks. To this purpose, we generated a constitutive IL6R knock out mouse (IL-6RA-/-) in order to obtain a complete ablation of IL-6 receptor alpha, that mediate IL-6 signaling. Body weight and tibial length measures of IL-6R-/- mice were compared to wild type mice. No differences between wild type and IL-6R-/- mice have been evidenced in body and muscle weight during the early post-natal life and in tibial length in adulthood. Furthermore, we did not observe skeletal muscle alterations in IL-6R-/- mice, in terms of skeletal muscle growth, functionality and robustness, as shown by ex-vivo and in-vivo functional tests and EBD injection, a stain that detect necrotic fibers, after exercise.

Altogether these data strengthen the rationale of our study, excluding the confounding action of potential alterations of general well-being and muscle physiology induced by IL-6 signaling blockade. We then analysed the impact of IL-6R blockade on dystrophic mouse model. Preliminary data revealed that modulation of IL-6 signaling in mdx dystrophic mice did not affect animal growth and muscle mass. Furthermore, the analysis of the maximal hindlimb grip strength measured during the progression of the pathology showed an increased strength values in mdx in which IL-6 signaling were inhibited compared to mdx controls and a reduced loss of muscle strength during disease progression.

The continuation of the project will provide new insights into the physiopathologic mechanisms underlying IL-6 activity.

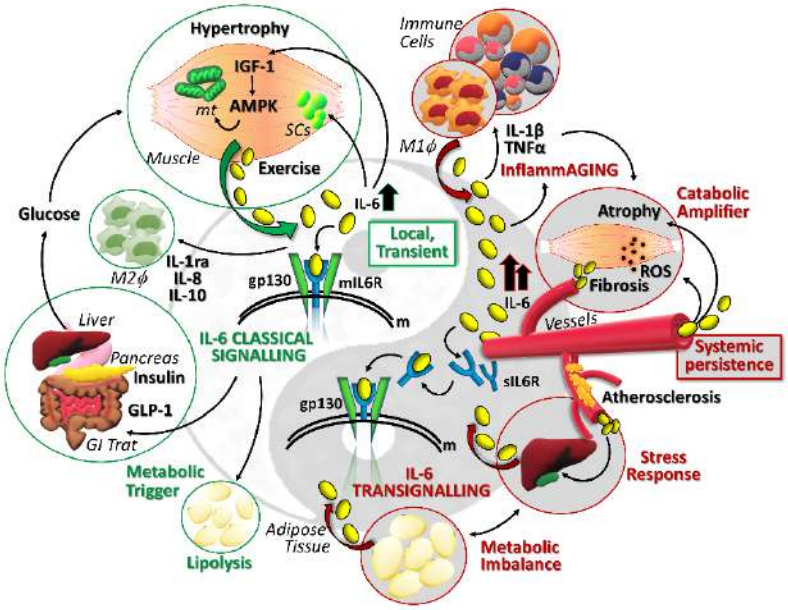


Figure 1 Schematic representation of the "yin e yang" IL-6 activities. The transient production of IL-6 positively influences muscle homeostasis and whole-body metabolism, whereas the persistence of high-level IL-6 is associated with muscle atrophy, redox imbalance, insulin resistance and chronic inflammatory response (from Forcina et al. Ageing Res Rev. 2022).

Publications

Forcina L, Franceschi C, Musarò A. **The hormetic and hermetic role of IL-6**. *Ageing Res Rev.* 2022; 80:101697. doi: 10.1016/j.arr.2022.101697. (IF: 11.78)

Research Group

Marianna Cosentino, Laura Barberi, Emanuele Rizzuto, Researchers; **Caterina Boccia,** PhD student; **Laura Forcina, Carmine Nicoletti,** Technician

Collaborations

Scicchitano Bianca Maria, Università Cattolica del Sacro Cuore, Roma; **Zaccaria Del Prete,** Sapienza Università di Roma

NUCLEAR ENVELOPE AND TELOMERE INSTABILITY IN LYMPHOMAGENESIS

ISABELLA SAGGIO

RESEARCH AREA: Genetics, biology and pathophysiology of eukaryotes

Department of Biology and biotechnology "Charles Darwin"
isabella.saggio@uniroma1.it

The nuclear envelope is a bilayer of membrane that surrounds the nucleus, organizing, separating and protecting the genome from the cytoplasm. When the envelope is fragile, the genome becomes unstable. These two intertwined processes favour the progression and the spreading of the cancer. We worked on a protein, AKTIP in humans and Ft1 in mice, which is highly concentrated in the nuclear envelope. We have also demonstrated that AKTIP is important for the stability of the telomeres, contributing in their correct maintenance. In vivo, we observed that the lack of AKTIP/Ft1 increases the spreading of lymphomas generated by p53 knock out. Recently, we have demonstrated that AKTIP is associated with the ESCRT machinery, a multi-protein complex that allows membrane repair, including nuclear envelope. The main aim of this project is to understand the relationship linking telomere maintenance, genome stability and ESCRT machinery and its implication in cancer progression.

WORK PACKAGE I: **Dissection of the ultrastructural organization of AKTIP and ESCRT complexes at the NE.**

Deliverables: Identification of new ESCRT players at NE and assessment of AKTIP physical proximity with ESCRT members. Exploiting Super Resolution Microscopy, we found that TSG101, an ESCRT I protein that shares similarities with AKTIP, is stably concentrated to the nuclear envelope (Fig 1A-C). This is the first time that an ESCRT protein belonging to complex I is demonstrated to be enriched at nuclear envelope. Through computational analysis of Super Resolution images, we found that 20% of TSG101 spots are closer than 0.5µm to lamin A (Fig 1B). We also analyse the reciprocal localization of AKTIP and TSG101. The two proteins share a similar cellular distribution and focusing on nuclear envelope we found that 20% of AKTIP and TSG101 are closer to each other less than 0.5µm (Fig 1D-E). This indicates that AKTIP and TSG101 are closely associated at nuclear envelope. Further analysis will be conducted to investigate the physical interaction between TSG101 and lamin A and between TSG101 and AKTIP at nuclear envelope.

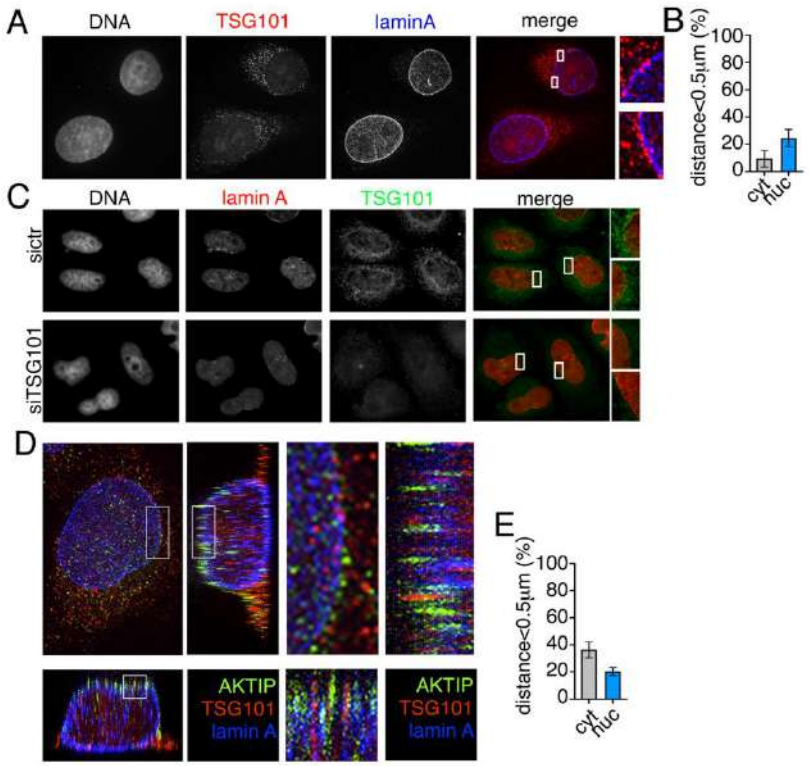


Figure 1: TSG101 and AKTIP localization at the NE: **A**: SIM images of HeLa cells showing the proximity of TSG101 to lamin A. **B**: Imaris quantitative analysis showing the percentage of TSG101 spots closer than 0.5µm to lamin A. **C**: Representative images of control and TSG101 depleted HeLa cells showing a reduction in the TSG101 signal at nuclear periphery. **D**: Projections of an extended section viewed from orthogonal planes and magnified sections from super-resolution images of a HeLa nucleus labeled with anti-lamin A, TSG101, and anti-AKTIP antibodies. **E**: Imaris quantitative analysis showing the percentage of TSG101 spots closer than 0.5µm to AKTIP at nuclear rim.

WORK PACKAGE 2- Analysis of the mechanistic implication of AKTIP and ESCRTs in NE Integrity

Deliverable: Definition of AKTIP role in the control of NE. We exploited Super Resolution Microscopy to investigate the localization of AKTIP in cellular conditions with wild type or altered nuclear envelope composition to identify the determinants of AKTIP recruitment at nuclear envelope. We analysed the distribution of AKTIP at nuclear rim in three cancer cell lines, HeLa, A549, MCF7. These three cell lines, express wild type p53, but differs for lamin A expression that is expressed in both HeLa and A549, but it is reduced in MCF7. We found that AKTIP is present at the rim in the three cell types, but also exhibit a specific pattern in MCF7. Indeed, the portion of nuclear rim occupied by AKTIP is significantly reduced in MCF7, that exhibit also multiple nuclear defects, wrinkles, blebs, and rim interruptions, compared to HeLa and to A549 (Fig 2A). To clarify if AKTIP mislocalization could be influenced by alterations of

lamins we used two cell models with known LMNA mutations: non-transformed fibroblasts derived from HGPS patients with LMNA c.1824C>T p.Gly608Gly and from EDMD2 patients with LMNA c.775 T>G. We found that AKTIP rim localization is significantly lost in HGPS cells, and modestly impaired in EDMD2 nuclei (Fig 2B). We analyzed the correlation between lamins proteins level, aberrant nuclear morphology and AKTIP mislocalization founding that it is the combined alteration of lamins expression and nuclear morphology that affects the localization of the cancer-associated factor AKTIP (Fig 2C-D).

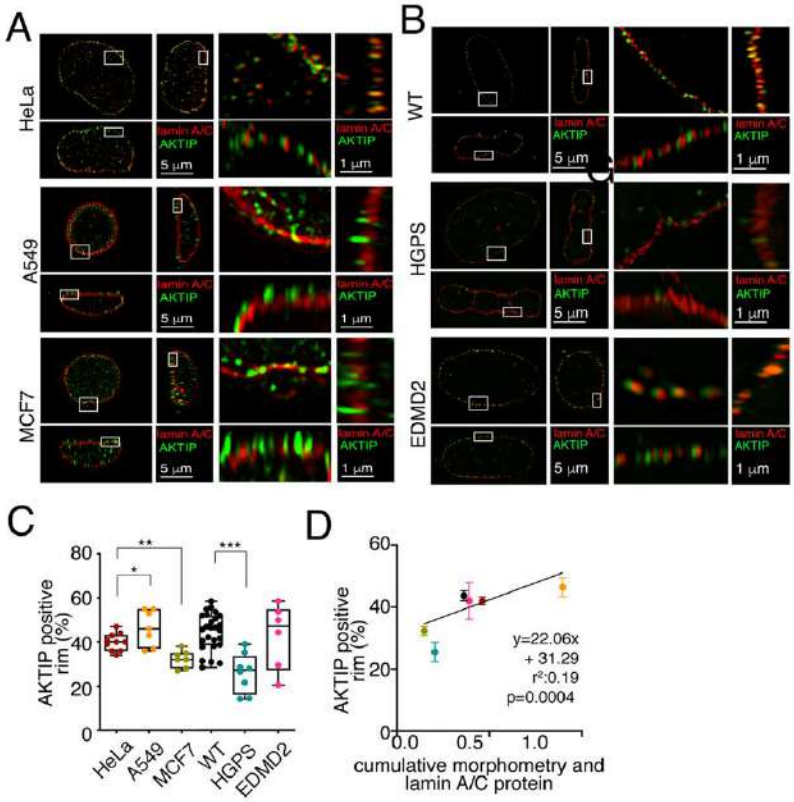


Figure 2: The localization of AKTIP at nuclear envelope is influenced by the combined alteration of lamin and nuclear morphology. **A-B**: Projections of an extended section of Hela, A549, MCF7, wild type (WT), HGPS and EDMD2 fibroblasts nuclei labeled with anti-lamin A/C and anti-AKTIP antibodies, viewed from orthogonal planes and magnified sections. **C**: Percentage of AKTIP positive rim in cells from F. **D**: Correlation between AKTIP positioning at the rim and cumulative parameter obtained merging for each type the ratio between lamin A and lamin C proteins levels from WB quantification and morphometric. Linear regression values are shown. Mean ± SEM is shown. ***p < 0.001 in unpaired Student t-test

WORK PACKAGE 3 – Dissection of the relative impact of NE integrity, AKTIP and ESCRTs in telomere homeostasis and genetic rearrangements

Deliverables: Definition of ESCRT and of NE role in AKTIP impact on telomeric function. We focused on the analysis of the connection between nuclear envelope

integrity and telomere maintenance. We used two different approaches. The first one consists in the analysis of the impact of nuclear instability on the telomeres aberrations generated by AKTIP downregulation. We generated nuclear instability through the expression of a lamin A mutant progerin. In this condition, we depleted AKTIP/Ft1 observing that both mutations trigger telomere aberrations. Interestingly, however, we found a different prevalence of specific telomeres aberrations in each condition (Fig 3). The second approach we followed was to investigate the impact on telomeres of the downregulation of nuclear envelope ESCRTs. To address this deliverable, we first analysed comparatively the impact on telomeres of the ESCRTs CHMP7, IST1, TSG101, and VPS28. The data show that CHMP7 and TSG101 trigger telomere aberrations, while the depletion of IST1 does not. We will explore in next months the mechanism of action of ESCRTs in telomere function investigating the effect of combined downregulation.

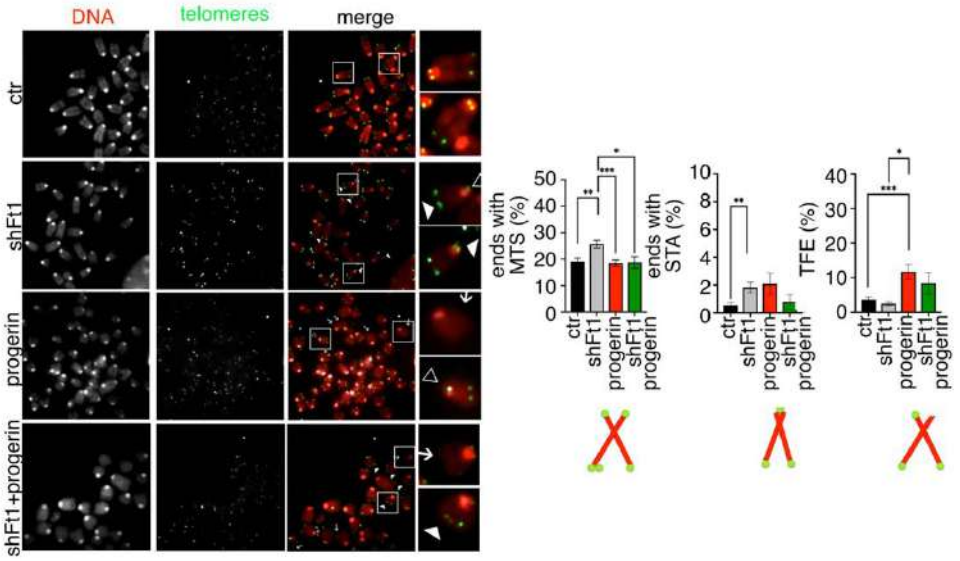


Figure 3: Nuclear envelope integrity impacts on telomere aberrations induced by AKTIP/Ft1 depletion: Representative telomeric FISH (A) of metaphase spreads of control, shFt1, progerin expressing and progerin expressing and shFt1 p53ko MEFs and the respective quantification of telomeric aberrations. STA: sister telomeric associations, MTS: multiple telomeric signals; TFE: telomere free ends. Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001 in t-student test.

Publications

- Mattia La Torre, Eleonora Centofante, Romina Burla, Carmine Nicoletti, Alessandro Giampietro, Antonio Musarò, Isabella Saggio. **The depletion of the telomeric factor Ft1 generates age and cell type-dependent DNA damage and cardiac defects** (in preparation)

- Romina Burla, Mattia La Torre, Eleonora Centofante, Stefania Petrini, Luciana Dini, Graham Wright, Brian Burke, Isabella Saggio. **Nuclear Envelope acting ESCRTs are needed for telomere maintenance** (in preparation)

Oral Presentation

Romina Burla, Mattia La Torre, Klizia Maccaroni and Isabella Saggio. **The nuclear envelope-associated ESCRT factor CHMP7 is needed for telomere integrity XVI FISV Congress**. Reggia di Portici, Naples, Italy 14-16 September 2022- selected oral presentation

Research Group

3 Researchers;
2 PhD students;
2 Fellows

Collaborations

Silvia Soddu, Regina Elena National Cancer Institute IRCCS, Rome, Italy
Wenting Zhao, School of Chemistry, Chemical Engineering and Biotechnology, Nanyang Technological University, Singapore
Brian Burke, A*STAR Skin Research Labs (ASLR), Agency for Science, Technology and Research, A*STAR, Singapore
Graham Wright, A*STAR Microscopy Platform, Research Support Centre, Singapore
Petrini Stefania, Ospedale Pediatrico Bambino Gesù, Rome, Italy

“UNDER 45” RESEARCH PROJETCS

2 YEARS PROJECTS LED BY UNDER 45 YEAR OLD RESEARCHERS – CALL 2020

FIRST YEAR REPORTS

EXPLORING PATHOGENICITY AND TUMORIGENIC POTENTIAL OF ANISAKIS PEGREFFII USING INTESTINAL ORGANOIDS

SERENA CAVALLERO
RESEARCH AREA: Parasitology

Department of Public health and infectious diseases DSPMI
serena.cavallero@uniroma1.it

The project aimed to expand the knowledge on the potential effects of *Anisakis* infection on human host, with particular attention to the potential involvement in tumor microenvironment induction.
Anisakis can cause a fish-borne zoonosis called anisakiasis or anisakidosis if humans eat raw or undercooked seafood with infective third stage larvae (L3). The interaction with accidental host through parasitic somatic and excretory/secretory (ES) products can determine a panel of heterogeneous gastrointestinal and/or hypersensitivities symptoms, with features of acute and chronic inflammation at the gastric, intestinal, or ectopic levels. The chronic inflammation is of extreme interest, as it is a key factor in the initiation and development of tumor microenvironment often associated with suppressor-gene inactivation, oncogene activation and somatic mutations. Considering that human helminths can survive for years into natural hosts, mainly by modulating both the host's immune system and physiological state, ES products represent the focus of investigation for host's immunoregulation, due to their involvement in pathogenesis and disease progression, even promoting tumorigenesis. Among ES products, the recent discovery of extracellular vesicles (EVs) as an innovative mechanism to deliver messages between cells and organisms across kingdoms of life and their detection also in helminths have changed the paradigm in the study of host-parasite interactions. In this project we investigated the impact of *Anisakis* L3 derived EVs on human intestinal organoids cultures using a comparative transcriptomic approach, and estimations of gene expression by qRT-PCR and multiplex estimations of inflammation mediators.
The *Anisakis* L3 were collected by visual inspection of fish from the Mediterranean basin (Fig1), and then they were incubated to obtained EVs. EVs were isolated by commercial kit and characterized using nanoparticles tracking analyses, scanning and transmission electron microscopy (Fig2) to have estimations of number of particles and average size of diameters.

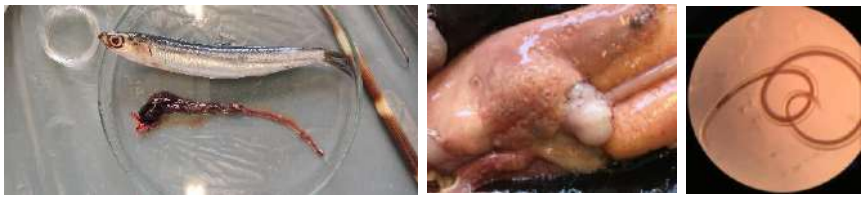


Figure 1: representative fish (*Engraulis encrasicolus*), fish visceral cavity infected and *Anisakis* L3 visualized at optical microscope.

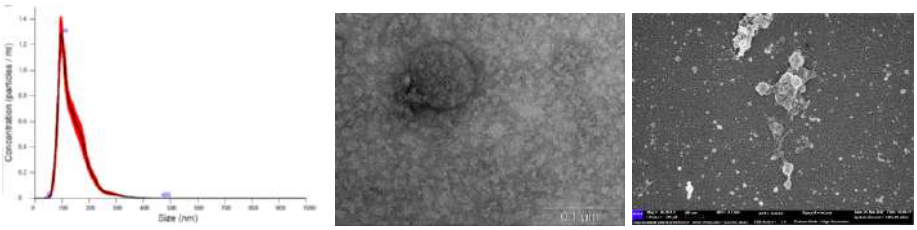


Figure 2: Averaged FTLA Concentration / Size of *Anisakis* derived EVs, images from SEM and TEM.
Thanks to the ethical committees approval from the HUB (Utrecth) and teaching hospital Policlinico Umberto I, threeD and 2D intestinal organoids were successfully obtained starting from colon biopsies. Before exposure to *Anisakis* derived EVs, 2-D intestinal organoids were induced to differentiate and immunostaining for Zonulin and Villin was performed, in order to confirm differentiation state (Fig 3 and 4). Once differentiated, human intestinal organoids were treated with *Anisakis* derived EVs and after 48h material were collected (total RNA and surnatants).

Figure 3: Human intestinal 3D and 2D cultures, starting by the isolation of intestinal crypts (B) from colon biopsy of a healthy donor (A). After the mechanical disruption of crypts, the material is seeded in Matrigel drops (C), intestinal organoids started to grow in 3D, cystic, conformation (D), and after a mechanical disruption they were seeded in 2D transwell.

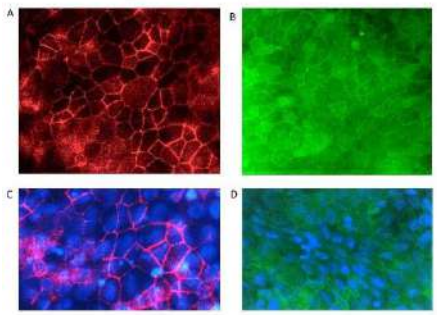


Figure 4: immunostaining of 2D organoids after differentiation of A. Zonulin (red) the tight junction protein as marker of enterocytes. B. Villin (green) a protein forming the structure of microvilli as a marker of the brush border C. D. merged pictures.

RNA from controls and treated biological triplicates were used for RNA-seq and an average of 87 million and 60 million 150bp-reads were generated, respectively (Table 1). Raw reads generated in the present study have been already submitted in the Sequence Read Archive (SRA) database of NCBI (http://www. ncbi.nlm.nih.gov/sra) under the Bioproject PRJNA942614.

The differential expression analyses (DEGs) between controls and treated provided some clues about the effect of *Anisakis* derived EVs on human intestinal organoids.

The top100 most abundant transcripts revealed the presence of several "structural molecule activity" (GO:0005198) as ribosomal proteins, in "binding" (GO:0005488) and "catalytic activity" (GO:0003824), as chaperones (Hsps) and major histocompatibility complex proteins (MHCs,) crucial for positive regulation of adaptive immune response (HLA-A, B, C and B2M). Other transcripts were involved in the tissue differentiation state and in regulation of apoptotic signaling pathway (LGALS3, RPS3 and TMBIM6). Around 30% of genes listed is involved in tumorigenic environment, according to literature, and few of them are included in pathways related to apoptosis, inflammation mediated by chemokine and cytokine, T-cell activation and TGF-beta signaling.

In addition to the most abundant transcripts, we focused on those found up or downregulated at the pairwise comparison with ii) **significant FDR** and ii) without significant FDR, but **with log2FC>2**.

Among the **95 upregulated in treated organoids**, transcripts related to GPCRs genes (protein-coupled receptor activity and chemokine receptor activity); RNA component of mitochondrial RNA replication; RAET1 family (major histocompatibility complex (MHC) class I-related genes); member of the lysyl oxidase gene family, essential to the biogenesis of connective tissue and WNT gene family, implicated in oncogenesis and in several developmental processes, including regulation of cell fate, were the most interested. Regarding the **210 downregulated in treated organoids**, the most interesting transcripts showed a role in cell division or death regulation. In particular, EXO1 (functions in DNA mismatch repair MMR); CDC25C, a conserved protein that

plays a key role in the regulation of cell division, as it suppresses p53-induced growth arrest; SKA (normal chromosome segregation and cell division with microtubules involvement); DDIAs, negative regulates intrinsic apoptotic signaling pathway in response to DNA damage.
The **seven statistically relevant DEGs** included two upregulated transcripts (NUPR1 and H2BC5) and five downregulated transcripts (LEFTY1, TACC1, MYBL2, MKI67 and EPHB2). Panther search for molecular function (Fig 5) identified LEFTY1 and EPHB2 as the most involved in transmembrane receptor protein tyrosine kinase and cytokines activities and in two important signaling pathways (TGF-beta PDGF), respectively; while the search for biological process identified MKI67, NUPR1 and MYBL2 involved in cell cycle regulation.

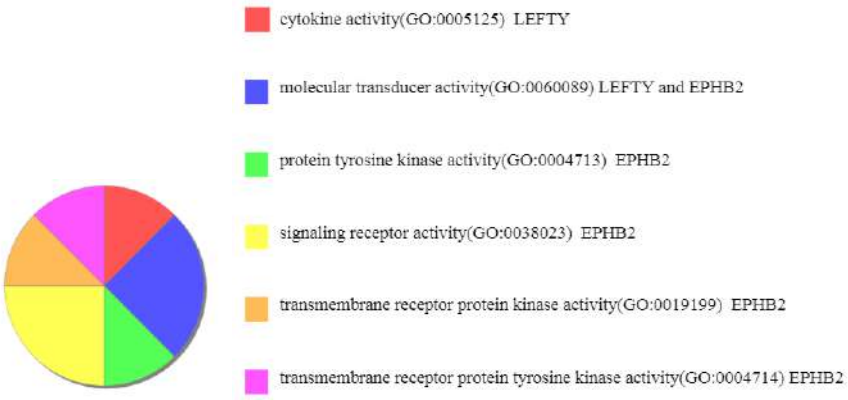


Figure 5: Results of Panther search for molecular function query among the 7 DEGS of human intestinal organoids treated with *Anisakis* derived EVs.

Relative quantifications of gene expression in transcripts of interest confirmed bioinformatics data, both in terms of expression trend and statistical significance in pairwise comparisons (controls vs treated).
Considering the potential ability of *Anisakis* derived EVs to impact gene expression in host intestinal tissue, we tested if putative gene target among transcripts here analysed were retrieved in the 13 *Anisakis* derived miRNAs selectively packaged into EVs identified in our previous work (Cavallero et al 2022). 10 out of 13 showed putative gene targets included in the list of transcripts of our interest, of which two significant DEGs downregulated in treated organoids: ape-lin-4-5p is associated with the putative target EPHB2 (score 68) and ape-miR-72-5p with the putative target TACC1 (score 81).
Further quantification of relative gene expression and multiplex protein profiling with Luminex is still ongoing.

Major achievements so far obtained: 1) assessment of human intestinal organoids culture (3D and 2D) starting from colon bioptic material; 2) isolation of infective third-stage *Anisakis* larvae EVs; 3) assessment of a model of parasitic infection based on

organoids and EVs; 4) identification of a shortlist of transcripts regulated by messengers selectively packaged into *Anisakis* EVs; 5) focus on transcripts related to tumors, inflammation and immune response

Results obtained will be the core of an original research article in preparation, to be submitted in highly ranking internationally relevant journals. The first attempt will be to Nature Communication (IF 17.76) or to Journal of Extracellular Vesicles (IF 17.34), given the potential high relevance of tumorigenic potential of a zoonotic parasitic nematode.

So far, we have disseminated results at national and international congress, as detailed below:

National congress

Italian Society of Parasitology SOIPA (Naples, 27-30 June 2022)
Invited speaker at the "Genomics and the other omics in parasitology: from epidemiology to functional studies" session. Cavallero S and D'Amelio S. Transcripts and miRNA from *Anisakis pegreffii* infective larvae and their released exosomes: from the pathogenic repertoire to host-cellular response.
Collaborator to the oral communication Bellini, D. Scribano, M. Sarshar, C. Ambrosi, A. Pizzarelli, A. Pronio, A.T. Palamara, S. D'Amelio, S. Cavallero (2022). Anisakiasis: expanding the repertoire of potential *Anisakis* inflammatory modulation strategies on human first line of defense.

XIII seminar Phd Day:An empathic approach to science: how to rebuild communities? (Rome, Istituto Superiore di Sanità). Bellini, D. Scribano, M. Sarshar, C. Ambrosi, A. Pizzarelli, A. Pronio, A.T. Palamara, S. D'Amelio, S. Cavallero (2022). Anisakiasis: expanding the repertoire of potential *Anisakis* inflammatory modulation strategies on human first line of defense.

International congress and workshops

XXVII School of pure and applied biophysics on "Extracellular Vesicles: from biophysical to translational challenges" (Venice, February 6-10, 2023). Poster S. Cavallero, I. Bellini, D. Scribano, C. Ambrosi, M. Sarshar, A. Pronio, A.T. Palamara, B. Arcà, S. D'Amelio. "Tracing the pathways of *Anisakis* pathogenicity"

15th International congress of Parasitology ICOPA (Copenhagen, 21-16 August 2022) invited speaker Cavallero S, Bellini I, D'Amelio S. "Transcripts and miRNA from *Anisakis pegreffii* infective larvae and their released exosomes: from the pathogenic

repertoire to host-cellular response". Collaborator to the oral presentation: Whitehead B, Borup A, Boysen A, Sørensen-Rossen L, Mardahl M, Fromm B, Williams A, Thamsborg S, Hansen E, Cavallero S, D'Amelio S, Gasser R, Nejsum P. "Extracellular vesicles released by nematodes induce three distinct immune responses."

Research Group (DSPMI)

Ilaria Bellini PhD student
Serena Cavallero Researcher
Stefano D'Amelio Associate Professor
Annamaria Pronio Associate Professor
Daniela Scribano Researcher

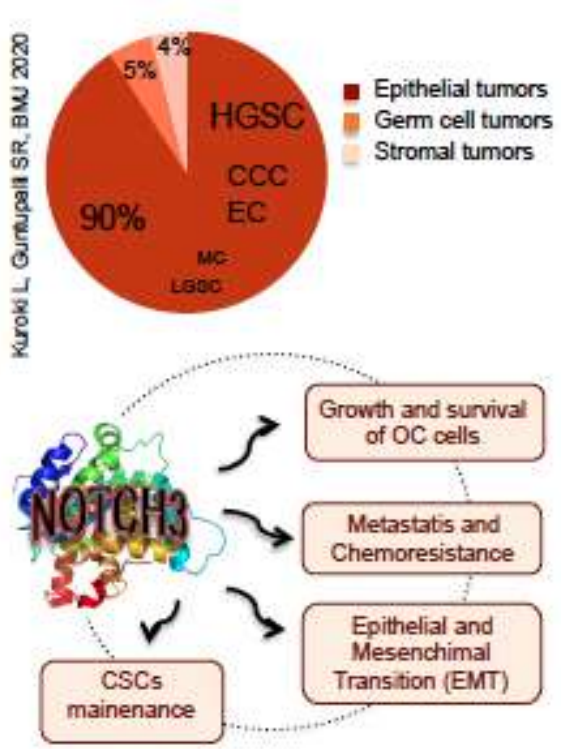
Collaborations

Cecilia Ambrosi Researcher at Dept. of Human Sciences and Promotion of the quality of life, San Raffaele Open University, IRCCS San Raffaele Roma
Agostina Pietrantoni Researcher at the Electronic Microscopy Unit of the ISS, Roma.

MOLECULAR DISSECTION OF NOTCH3-PIN1 CROSS-TALK: NOVEL APPROACHES OF TARGETED THERAPY IN OVARIAN CANCER

SAULA CHECQUOLO
RESEARCH AREA: Novel therapeutic interventions

Department of Molecular Medicine
saula.checquolo@uniroma1.it



Epithelial ovarian carcinoma (EOC) is the gynecological tumor with the highest death rate, mainly due to the rapid and silent dissemination of ovarian cancer cells in the peritoneum, the omentum and the organs located in the abdominal cavity, with up to 75% of patients presenting metastases at diagnosis. The standard care for EOC-bearing patients includes maximal surgical cytoreduction followed by Platinum (PT)-based chemotherapy. Unfortunately, despite a promising initial clinical remission, most of patients will develop chemotherapy resistance with recurrent metastatic disease, a frequent event that predicts poor prognosis. The majority of cases are diagnosed as high-grade serous ovarian carcinoma (HGSOC). Chemotherapy resistance relies on different converging signaling pathways that cooperate to build up precise transcriptional and proteomic profiles within tumor cells, resulting in effective strategy to escape chemotherapy response. Therefore, the identification of interconnected pathways and their common molecular profiles might allow the discovery of potential druggable targets to support clinicians in the early choice of the optimal therapy. In this scenario, the evolutionarily conserved Notch signaling pathway has emerged as a promising candidate given its multifaceted and well-documented role in tumorigenesis. Overall, due to the key role of Notch signaling in the development of the normal ovarian tissue as well as in the carcinogenesis and tumor progression of OC, an increasing number of studies have demonstrated its involvement in the promotion of drug resistance in OC, hence evaluating the efficacy of targeting this pathway. Among the four Notch paralogs encoded by the mammal genome, Notch3 has been reported as the oncogenic candidate

frequently over-expressed in a wide panel of OCs, which rely on Notch3 for their cellular growth and survival. In particular, it has been demonstrated that Notch3 activation renders OC cells more resistant to carboplatin and it is frequently associated with OC progression, tumor invasion and metastasis, which are of high relevance as most OC-bearing patients experience disease recurrence.
More recent studies suggested that Notch3 specific inactivation could be exploited to restore chemo-sensitivity in HGSOC. Therefore, selective targeting of Notch3 may represent a potential therapeutic approach for HGSOC-bearing patients aimed at overcoming the known off-target effects associated with pan-Notch inhibition. Notably, research is moving towards Notch-specific targeted therapies, among which the modulation of its positive regulators. One appealing candidate as a fine-tuner of Notch3 might be the peptidyl-prolyl cis/trans isomerase Pin1, that we have demonstrated to positive influence Notch3 protein expression and function in T-cell acute lymphoblastic leukemia (T-ALL), thus modulating the Notch3-dependent aggressive properties of T-ALLs. Several studies documented that aberrant function of Pin1 has been implicated in tumor initiation and progression through the regulation of several oncogenic pathways, including Notch. In keeping with these observations, genetic or pharmacological Pin1 targeting is strongly correlated with recovered sensitivity to chemotherapeutic drugs, through breast CSC exhaustion. Therefore, from a clinical perspective, Pin1 targeting might be exploited to hit Notch3 signaling pathway in HGSOCs.
Our preliminary data showed the Notch3/Pin1 correlation in a cohort of HGSOC-bearing patients, significantly related with an advanced tumor stage, thus suggesting that Notch3/Pin1 axis might be involved in the acquisition of aggressive phenotype also in HGSOC context. Therefore, we expect to identify therapeutic options based on Pin1 inhibition aimed at targeting Notch3, and finally restoring OC chemo-sensitivity and inhibiting metastatic spread.

During the first year of the funding (2022), our project had essentially two main aims:
1. Evaluating the biochemical mechanism(s) linking Pin1 activity on Notch3IC protein (Mechanistic studies)
2. Analysing the functional significance of the Pin1 inhibition on Notch3-dependent resistance properties in HGSOC (functional studies)
By using different experimental approaches on HGSOC cells (Mass Spectometry analysis, Peptides Synthesis and Binding simulation, biochemical assays) we identified the specific phospho-sites targets of both Pin1 isomerase (positive regulator) and GSK3□ kinase (negative regulator), overlapped inside the same region of the intracellular domain of Notch3IC, and whose differential activation results in antagonistic effects on Notch3IC protein. Indeed, depending on the Pin1/GSK3□ competence inside the cell, the imbalance between these two Notch3 regulators leads to Notch3 protein stabilization or proteasomal degradation, thus amplifying or blocking the Notch3-

dependent tumorigenic effects. The data presented are graphically summarized in Figure 1.

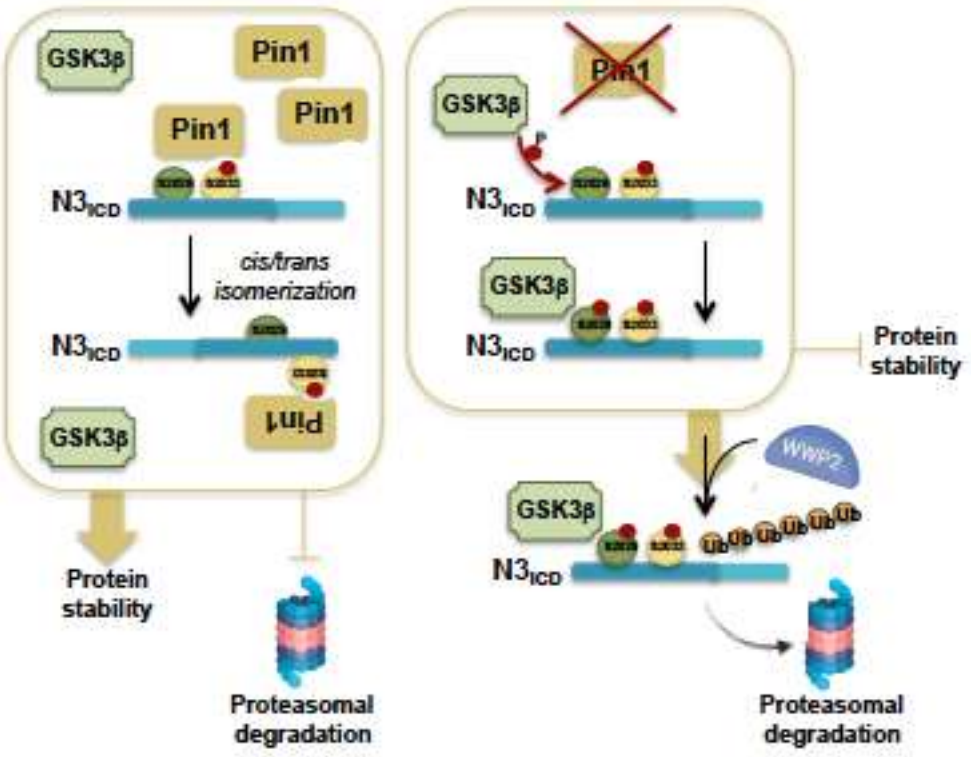


Figure 1. Schematic model depicting the antagonistic role of Pin1 and GSK3□ on Notch3IC protein in HGSOC cells.

Functionally, we observed that the genetic ablation of Pin1 in HGSOC cells overexpressing Notch3 is correlated with the resensitization to PT-based drugs (Carboplatinum and Cisplatin) *in vitro*, thanks to the decrease in Notch3 protein expression that become detectable mainly under chemotherapeutic pressure. Regarding the *in vivo* studies, we generated HGSOC_luc cells, differentially expressing Notch3 protein (SKOV3 vs SKOV3_N3), further intraperitoneally injected in NSG mice and followed for their growth and dissemination by using IVIS technology at various time points after cells implantation. We were able to establish an interesting murine model of metastatic ovarian cancer in order to evaluate the response to PT-based drugs, in combination or not with Pin1 inhibitors. To date, we demonstrate that the presence or absence of Notch3IC in HGSOC correlates with an increased Carboplatinum resistance. In order to confirm the functionality of our Pin1/Notch3 axis, i*n vivo* experiments with Pin1-silenced_luc cells, under Carboplatinum treatment, are now ongoing. The data presented are summarized in Figure 2 (Giuli MV et al, under preparation).

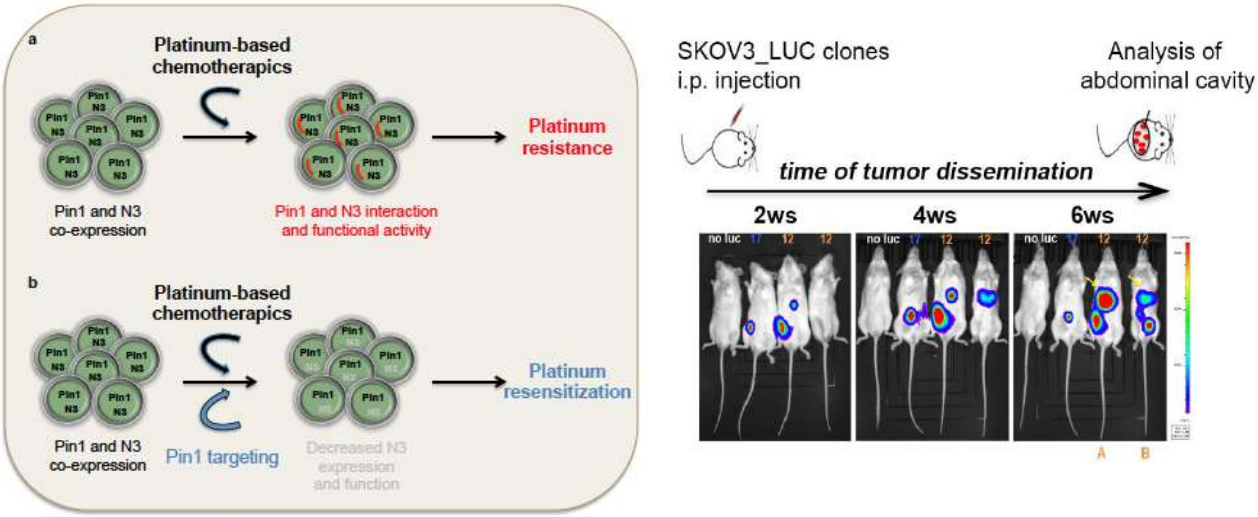


Figure 2. Schematic model of Pin1/Notch3 axis targeting resulting in the Platinum resensitization of HGSOC cells *in vitro* (left). Metastatic ovarian cancer xenografts model (right). Clones: #17: SKOV3 vs #12: SKOV3_N3.

Overall, this research program has the ambition of scientific and translational impacts. We expect to improve the knowledge of HGSOC heterogeneity, thus identifying novel mechanisms and regulators underlying Pin1/Notch3-driven tumorigenesis, predictive of poor PT-response, potentially aimed at ameliorating the OC outcome.
In this scenario, our research activity is also focused on evaluating 1. the functional activity of Pin1/Notch3 axis on primary HGSOC tumors by using other pre-clinical models (i.e. organoids) and 2. the effects of the pharmacological targeting of Pin1 by using different Pin1 inhibitors (ATRA, SulfoPin, KPT6566), currently tested in both *in vitro* and *in vivo* studies.

Publications

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* corresponding author.

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Research Group
Maria Valeria Giuli, Post-Doc
Angelica Mancusi, Post-Doc
Bianca Natiello: PhD student

Collaborations
Giannino Del Sal, University of Trieste
Stefano Indraccolo, University of Padova
Daniel D'Andrea, University of Nottingham
Nunzianna Doti, CNR Napoli
Domenico Raimondo, Sapienza University
Giulia d'Amati, Sapienza University
Giulio Caracciolo, Sapienza University
Diana Bellavia, Sapienza University