PhD program in Cellular and Molecular Biology

Coordinator: G Capranico

ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

10th PhD Workshop

6-7 October 2016, Polo Didattico Aule Navile, Via della Beverara 123/1, Bologna

Abstracts (by author in alphabetical order)

Borghi Sara

Supervisors: Delany/Scarlato

Regulation of Neisserial Heparin binding antigen (NHBA) during meningococcal colonization and invasion.

Neisseria meningitidis (Nm) colonizes the nasopharynx of humans and pathogenic strains can disseminate into the bloodstream causing septicemia and meningitis. Neisserial Heparin Binding Antigen (NHBA) is part of a multicomponent vaccine against Nm serogroup B, Bexsero™. NHBA is a surface-exposed lipoprotein which is expressed by all Nm strains in different isoforms. NHBA harbors an arginine-rich motif through which it is able to bind heparin-like molecules, increasing adherence to host tissues and heparin-mediated serum resistance.

We determined that temperature controlled the expression of NHBA in all strains tested, regardless of the clonal complex or peptide isoform expressed. NHBA expression was significantly increased at 30-32°C compared to 37°C, the temperature standardly used for *in vitro* culturing. An increase in NHBA expression at lower temperatures was measurable both at protein and RNA levels and was also reflected by a higher surface exposure of this antigen. A detailed molecular analyses and the comparison of RNA steady state levels in cells cultured at 30°C and 37°C indicated that the multiple regulatory mechanisms exerted their role at RNA level, mainly through an increased RNA stability/translatability at lower temperatures. Furthermore, the protein stability was also impacted resulting in higher NHBA stability at lower temperatures. Finally, the increased NHBA expression resulted in more efficient killing as shown by serum bactericidal assay (SBA). We propose a model in which NHBA regulation in response to temperature downshift might reflect the bacterial adaptation during the initial step of host-bacterial interaction and might also explain higher susceptibility to anti-NHBA antibodies in the nasopharynx niche.

Bexsero is a trademark of the GSK group of companies.

Borrachero Conejo Ana Isabel

Supervisors: Monti/ Caprini/ Benfenati

Stimulation and recording of astroglial cells by the use of organic bioelectronic devices

Astrocytes are one of the most numerous types of cells in the central nervous system (CNS) and have a key role for many important processes such as maintaining brain homeostasis and regulating synapse transmission [1] [2] [3]. Despite being under intensive study, there is still a lot to understand about how they work and their role in

the physiology and pathophysiology of the brain [4]. Investigations of neural cells electrophysiological properties have been relied mainly on single cells recordings. However, neural circuits surely include millions of cells making necessary the development of new tools to study these circuits on a bigger scale [5]. Developing new biocompatible tools for their study is therefore, one of the challenges that scientist are trying to meet nowadays. On this regard, organic materials and electronic devices offer promising feature for the development of an alternative technology to interface the brain with electronic instrumentation. These molecule-based platforms can bring transparent and flexible electronic sensors, actuators and circuitry closer to biological tissue as it can show high biocompatibility.
Here we present two bioelectronic devices, an OFET (Organic Field Effect Transistor) and an OECT (Organic Electrochemical Transistor) that are capable to excite and record signaling from astroglial cells. The effects are characterized by patch clamp, calcium imaging and extracellular recording techniques. Collectively the results show very promising properties of organic bioelectronic device for the study of astroglial cells in vitro.

Bibliography

[1] H. K. Kimelberg, «Functions of Mature Mammalian Astrocytes: A Current View,» The Neuroscientist, vol. 16, pp. 79-106, 2010.

[2] M. Nedergaard, B. Ransom y S. A. Goldman, «New roles for astrocytes: Redefining the functional architecture of the brain,» TRENDS in Neurosciences, vol. 26, 2003.

[3] V. Benfenati y S. Ferroni, «Water transport between CNS compartments: functional and molecular interactions between aquaporins and ion channels,» Neuroscience, vol. 168(4), pp. 926-940, 2010.

[4] I. Markiewicz y B. Lukomska, «The role of astrocytes in the physiology and pathology of the central nervous system,» Acta Neurobiol Exp, vol. 66, pp. 343-358, 2006.

[5] A. P. Alivisatos, M. Chun, G. M. Church, R. J. Greenspan, M. L. Roukes y R. Yuste, «The Brain Activity Map Project and the Challenge of Functional Connectomics,» Neuron, vol. 74, pp. 970-974, 2012.

Brignoli Tarcisio

Supervisors: Delany/Scarlato

A high throughput qRT-PCR method to study transcription of virulence factors in *Staphylococcus aureus*

Staphylococcus aureus is a major human pathogen, responsible for a wide range of diseases. Its remarkable ability to develop resistance to antibiotics made *S. aureus* a worldwide issue in clinical medicine. One of the causes of its success as a pathogen is the peculiar array of immune evasion factors that enable the bacterium to avoid host defenses. One of these factors, the staphylococcal protein A (SpA), is able to bind the Fc region of IgG, hence preventing recognition of the Fc by the host immune system and allowing escape from antibody-mediated neutrophil phagocytosis. We analyzed a panel of circulating strains and for about 10% of the isolates SpA is not detectable by Western blot, despite the presence of the gene. Furthermore, the analysis of a subset of isolates belonging to the USA100 lineage showed that SpA negative strains express significantly lower amounts of *spA* transcript than SpA positive isolates. A high-throughput qRT-PCR analysis on a set of 90 virulence factors and regulators showed a negative Spearman correlation coefficient between *spA* transcript levels and capsule biosynthesis-related genes (-0,74 for *capA*, -0,62 for *cap5H*). In other words, strains that have low amount of *spA* mRNA, have high amount of *cap* operon mRNA. The capsule is another important virulence factor that inhibits opsonophagocytosis, hence we can speculate that this immune evasion mechanism is enhanced in strains that express low amount of SpA.
Our data suggest a tight regulation between *spA* and capsule, and let us hypothesize a different relevance of these two immune evasion mechanisms in different *S. aureus* strains.

Cameli Cinzia

Supervisor: Maestrini

A genome-wide association study of common SNPs and rare protein altering variants influencing risk for cluster headache

Cluster Headache (CH) is a primary headache with a mean prevalence of 0.1% among general population and a clear male predominance. It is characterized by severe unilateral retro-orbital or fronto-temporal pain, lasting from 15 to 180 minutes. Pain attacks show a circadian and seasonal rhythmicity and smoking is the most frequent life-time habit in CH patients with a prevalence over 80%.

The biology of CH is poorly understood. Current hypotheses are focused on vasomotor changes, inflammation, immune changes, autonomic system imbalance and hypothalamic dysfunction.

Twin and family studies have indicated the importance of genetic factors in CH; however genetic predisposition seems to be complex, with the environment and genetic factors interacting together[1].

Given the largely unknown pathophysiology of CH, We have performed a genome-wide association study (GWAS) in a clinically well-defined cohort of 99 Italian patients with CH and in a control sample of 360 age-matched sigarette smoking healthy individuals, using the Infinium PsychArray (Illumina), which combines common highly-informative genome-wide tag SNPs and exonic SNPs. Genotype data were used to carry out a genome-wide single marker case-control association analysis using common SNPs, and a gene-based association analysis focussing on rare exonic variants in 745 candidate genes with a putative role in CH.

Although no single variant showed statistically significant association at the genome-wide threshold, we identified an interesting suggestive association (P=9.1x10-6) with a common variant of the PACAP receptor gene (*ADCYAP1R1*). Furthermore, gene-based analysis provided evidence for a rare variant of the *MME*. Both gene products are known to have a pivotal function in pain mechanisms, thus making these association particularly stimulating.

Capriotti Luigi

Supervisors: Norais/Scarlato

Applying Mass Spectrometry to understand glycoconjugate-MHCII interactions

Glycoconjugates based vaccines are among the safest and most efficacious vaccines developed so far. In spite of their great effectiveness, their mode of action and in particular their presentation to the immune system are not fully understood. Dissecting the mechanism that controls glycoconjugates/peptide-MHCII interactions will allow to define a rational for a better design of glycoconjugate vaccines. It is well accepted that polysaccharides function as T cell–independent antigens, since they fail to induce T cell–mediated immune responses (IgM-to-IgG class switching, booster antibody response and T cell memory). On the contrary, when a polysaccharide is linked to a carrier protein, the protein provides the T cell epitopes that engage the T cell receptor (TCR) and trigger the release of cytokines that help the B cell to differentiate and proliferate. With the aim to analyze glycoconjugate/MHCII interactions and evaluating the efficacy of glycoconjugate MHCII processing and presentation, different glycoconjugates were synthesized. ß 1,3 glucans and meningococcal serogroup C oligosaccharides were covalently linked to the lysine side chains of recombinant proteins from *Neisseria meningitids* and *Streptococcus pneumoniae*. Testing the glycoconjugates in mice, we highlighted differences in the immune response probably due to different pattern of glycosylation that can lead to a different MHCII-peptide interaction. Using a proteomic and glycoproteomic approach we evidence differences in the pattern and extent of conjugation. Mutated recombinant carriers lacking of the identified conjugation-sites were produced to be conjugated and tested as carrier in mice. This represents a first step in the design of experiments that will provide insight in the understanding of the peptides/glycopeptides-MHCII interaction.

De Magis Alessio

Supervisor: Capranico

Stabilization of R-loop and G-quadruplex structures in cancer cells treated with G-Quadruplex binders

The aim of my PhD project is to define the mechanism of G-quadruplex (G4) binder-mediated induction of genome instability and alteration of gene expression in cancer cells.

G4 and R-loop are non-B DNA structures that have become the topic of intensive research from scientist, over the last few decades, because both structure can block transcription and DNA replication, creating replicative stress and potentially causing further DNA damage.

We have used different G4 binders such as the well-known G4 binders Pyridostatin (PDS) and Braco-19, and two new compounds synthesized by the Rambaldi laboratory (FABIT-Unibo) called FG and FA. Our recently published data show that FG but not FA stabilize G4 in living human cells, as the absence of the guanylhydrazone group in the FA structure relative to FG determines a decreased interaction with G4s. For this reason, I use FA as a negative control.

Immunofluorescence experiments using an antibody that specifically recognizes G4 (BG4) show an increase signal after PDS, Braco-19 and FG treatments of 24 hours. In contrast, no differences are observed in cells treated with FA. Moreover, several experiments demonstrate that R-loop structures also increase following treatments of cells with the studied G4 binders, but not with FA. R loops are detected by immunofluorence microscopy using the specific S9.6 antibody. The data may suggest that G4 stabilization by the compounds increases the stability of R loops.

Further experiments aimed at determining G4 binder effects at cellular levels showed that cells accumulate in G2/M of the cell cycle following treatment. Moreover, FG and PDS induce a decrease of Ki-67 signal and an increase of γH2AX levels suggesting that they halt cell cycle progression inducing DNA breakage of the genome.

The current results show that G4 binders stabilize, not only G4 structures, but also R loops in the nuclear genome of human cells. It will be interesting to map R loops in the genome along with γH2AX to establish molecular aspects of G4 binder-induction of DNA damage and genome instability.

Ferrucci Francesca

Supervisor: Perini

Modulation of MYCN/MAX/MAD network

The Myc oncoproteins belong to a class of basic-region/helix-loop-helix/leucine-zipper (BR/HLH/LZ) proteins. This oncoproteins can dimerize with MAX forming a functional transcriptor factor that can bind DNA at specifical site called E-box and carry out its functions as positive modulator of gene involved in proliferation and self-renewal. Because MAX was found to be present under conditions in which MYC proteins are not expressed, it was considered that MAX may bind other bHLHLZ containing proteins. Indeed, among the member of the network which MYC belongs, other MAX interacting protein were identified: MXD1 and MNT, belonging to the MXD protein family. Importantly, both MXD1-MAX and MNT-MAX heterodimers recognize the same E-box consensus sequence as MYC-MAX heterodimers, but function as transcriptional repressor, further strenghten the model in which MXD proteins antagonize MYC in regulating many aspects of cell biology. (1)(2)

Overexpression of the MYC genes plays a prominent role in the etiology of many types of tumor and therefore MYC overexpression have been the focus of several reviews.

It is well established that switching off c-MYC expression results in tumor regression, suggesting that a tumor cell requires continuous c-MYC expression. (3) Despite knowing about the existence of MYCN for nearly thirty years, the majority of functional studies involving MYC family members have focused on c-MYC due to the limited expression profile of MYCN in human cancers, and also in part due to the existence of highly conserved functional domains between c-MYC and MYCN. (4) The MYCN gene is found amplified in several types of

childhood tumor of mostly neuroendocrine origin, including about 25% of neuroblastomas that represent a subset of aggressive neuroblastoma with a poor prognosis. (5)
Previous studies have shown that downregulating MYCN expression, via antisense oligonucleotides, resulted in lower tumor incidence and decreased tumor mass in murine neuroblastoma tumor model. Together, these observations suggest that blocking MYCN expression may be beneficial for neuroblastoma patients. (6)
My Ph.D. project was therefore divided into two parts, one of which was the evaluation of the oncogenic behaviour of neuroblastoma cells after modulation of MYCN functional antagonists proteins MAX and MNT.
Using ShRna and transient inducible overexpression of MYCN antagonists MNT and MAX, I generated different pools of neuroblastoma MYCN-amplified and MYCN-not amplified cell lines characterized by the overexpression and downregulation of both MAX and MNT proteins. Through analysis of oncogenic behaviour such as motility, growth rate and transcription profile, I suggested that overexpression of MAX and MNT in MYCN-amplified neuroblastoma cell lines lead to a more malignant phenotype while downregulation bring to a mild oncogenic phenotype. Interestingly seems that MNT has an essential role in the modulation of proliferation and cell motility while MAX is required for both differentiation and proliferation with a distinct role depending on MAX isoforms. Data were confirmed also in MYCN-not amplified cell lines.
The second part of my Ph.D. project arised from a collaboration with Australia Children's Cancer Institute and was focused on characterization of a new compound named A9 able to downregulate MYCN expression.
From evaluation of protein and transcript stability, it was excluded that the drug A9 acts on stability and it was therefore hypotesized that it can act at transcriptional level: aim of this second part of my project was to identify the mechanism(s) through which the drug downregulates MYCN protein level. Using a reporter gene assay, it was identified the minimum responsive region to the drug A9. This region spans 33bp immediately upstream of the multiple transcription start site, which is highly conserved in the human and mouse MYC genes and control basal promoter activity. (3) In silico analysis have revealed the presence of three E2F sites, two of which are inversely oriented and overlapped, and one TIE sites, a Transforming Growth Factor B inhibitory element. E2F proteins are already known to be involved in the regulation of MYCN expression in Neuroblastoma (3-4) and actually my purpose is to verify their role in the A9-mediated downregulation of MYCN.

References

1. Grandori, C., Cowley, S.M., James, L. P. and Eisenman, R.N. The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu. Rev. Cell Dev. Biol. 2000;16;653-699
2. Diolaiti D. et al. Functional Interactions Among Members of the MAX and MLX Transcriptional Network During Oncogenesis. Biochim. Biophys. Acta. 2015;1849(5);484-500
3. Strieder V, Lutz W. E2F proteins regulate MYCN expression in neuroblastomas. J Biol Chem 2003;278:2983–9.
4. V.H Cowling, and M.D Cole, Mechanism of transcriptional activation by the Myc oncoproteins Semin Cancer Biol 2006;16;242-252
5. Brodeur G.M., Seeger R.C., Schwab M, Varmus HE, Bishop JM. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 1984;224;1121-1124
6. Burkhart, C.A., et al., Effects of MYCN antisense oligonucleotide administration on tumorigenesis in a murine model of neuroblastoma. J Natl Cancer Inst, 2003;95(18);1394-403

Fogazza Mario

Supervisor: Rugolo

Generation and biochemical characterization of innovative cellular models for Dominant Optic Atrophy.

DOA is the most common hereditary optic neuropathy. There are two forms of DOA: the most common, characterized by degeneration of the retinal ganglion cells and optic nerve atrophy, and a syndromic form, named DOAplus, with the involvement of other organs and a much more severe phenotype. More than 200

pathogenic mutations in the OPA1 gene have been described so far associated with DOA, spread throughout the gene. OPA1 is a conserved dynamin-related GTPase essential for mitochondrial inner membrane (IMM) fusion and cristae morphology, and is involved in oxidative phosphorylation efficiency and mitochondrial DNA (mtDNA) maintenance. The protein is localized in the mitochondrial intermembrane space, anchored in the IMM.

In order to better understand the mechanisms behind the pathology, we collected fibroblasts from DOA patients, introduced the same mutations in MEFs, and performed a deep biochemical characterization of both cell models, analyzing mitochondrial network morphology and fusion/fission proteins expression, mtDNA content, ATP levels and synthesis rate and respiratory supercomplexes assembly. Furthermore we are now introducing OPA1 mutations associated with DOA and DOAplus on HeLa cells through CRISPR/Cas9 technology. We will characterize these new cell lines like we already did with fibroblasts and MEFs and moreover, in collaboration with the Mitochondrial Medicine Research Centre of the University Hospital of Angers, we will study the effects of these mutations and of the deletions of the exon 4, 4b and 5b on the cells metabolome

Formaggio Francesco

Supervisors: Monti/ Caprini/ Benfenati

A Swell channel indeed: expression of Leucine-rich repeat containing 8 member A (LRRC8A) in the adult mouse brain.

Vertebrate cell volume regulation involves the rapid adjustment of cellular volume in response to external challenges or during the execution of cellular functions. In almost all cells, osmotic swelling is followed by the cell-intrinsic regulatory volume decrease (RVD), which tend to restore the initial cell volume. Volume regulated anion channels (VRAC) regulate the efflux of Cl- which is an important element of the RVD. In the brain cell volume regulation is critical and is precisely maintained within the homeostatic parameters. This task is especially carried out by astrocytes, which are responsible for the maintenance of the external environment surrounding neurons.

Leucine-rich repeat containing 8 member A (LRRC8A), a protein that contains 17 leucine-rich repeats, has been discovered as en essential sub-unit of VRAC in 2014 (Voss et al.). Here we show the production of an antibody directed against LRRC8A and the expression of this protein in cell lines, cultured astrocytes, mouse brain and other organs. In brain edema and herniation, for instance, the cell volume regulatory mechanisms are overwhelmed and disrupted by the action of excitatory amino acids released from injured cells. The study of this protein, intimately involved in cell volume regulation, may be an important contributions towards the clinical treatment of these pathological conditions.

References:

Voss FK, Ullrich F, Münch J, Lazarow K, Lutter D, Mah N, Andrade-Navarro MA, von Kries JP, Stauber T, Jentsch TJ. Identification of LRRC8 heteromers as an essential component of the volume-regulated anion channel VRAC. Science. 2014 May

Gasperini Gianmarco

Supervisor: Aricò/Scarlato

OMV-based and proteomic-driven antigen selection identifies BrkA as a novel factor able to prevent B. pertussis adhesion to epithelial cells

Bordetella pertussis is a gram-negative bacterium, obligate human pathogen and causative agent of whooping cough, a greatly contagious disease which is recently increasing in occurrence despite high vaccination coverage world-wide. The resurgence of pertussis over the last two decades made it clear that a new generation vaccine against pertussis able to prevent colonization and to induce a longer lasting protective immunity is needed.

The present study aims at the identification and further characterization of novel virulence factors both able to contribute to *Bordetella pertussis* adhesion to the human upper respiratory tract and to induce a strong and protective immune response.

In order to identify new key adhesins we used spontaneously released outer membrane vesicles (OMV) to study the protein decoration of the pathogen in its virulent or avirulent phase. We started by employing a proteomic approach to identify and quantify all the proteins in OMV, especially focusing on outer membrane proteins found exclusively in the virulent phase. Based on their high abundance in OMV and on their peculiar autotransporter structure, we focused on 5 promising candidates to be assessed for their adhesive properties and vaccine potential. We generated *E. coli* strains constitutively expressing the selected full length proteins and checked for their ability to bind to A549 respiratory epithelial cells as compared to wild type *E. coli*. We found that 4 out of 5 proteins conferred adhesive ability to *E. coli*. To further characterize the immunogenic properties of the selected proteins we continued the analysis by identifying the proteins specifically detected by anti-OMV serum: only 3 out of 5 of the selected antigens were able to elicit an antibody response when displayed in the complexity of the OMV. The ability of anti-OMV serum to inhibit the adhesion of *B. pertussis* and recombinant *E. coli* on A549 cells was checked: anti-OMV serum proved to be extremely powerful in preventing *B. pertussis* adhesion to a respiratory epithelium but only BrkA-expressing *E. coli* was inhibited. To finally analyze the protective potential of the selected vaccine candidate, recombinant His-tagged BrkA was expressed, purified and administered to mice; stand-alone immunization with BrkA resulted in significant protection against lower respiratory tract infection of *B. pertussis*. Moreover, anti-BrkA serum was able to completely inhibit recombinant *E. coli* adhesion to A549 cells.

To the best of our knowledge, this is the first time that B. pertussis autotransporters were rationally characterized for their adhesive properties exploiting a heterologous *E. coli* background. Also, taking advantage of the OMV structure, we were able to better simulate the natural antigen display and to identify immunogenic outer membrane proteins. Finally, with our adhesion inhibition assay we contributed to unravel the mechanism of protection induced by BrkA immunization and further supported its potential in the control of *B. pertussis* colonization.

Gugnoni Mila

Supervisors: Ambrosetti/Ciarrocchi

Cadherin 6 promotes EMT and cancer metastasis by restraining autophagy

The transdifferentiation of epithelial cells towards a mesenchymal condition (EMT) is a complex process that allows tumor cells to migrate to ectopic sites. Cadherins are not just structural proteins but they act as sensors of the surrounding microenvironment and as signaling centers for cellular pathways. However, the molecular mechanisms underlying these signaling functions remain poorly characterized. Cadherin 6 is a type 2 cadherin, which drives EMT during embryonic development and it is aberrantly reactivated in cancer. We recently showed that CDH6 is a TGFβ target and an EMT marker in thyroid cancer, suggesting a role for this protein in the progression of this type of tumor. Here, we assessed the role of CDH6 in the metastatic progression of thyroid

cancer, showing that loss of CDH6 expression profoundly changes cellular architecture, alters the inter-cellular interaction modalities and attenuates EMT features in thyroid cancer cells. Using a yeast two-hybrid screening approach, based on a thyroid cancer patients library, we showed that CDH6 directly interacts with GABARAP, BNIP3 and BNIP3L, and that through these interactions CDH6 restrains autophagy and promotes reorganization of mitochondrial network through a DRP1 mediated mechanism. Analysis of the LIR domains suggests that the interaction with the autophagic machinery may be a common feature of many cadherin family members. Moreover, the analysis of CDH6 expression in a unique cohort of human PTCs showed that CDH6 expression marks specifically EMT cells and it is strongly associated with metastatic behavior and worse outcome of PTCs. Furthermore, we attempted to characterize the molecular mechanisms through which CDH6 expression is controlled in thyroid cancer. Using the ENCODE annotation data we identified 12 regulatory regions with features of potential ENHs within the CDH6 locus. We used ChIP and luciferase analysis to investigate the function of these regions in aggressive thyroid cancer cells and in response to TGFβ, master regulator of CDH6 expression. In conclusion, our work provides new insights into the molecular mechanisms driving metastatic spreading in thyroid cancer.

Gurrieri Libero

Supervisors: Sparla/Trost

Exploring the connection between carbon metabolism and drought stress response in *A. thaliana*

Starch is a polymer of D-glucose also accumulated in leaves as transitory starch, typically synthetized during the day and degraded the following night to allow plant metabolism even in absence of photosynthesis. Recently it has been demonstrated that in response to osmotic stress (Zanella et al., 016) starch degradation could occur even during the light period, supplying part of the carbon skeletons required for the biosynthesis of proline. Proline is a well-known osmoprotectant, also involved in the scavenging of reactive oxygene species (Szabados and Savouré, 2009). Starting from this observation and in order to better characterize the connection between starch and proline, we outlined a set of *in vivo* experiments to address the question. To this aim *Arabidopsis thaliana* seeds, knock-out mutants impaired in starch, sugars and proline metabolisms were purchased from the European Arabidopsis Stock Centre (NASC, Nottingham, UK) and screened by PCR to select homozygous lines. On the first available homozygous lines, a preliminary screening with Lugol's solution has been performed, comparing with wild-type plants the level of leaf starch in response to stress. Two mutants were deeper analysed: *gwd2* and *p5cs1* mutants. Glucan, water dikinase isoform 2 (GWD2) is a cytosolic enzyme suggested involve in sugars mobilization (Glaring et al. 2007); δ1-pyrroline-5-carboxylate synthase 1 (P5CS1) is also a cytosolic enzyme that translocates into the chloroplast in response to stress and responsible of the last step of proline biosynthesis (Székely et al., 2008). Our preliminary data suggest that in response to osmotic stress both mutants degrade less starchthan wild-type plants, however only *gwd2* mutant shows an higher level of lipid peroxidation in comparison with both wild-type and *p5cs1* plants.

Ignesti Marilena

Supervisor: Gargiulo

Analysis of intracellular and extracellular Awd distribution in *Drosophila*

The *abnormal wing discs* (*awd*) gene is the *Drosophila* homologue of the human group I *Nme* genes coding for metastasis suppressor proteins with NDPK activity. Beyond its NDPK function, many other cellular activities have been assigned to Awd, including a role as endocytic mediator. Earlier genetic studies in *Drosophila* suggested that Awd could act as a supplier of GTP for Dynamin, encoded by the *shibire* (*shi*) locus. Subsequent studies demonstrated the presence of Awd and Nme23 in the extracellular environment, both in cell cultures and in the whole organisms. While no role has been inferred to extracellular Awd, elevated Nme1 level in cancer patient serum is an unfavourable prognostic marker. Moreover, it has been shown that Nme1, once internalised, can inhibit metastasis-associated phenotypes in tumour cell lines.

I investigated Awd secretion and internalisation in *Drosophila* fat body. This larval tissue is an excellent model to study endo- and exo-cytosis since, to absolve its numerous functions, appropriate trafficking machineries are strictly required. My genetic analysis revealed that in shi^{ts} mutants exposed at the restrictive temperature Awd haemolymph levels are significantly enhanced, while conditional block of Shi activity in adipocytes greatly reduces intracellular amount of Awd. This indicates that Shi plays a key role in controlling the balance between intracellular and extracellular Awd. I then analysed the co-localisation of Awd with exosome markers in wild type and Shi-defective adipocytes. I found that Awd secretion does not involve CD63- or Evi- positive exosomes nor Rab11 recycling vesicles. A lot of trafficking pathways have been already characterised in *Drosophila*. A key step in many endocytic routes is the trafficking through Rab5 positive early endosomes. I then decided to investigate the effect of *rab5* mutation on Awd endocytosis by generating $rab5^{2}$ loss-of-function cell clones. The endocytic route through which Awd enters into the cells seems not to encompass Rab5 early endosomes, since its absence does not affect Awd intracellular amount. Collectively, these data allowed me to draw a possible shuttling pathway of Awd through the inside and the outside of the cell.
These findings draw a tight intertwined relationship between Awd and Shi and add new data on Awd trafficking between the intracellular and extracellular milieu.

Karges Saskia Katharina

Supervisors: Monti/ Caprini/ Benfenati

Impact of an OFET organic interface on the physiology of astroglial cells *in vitro*.

Astroglial ion channels and calcium signalling play a central role in the physiology and pathophysiology of the Central Nervous System. In this context, increasing efforts are needed to generate innovative tools for monitoring astrocytes biochemical or bioelectrical activity *in vitro* and *in vivo*.
My group previously reported on transparent Organic Cell Stimulating and Sensing Transistors (O-CSTs), an organic field effect device, capable of providing bidirectional stimulation and recording of primary DRG neurons. Here we explore O-CST's functionality to stimulate, evoke and control the whole cell conductance in primary cultured astrocytes.
We found that the perylene based capping layer material P13 is biocompatible with primary astroglial cells, enabling their adhesion and growth. Furthermore, the organic material preserves the astrocytic electrophysiological properties. By means of patch-clamp analyses, we explore the effect of the stimulation provided by the O-CST on the whole-cell conductance of the patched astrocytes. We found that the stimulation by O-CST lead to an exclusive increase in the inward current that could be prevented by application of Ruthenium Red prior to stimulation and partially be suppressed by the TRPV4 specific blocker RN1734, suggesting a contribution of the transient receptor potential (TRP) channels, of which TRPV-4 has been shown in former studies to mediate Ca2+ influx in astrocytes.
The results suggested that the O-CST is capable to modulates the specific conductance and calcium signaling in astrocytes and could become a powerful tool to unravel mechanisms underpinning astrocytic physiology and pathophysiology

Leone Giulia

Supervisor: Porcelli

Induction of pseudonormoxia as adjuvant therapeutic strategy for cancer

Mitochondrial complex 1 (CI) is the first component of electron transport chain and plays a key role in the generation of energy required for the cell growth and proliferation. This enzyme can be imagined as a hard-working machine that promotes the oxidation of NADH, produced through the tricarboxylic acid (TCA) cycle,

and simultaneously the proton translocation across the inner membrane, generating the mitochondrial membrane potential.
A prominent role of CI in cancer progression is currently emerging since several somatic mtDNA mutation have been reported in different types of tumors and their ability in the modulation of tumor progression is still under discussion.
Our research group has demonstrated that severe mtDNA mutations that induce the disassembly of CI display an antitumorigenic potential. Mutations that impair CI structure and function are correlated with NADH accumulation and alteration of α-ketoglutarate (KG)/succinate (SA) balance. This condition leads to a chronic Hypoxia-Inducible factor 1 α (HIF-1α) destabilization even when O_2 is available, a condition that we termed pseudonormoxia, correlated with a decrease in tumor growth *in vitro* and *in vivo*.
My PhD project is based on the idea that pseudonormoxia can be induced by unbalancing the αKG/SA ratio mimicking a normoxic condition and leading to the block of tumor progression. The link between HIF1α stabilization and mitochondrial function opens to new metabolic-based therapeutic approaches for those cancers for which adaptation to hypoxia is a step that must be overcome to progress toward malignancy. In fact, during the past years, many efforts have been made to identify small molecules that inhibit HIF-1α signaling through different mechanisms, but these inhibitors are often cytotoxic or not specific.

Loibman Stefany

Supervisors: Scarlato/Roncarati

Functional analysis of heat shock regulator HrcA in *Helicobacter pylori*

Under conditions of heat shock, bacteria induce the synthesis of a class of highly conserved proteins, called Heat Shock Proteins (HSPs). In *Helicobacter pylori*, the major heat-shock genes are negatively regulated by two transcriptional repressors, one of which HrcA. To characterize the HrcA regulons, we use a strategy that combines whole transcriptome analysis (through RNA sequencing) with chromatin immunoprecipitation followed by deep-sequencing (ChIP-seq), one of the most powerful approaches to characterize protein-DNA interactions *in vivo.* Specifically this approach is dependent on the cross-linking of proteins to specific genomic targets, followed by antibody enrichment of the protein–DNA complexes and high-throughput sequencing of the recovered DNA fragments. However, this kind of analysis has been hampered so far by the fact that the HrcA protein, is characterized by a peculiar instability *in vitro* and by a particularly low immunogenicity; as a consequence, any attempt to generate a specific α-HrcA antibody in animals was unsuccessful. To overcome this limitation, it has been engineered a strain of *H. pylori* able to express a fusion protein with an epitope-tag, that can be immunoprecipitated in ChIP using a commercial antibody. Specifically, the aim of this work was to obtain an HrcA-3XFLAG-tagged strain and to analyze the functionality and expression of this tagged-protein, to assess the functionality of the HrcA-3XFLAG fusion protein expressed in the generated strain, the HrcA-dependent regulation of heat shock genes in these bacteria was analyzed. To further confirm that the HrcA-3XFLAG fusion protein was expressed and to evaluate the specificity of the commercial antibody, protein extracts were subjected to immunoblot analysis with an anti FLAG-tag antibody. The ongoing ChIP assay on the epitope tagged generated strain will allow to identify regulatory interactions of HrcA with other genes and to understand how this protein is possibly involved in other cellular processes.

Massaro Raffaele

Supervisor: Campadelli

Interaction of glycoprotein H/L from human and swine α-herpesviruses with integrins of the αV family

α-Herpesviruses are important human and animal pathogens.Examples include the human Herpes Simplex Virus 1(HSV1) Varicella Zoster Virus (VZV) and the swine Pseudorabies Virus (PRV). HSV1 enters receptor bearing cells through a species-specific tropism factor (gD, which binds host Nectin1) and by envelope fusion mediated by an evolutionary conserved glycoprotein trio (gB, gH and gL), sequentially engaged by cellular receptors. gH

heterodimerizes with gL (a conserved feature across the Herpesviridae family) and binds to αVβ6 or αVβ8 integrins, to promote virion entry through the endocytic pathway. Previously we showed that interaction with αVβ6 or αVβ8 integrins promote, in both virion adsorption and co-culture assay, the dissociation of gL and its release in the medium(Gianni et al. 2015).gL dissociation only takes place if gB, gH/gL, gD, Nectin1 and integrins are present, as it happens during infection, and is prevented by a neutralizing Mab to gH/L (LP11). Here we asked whether this mechanism is typical of HSV1 or common to other α-Herpesviruses, VZV and PRV. VZV encodes no gD, the receptor for VZV gB is MAG (Suenaga et al. 2010) and receptor for VZV gH is still elusive. Recently Yang et al. 2016 showed that RNA-i mediated down-modulation of αV integrin hampers VZV infection in melanoma cells. VZV(HHV-3) requires a Biosafety Level 3. In our laboratories (BL2) we performed a surrogate cell-cell fusion (co-culture) assay to investigate which β subunit is involved. When present, specific integrins increased the extent of fusion of VZV. PRV uses the same glycoprotein quartet as HSV1 for cell entry, receptors for PRV gH/L are unknown.We investigated integrin interaction with PRV glycoproteins by immune-precipitation and demonstrated that gL dissociation and its release in the medium take place only with integrin αVβ5 or αVβ8 in co-culture assay and during virion entry.

Massenzio Francesca

Supervisor: Monti

Reduction of DNA/ RNA binding protein (TDP-43) toxicity in primary motor neurons mediated by Glycogen Synthase Kinase (GSK)-3 inhibition.

Abnormal intracellular protein aggregates are a key characteristic in most neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS). The DNA/RNA binding protein (TDP-43) was initially recognized as a major constituent of pathological ubiquitinated protein aggregates in the brain and spinal cord tissue of patients with sporadic ALS (sALS). Dominant mutations in TDP-43 were subsequently identified in ALS patients with evidence that these mutations were indeed causative of ALS pathogenesis. The Q331K mutation (substitution of lysine for glutamine) is one of the most common TDP-43 mutations related to the onset of ALS. It is localized in a highly conserved region of the C-terminus of TDP-43 known to be involved in protein-protein interactions.

By using primary motor neurons from E12.5 mice embryos which overexpressed TDP-43, we observed a predominant nuclear localization in physiological conditions while, under pathological conditions, TDP-43 is characterized by a nucleus-to-cytoplasm translocation.

To identify the way to reduce TDP-43 toxicity, we discovered a mechanistic link between TDP-43 and Glycogen Synthase Kinase (GSK)-3. The inhibition of GSK-3 activity, mediated by two different compounds, restores the viability in motor neurons overexpressing Q331K TDP-43 compared to the control condition. The beneficial effects of GSK-3 inhibition seem to be related with the ability to promote the partial nuclear re-localization of Q331K TDP-43 and, at the same time, to activate a degradative pathway to reduce the intracellular level of the protein. While still preliminary, these data show that the GSK-3 pathway in ALS may be a potential therapeutical target and thus deserves further study.

Mazzei Luca

Supevisor: Ciurli

Old and new urease inhibitors: in search of a key to enzymatic control

Urease is a nickel-dependent enzyme that catalyses hydrolysis of urea, triggering an overall pH increase and causing negative effects for human health as well as agriculture.[1] Therefore, a tight control of its activity is required and several classes of inhibitors were studied in the last decades.[1]

Here, we present a structural and kinetic study of *Sporosarcina pasteurii* urease (SPU) inhibited by three chemical species: i) 1,4-benzoquinone (BQ), ii) catechols (CATs), and iii) N-(*n*-butyl) thiophosphoric striamide (NBPT). Both BQ and CATs act as irreversible SPU inhibitors by covalently binding to a conserved cysteine residue located on a flexible flap that controls access to the active site cavity.[2,3] Inhibition mechanism of BQ on SPU shows an exponential decay profile,[2] while CATs have a more complex behaviour.[3] On the other hand, the X-ray crystal structure of SPU inhibited by NBPT sheds light for the first time on the inhibition mode of this commercial product extensively used in agriculture.[4] Altogether, this data will be useful to develop novel and more efficient urease inhibitors necessary to modulate its activity and to counterbalance its negative effects.

References

1. M.J. Maroney, S. Ciurli – *Chem. Rev.* (2014), 114, 4206.
2. L. Mazzei, M. Cianci, F. Musiani, S. Ciurli – *Dalton Trans.* (2016) 45, 5455.
3. L. Mazzei, M. Cianci, F. Musiani, S. Ciurli – *submitted*
4. L. Mazzei, M. Cianci, F. Musiani, S. Ciurli – *in preparation*

Moramarco Filippo

Supervisors: Balducci/Scarlato

Molecular characterization of a toxin-like protein of *Bordetella pertussis*

Whooping cough is a contagious respiratory disease caused by the gram-negative bacterium *Bordetella pertussis (*Bp). It has been described that the switch from the whole cell to acellular vaccines raised the cases of infection with Bp. This increase is primarily due to waning of vaccine-induced immunity, to increased transmission and to a selection pressure of vaccine-adapted strains. Therefore, the functional and biochemical characterization of *Bordetella pertussis* proteins could improve our knowledge regards Bordetella pathogenesis and microbiology. BP1253 is a protein with unknown function that presents a 3D structure similar to the chain A hypothetical protein Tt1465 from Thermus Thermophilus Hb8 that resembles the pertussis toxin (PT) structure. In view of this interesting structural similarity, BP1253 was selected for preliminary characterization experiments to determine if this protein is indeed toxin-like. A cell proliferation assay was used to test cytotoxicity. Adenocarcinomic human alveolar epithelial cells (A549) were incubated with different concentrations of the protein, but toxic effects were not evidenced. Pertussis toxin carries out its toxic activity through the ADP-ribosylation of the alpha subunit of G-proteins. The analogy with PT prompted us to investigate the presence of catalytic activities typical of the members of this protein family. In agreement with the absence of the hallmark residues of the catalytic core all the experiments performed gave negative results. However, results obtained, in agreement with the presence of an adenylate binding fold (Rossmann fold), suggested a binding with NAD and ADP-ribose. New experimental strategies will be considered to verify possible existence of intracellular toxic effects and different approaches will be followed to prove the binding with different adenylic nucleotides.

Pasquini Miriam

Supervisors: Francia/Zaffagnini

Structural and functional features of chloroplastic transketolase from *Chlamydomonas reinhardtii*

The biochemical and structural properties of the chloroplastic transketolase from *Chlamydomonas reinhardtii* (CrTK) were investigated, with a particular emphasis on its redox regulation involving thiol-based Post-Translational Modifications (PTMs). Our *in vitro* activity assays showed that the enzyme reconstituted with both its cofactors, thiamin pyrophosphate (TPP) and magnesium, is resistant to oxidant treatments, while the apo-enzyme showed a redox-sensitivity, suggesting the presence of regulatory disulfide bond(s). Through Mass Spectrometry (MS), a disulfide bond between the residues Cys470 and Cys484 has been detected. Inspection of the protein three-dimensional structure, solved at a 1.73 Å resolution, shows that Cys470 and Cys484 are indeed

at suitable distance for a disulfide bond formation. Circular dichroism analysis highlighted the importance of the TPP and magnesium in the formation of the final active enzymatic complex (holo-enzyme).
The importance of thiamin (a TPP precursor) and of the compounds belonging to the TPP biosynthesis pathway has been tested *in vivo* on *Chlamydomonas reinhardtii* coltures. Interestingly, the exposition of the coltures to these compounds induces a decrease of the transketolase gene expression.

Pelliciari Simone

Supervisors: Scarlato/Roncarati

Identification of HsrA (HP1043) regulon through ChIP-seq analysis reveals its role in controlling crucial cellular functions of *Helicobacter pylori*

The HP1043 protein of *Helicobacter pylori* is an essential transcriptional regulator and is expected to play a role in the regulation of crucial cellular processes. Even though detailed structural informations are available for HP1043, a deep understanding of its function and the definition of its target genes have been hampered so far by the fact that the *hp1043* gene cannot be deleted, nor the amount of protein modulated.
Using Chromatin Immunoprecipitation-sequencing (ChIP-seq), one of the most powerful approaches to characterize protein-DNA interactions *in vivo*, we were able to identify genome-wide several new HP1043 binding sites. Moreover, *in vitro* DNA binding assays enabled precise mapping of the HP1043 binding sites on new targets, whose analysis revealed the presence of a conserved nucleotide sequence motif. Intriguingly, a significant fraction of the newly identified motifs overlaps promoters associated to genes involved in the process of translation. Interestingly, when protein translation was experimentally blocked, a significant induction of almost all HP1043 target genes was detected, suggesting the idea that the protein can act as a transcriptional activator. To assess this hypotesis, *in vitro* transcription assay were performed on some HP1043 bound promoters: results suggesting a positive regulation of transcription directly mediated by HP1043 regulator will be presented.

Pepe Simona

Supervisors: Scarlato/Roncarati

Heat shock response in *Helicobacter pylori*: unravelling the role of the heat-shock repressor HspR

The ability of pathogens to cope with different environmental stresses is a crucial feature for bacterial survival; in this respect, the heat-shock response (HSR), a universal cellular response, allows cells to adapt to hostile environmental conditions and to survive during stress. In the major human pathogen *Helicobacter pylori* the expression of chaperone-encoding operons is under control of two transcriptional auto-regulated repressors, HspR and HrcA, with the HspR repressor acting as the master regulator of the regulatory circuit. To deepen our understanding of HSR in *H. pylori* and to further characterize HspR regulon, we used global transcriptome analysis (RNA-sequencing) in combination with Chromatin Immunoprecipitation coupled with deep sequencing (ChIP-sequencing) of HspR genomic binding sites. The data from RNA-seq analysis showed that HspR is directly or indirectly involved in the regulation of different cellular crucial functions. On the other hand the data from ChIP-seq analysis showed that HspR directly controls a limited set of target genes. Moreover, we further characterized HspR-DNA interactions on its genomic targets through hydroxylradical Footprinting assay: this analysis revealed a peculiar pattern of DNA protection, suggesting new structural insights of HspR-promoter interactions. An important role in resistance against environmental stresses and in the control of bacterium cellular adaptations is also played by the ATP-dependent caseinolytic proteases (Clp), a class of serine proteases, involved in protein quality control as well as in degradation of regulatory proteins. To directly identify ClpP protease substrates, we are implementing a strategy to express *in vivo* a proteolytic inactive form of ClpP that will retain but not degrade substrates translocated into its proteolytic chamber.

Petralla Sabrina

Supervisor: Monti

***In vivo* studies of oligodendrocyte precursor proliferation in the rare demyelinating disease AGC1 deficiency.**

The mitochondrial aspartate-glutamate carrier isoform 1 (AGC1) deficiency is a rare syndrome caused by mutations in the SLC25A12 gene which lead to profound developmental delay, epilepsy, abnormal myelination and low levels of the myelin precursor N-acetyl aspartate (NAA) in the CNS (Wibom 2009). To understand the alterations involved in the synthesis of the myelin sheath in this disease, we previously analyzed the effect of AGC1 silencing on the proliferation and differentiation of OPCs (Oligodendrocyte Precursor Cells). In particular the analysis of PDGF-alpha and TGF-beta pathways showed alterations in AGC1 silenced cells. We therefore hypothesized that alterations of PDGF-alpha and TGF-beta pathways, in the presence of impaired AGC1, may disrupt the physiological cross-talk between neurons and OPCs, crucial for OPC proliferation and differentiation into oligodendrocytes and for myelin synthesis in the CNS. In order to confirm these in vitro data, we initiated in vivo studies after setting up a mouse colony (C57BL6/N background). Since AGC1 deficiency patients are characterized by reduced carrier activity and not lack of this carrier, we decided to focus on heterozygous mice which may better reflect in vivo disease mechanisms. Because our aim is to study proliferation defects in oligodendrocyte precursors, in addition to analyzing adult animals we focused on the study of mice at 21 days after birth since according to the literature, oligodendrocyte precursors reach a peak in proliferation at this stage. Our in vivo results (western blot analysis, immunohistochemistry, CNPase activity) reflect our previously obtained in vitro data. During the next year we aim to confirm these data also on human iPS (induced pluripotent stem) cells in the framework of the project "Biochemical changes in the rare genetic demyelinating and neurodegenerative disease AGC1 deficiency: a study on the different brain cells derived from human iPS" from the Italian Ministry of Foreign Affairs (MAE) for Italy-USA cooperation (2016-2017).

Petrovic Biljana

Superviror: Campadelli

Oncolytic Herpes Viruses retargeted to cancer-specific receptors

The aim of my PhD project is to optimize and produce safe and effective oncolytic vectors based on Herpes Simplex Virus type 1. The strategy pursued by Campadelli's group was to insert a scFv specific for cancer receptors in the envelope glycoprotein D (the major determinant of HSV-1 tropism), lacking its binding sites to HSV natural receptors. In this way they attained HSV-1 retargeted to cancer specific-receptors and de-targeted from HSV natural receptors. My work, this year, covered three lines of research, closely interconnected.

1. In this last year of my PhD, I continued to explore novel retargeting strategies for the design of oncolytic HSVs. I studied HSV-1 retargeting by inserting a scFv directed against overexpressed cancer receptors in a new position of the HSV-1 genome. I performed neutralization assays to confirm that the recombinant virus uses the cancer receptor during the entry. I measured the virus produced by the cancer cell line and set up in vitro cytolitic assay for the cancer cells. Furthermore, I investigated how the scFv mediates entry of the new retargeted HSV, trying to shed light on the mechanisms involved.

2. To enable the production of clinical grade virus, we constructed a bifunctional HSV-1 able to use alternatively HER2 (to infect the cancer cells) and an artificial receptor (to infect the non-cancer cells employed for virus production). I engineering the bifunctional HSV-1 trough galK recombineering in E. Coli, transfected it and growth it in mammalian cells. Then I tested it in vitro for its double entry apparatus by means of neutralization assays and measured the yield of the virus in the cancer and non-cancer cell line.

3. Therapies relying on just one target carry the risk of resistance development and don't take into account the heterogeneity of cancer. Targeting two tumor receptors could help to overcome these issues. During the 2nd year of my PhD I tested the double retargeted virus for the ability to infect and kill in vitro several human cancer cell lines with different expressions of the two receptors. In this last year, to evaluate the in vivo antitumor efficacy of the double retargeted virus, I tried to develop a suited experimental model. In collaboration with Primm s.r.l. of Treviso, I first set up a tumor induction experiments in nude CD1 mice to evaluate the growth rate of the selected cell lines and then I proceeded with the testing of anti-tumor efficacy, treating the xenotraplanted mice with the virus.

Pigini Paolo

Supervisor: Perini

The Ornithine Decarboxylase G317A polymorphism differentially affects gene expression and is potentially prognostic of outcome in Neuroblastoma

Polyamines are highly regulated cations derived from amino acids and found in all organisms. Polyamines are known to regulate cell cycle and high levels are characteristic of rapidly proliferating tissues and also different types of cancer, including Neuroblastoma (NB). Ornithine decarboxylase (ODC1) is a rate-limiting enzyme of polyamine synthesis and the overexpression is an independent prognostic marker in NB. In addition, ODC1 is transcriptionally regulated by MycN, whose amplification has a central role in NB development. In this work, we examined the prognostic significance of a single nucleotide polymorphism (SNP) within the ODC1 promoter (G317A, rs2302615). Previous analyzes, conducted by genotyping 839 primary NBs, showed that, in MYCN amplified patients, the GG genotype strongly predicted poorer outcome, whilst, in non-amplified patients, the presence of at least one G allele was enough for a worse outcome. At the same time electrophoretic mobility shift assays (EMSAs) showed a 3/5-fold reduced binding of MycN:Max to the SNP-closest Ebox when the SNP genotype was A rather than G. We therefore supposed that the SNP genotype could affect the MycN-mediated regulation of ODC1 expression in NB, and the disease outcome afterwards. We then demonstrated that the A allele is associated to a reduced promoter activity in MycN-inducible NB cells through dual-luciferase assays. We also edited BE(2)-C cells (a NB line with GG genotype) by CRISPR-Cas9 to finally obtain AG clones. Following analyzes showed a reduced chromatin acetylation and transcription for the ODC1 gene when the AG clones were compared to the wild-type line. Our results overall demonstrate the functional relevance of the G317A polymorphism in the regulation of ODC1 expression, and provide a mechanistic explanation for how the SNP can influence the NB progression. However further studies will be conducted in order to better understand the mechanism lying at the base of this process.

Ricchetti Beatrice

Supervisors: Scarlato/Delany

Investigation of lipoproteins translocation system in *N. meningitidis*

Lipoproteins (LPs) of pathogenic Gram-negative bacteria are involved in different biological processes ranging from outer membrane stabilization to activation of the immune response. For some Gram-negative bacteria, like *E. coli*, the lipoprotein translocation machinery is well characterized and, for *N. meningitides* (*Nm*), homologues of these proteins can be identified from the genome. *Nm*, unlike *E. coli*, displays many lipoproteins on its surface suggesting the presence of one or more unknown factors involved in the surface exposure of LPs [1]. An additional translocation component, Surface lipoprotein assembly modulator (Slam), involved in the surface exposure of specific *Nm* lipoproteins has been recently identified [2].

In this work we have generated Slam knock-out mutant in different *Nm* strains. We analyzed the surface exposure of NHBA (Neisserial Heparin Binding Antigen) and fHbp (factor H binding protein) as representatives of *Nm* surface exposed LPs, which are components of a novel vaccine against *Nm* serogroup B (MenB). FACS analysis showed that the deletion of Slam affected the surface exposure of the analyzed LPs in the selected *Nm* strains. In particular, the absence of Slam resulted in no detectable NHBA on the surface and NHBA accumulation inside the bacteria. On the other hand, we observed a reduction in fHbp levels on *Nm* surface that corresponded to an overall decrease in fHbp amout in Slam deletion mutant as compared with the wt isogenic strain.
In addition, we tested heterologous expression of *Nm* surface lipoprotein in the *E. coli* background. Co-expression of fHbp with a functional Slam protein resulted in significantly higher levels of fHbp expression and its surface exposure, confirming that Slam was involved in efficient expression and surface localization of fHbp.
In conclusion, our results validated the role of the surface lipoprotein assembly modulator Slam in outer membrane translocation mechanism in *Nm*. In addition, we showed that Slam was necessary for surface exposure of NHBA and had an impact in fHbp protein expression/stability in *N. meningitidis*, suggesting that it may play a differential role in the correct surface assembly of lipoproteins, depending on the nature of the lipoprotein.

1. Bos, M.P., V. Robert, and J. Tommassen, *Biogenesis of the gram-negative bacterial outer membrane.* Annu Rev Microbiol, 2007. **61**: p. 191-214.
2. Yogesh Hooda, C.C.-L.L., Andrew Judd, Carolyn M. Buckwalter, Hyejin Esther Shin, Scott D. Gray-Owen and Trevor F. Moraes, *Slam is an outer membrane protein that is required for the surface display of lipidated virulence factors in Neisseria.* Nature microbiology, 2016. **1**.

Sandri Federica

Supervisor: Zannoni

***Pseudomonas pseudoalcaligenes* KF707: role of terminal oxidases in the growth on different carbon sources**

During this year I focused my studies on the bacteria *Pseudomonas pseudoalcaligenes* KF707 and the role of its terminal oxidases in the growth on different carbon sources. KF707 is a Gram-negative proteobacteria. It was isolated in 1986, in Japan, in a site contaminated by polychlorinated biphenyl (PCB), a particular class of toxic and aromatic compounds. This bacterial strain represents a promising tool for bioremediation applications, because of its tolerance and its ability to degrade different compounds.

KF707 owns an aerobic energy metabolism and a membrane redox chain formed by five terminals oxidases: one quinol oxidase (*QXO*) *CIO* and four cytochrome oxidases (*COX*) cbb_3I, cbb_3II, aa_3I and aa_3II.

To study how each terminal oxidases is expressed with different carbon sources used for the growth, we constructed two categories of mutants:

- knockout mutants, for gene clusters coding for one or more oxidase;
- translation fusion mutants, in which the promoter of each oxidase has been fused to the *lacZ* gene, in order to evaluate the expression using the β-galactosidase assay.

Phenotypic and expression analysis, in minimal medium with addition of single carbon sources, demonstrated the existence of a relationship between the carbon source used during growth and the expression of specific terminal oxidases.

In the future it will be useful to complete the range of knockout and fusion mutants, in KF707, and characterizing them phenotypically. Furthermore, to complete this study, it will be necessary to analysed a series of proteins that regulate the biogenesis of cytochrome oxidases.

Sollazzo Manuela

Supervisor: Pession

Social cell biology in cancer: cell-cell communication in malignant evolution.

Cancer develops in a complex area where interactions between normal, cancer and stromal cells can modify local microenvironment. Well before turning into cancer derivatives, the epithelial cells sense the surrounding field, which can be either normal or mutated, as suggested by the Slaughter's *field cancerisation* theory, while comparing their fitness with that of the adjacent normal cells, according to the *cell competition* hypothesis. Some of them may acquire the ability to raid the basement membrane and enter the stromal compartment, where mesenchymal cells, extra-cellular matrix and blood vessels all contribute to malignant progression.

The term *field cancerisation* describes a pre-cancerous area in which genetically altered but histologically normal tissues proceed towards the development of multiple malignant *foci*. In the early stages of tumour progression cells may indeed acquire genetic damages that allow them to proliferate in patches of altered cells, gradually replacing normal tissue. This mechanism recalls *cell competition*, a phenomenon resting on fitness confrontation between cells sharing the same tissue. It is well-known that cells expressing high MYC levels acquire competitive behaviours if neighbours express lower MYC levels: as a consequence, these latter succumb by apoptotic cell death and are replaced by high MYC-expressing cells which eventually colonise the whole tissue. These intrinsic features of cell competition make it a candidate mechanism pioneering field cancerisation.

Based on these premises, we mimicked field formation by overexpressing MYC in a larval epithelium of *Drosophila*. Analysis of specific markers usually found in mammalian pre-cancerous areas confirmed that MYC overexpression is sufficient to trigger specific cellular responses. Moreover, following induction of different second hits, MYC-expressing fields were susceptible to the development of multifocal tumours, a typical trait associated with mammalian field cancerisation. In summary, our study identified an undescribed early genetic change implicated in field cancerisation and established a genetically amenable model which may help study the molecular basis of the initial tumourigenic events.

Vatrinet Renaud

Supervisor: Porcelli

Targeting respiratory Complex I: a metabolic strategy to prevent cancer progression.

Tumor cells exhibit profound bioenergetic changes with respect to the original non-transformed cell types. One of the main driving mechanism leading to such a metabolic alteration is triggered by hypoxia. Hypoxia is experienced by cancer cells during tumor progression and leads to a significant enhancement of glycolysis in order to sustain tumor growth and survival. The transcription factor hypoxia-inducible factor-1 (HIF1) is the master regulator of cell adaptation to low oxygen condition, and thus of tumor progression. Our group described the lack of the subunit HIF1a in low-proliferative oncocytic tumors, which are characterized by the loss of the mitochondrial respiratory Complex I (CI). Hence, we hypothesized that severe CI dysfunctions prevent HIF1a stabilization and impair tumor adaptation to low oxygen levels. Using the zing finger nucleases technology, we generated NDUFS3-deficient cancer cells that display acute CI deficiency. Engineered CI-defective cancer cells showed a lack of HIF1a stabilization in hypoxic condition, together with a significant reduction of the expression of HIF1a-responsive genes involved in the glycolytic machinery and tumor vascularization. *In vivo* experiments showed that the lack of CI was associated with an impairment in the growth of the xenografts, together with the lack of HIF1a activation, suggesting an inability of NDUFS3-KO tumors to face hypoxic conditions. Accordingly, further analysis showed that CI-deficient xenografts have developed a tailored structure to sustain oxygen supply and growth. Using a Tet-Off expression system, we thus generated NDUFS3- knock out inducible clones, allowing us to target CI during xenografts growth. In conclusion, these data showed that cancer cells are unable to sustain proliferation when functional CI is lost during tumor progression.

Supported by EU FP7 Marie Curie ITN MEET (grant No. 317433)