Abstracts - 18° PhD Workshop

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Organizers: V Scarlato, D Roncarati

Eva Donini (37-PON, Cappelletti)

Biochar and microorganisms: a synergy for the sustainable treatment of contaminated waters and filters

Biochar is a porous carbonaceous adsorbent generated through pyrolysis of organic biomasses (like agricultural wastes). It can be used as sustainable and low-cost alternative of activated carbon (AC) filters in wastewater treatment plants. Bacteria can establish biofilms on filter surface playing a role in the filtering performance, but the mechanisms of their interaction and the evolution of communities on biochar used as filter are largely unexplored. This PhD project (PON Ricerca e Innovazione 2014-2020, D.M. 1061/2021, financed by FSE REACT-EU) was aimed at i) investigating the metabolic activity, composition and dynamics of the microbial communities colonizing experimental filters (AC and biochar) applied in different water treatment plants, ii) studying the possibility to recover filters at the end of their lifecycle by treating them with synthetic microbial consortia or adapted communities, and iii) comprehending the molecular mechanisms involved in bacterial adhesion. Illumina sequencing data showed that microbial communities' composition differed depending on the treatment plant under analysis and the contamination present. Enrichment of specific taxa associated with biofilm growth, stress resistance and organic contaminant biodegradation was observed on filters with similarity between AC and biochar used in the same treatment plant.
Synthetic microbial consortia and adapted communities were applied to regenerate filters saturated with different types of contaminants. GC-FID analyses of regenerated filters demonstrated a better performance of contaminant removal on biochar filters by microbial communities as compared to AC, providing good indications on the possibility to utilize biological treatment to recover biochar filters.

Elena Chiti (38, Roncarati / Scarlato)

Investigating *Helicobacter pylori*'s transition to the coccoid form: insights into chromatin dynamics and regulation by HP1043

Helicobacter pylori, a WHO classified class I carcinogen, is a widespread human pathogen associated with gastrointestinal diseases such as peptic ulcer, gastric adenocarcinoma and MALT lymphoma. Under metabolic stress and extreme conditions, it adopts a viable non-culturable (VBNC) state, known as the coccoid form, which favours survival and dissemination. This transition involves specific morphological, metabolic and chromosomal remodelling events. Using SAMMY (Sequential Analysis of MacroMolecules accessibilitY) technology, which isolates and sequences chromatin fractions with different DNA accessibility, we investigated the regulation of nucleoid structure and genomic changes during the vegetative-coccoid transition of *H. pylori*. The master regulator HP1043 is hypothesised to play a key role in this transition, directly regulating essential genes and presumably controlling bacterial growth phases towards the coccoid form.

Furthermore, knockdown of HP1043 using peptide nucleic acids (PNAs) induced a shift towards the coccoid form. To further investigate the function of HP1043, we employed a reporter system controlled by HP1043-targeted promoters (wild-type or mutated). Reporter expression analysis revealed the dual role of HP1043 in gene regulation, supported by in vitro binding assays. In addition, to generate conditional silencing of HP1043, a paired-termini RNA (PTasRNA) against *hp1043* mRNA was designed in a heterologous *E. coli* system, resulting in *hp1043* repression upon system induction.

Clelia Manna (38-PNRR, Roncarati / Vannini)

Characterization of engineered bacterial outer-membrane vesicles (OMVs) and their effect as specific carriers for RNA-based therapy against osteosarcoma

Osteosarcoma is a malignant tumor primarily affecting long bones, and it is the most common primary bone sarcoma in children and adolescents. Despite advances in prognosis, there is room for improvement through new chemotherapeutic agents. A novel strategy for targeted drug delivery involves using biologically derived vesicles. Specifically, we are focusing on engineering outer membrane vesicles (OMVs), which contain proteins, lipids, and RNAs, from Gram-negative bacteria like *Helicobacter pylori* and *Escherichia coli*. Our first objective is to characterize OMVs, particularly their RNA content, using RNA sequencing and RT-qPCR.
Moreover, we aim to direct *H. pylori* and *E. coli* OMVs to specifically interact with osteosarcoma cells. To achieve this, we are employing single-chain variable fragments (scFv) targeting osteosarcoma-associated antigens while removing natural binding domains to minimize adhesion to non-target cells. As a proof of principle, we selected an scFv targeting HER-2, overexpressed in certain tumors including breast, ovarian, and less frequently, osteosarcoma. Furthermore, we are investigating additional osteosarcoma surface proteins with a scFv antibody specific to osteosarcoma cells, designated TP3, targeting ALPL-1. These scFvs will be expressed with outer membrane proteins *E. coli* OmpA and *H. pylori* BabA and presented on the OMV surface to deliver therapeutic agents to target cells.

Francesco Flandi (38, Oggioni)

Human spleen macrophages clear the pneumococcus by apoptosis not by phagolysosomes

The bacterium *Streptococcus pneumoniae* is one of the major causes of serious diseases and death. *S. pneumoniae* is exposed to phagocyte-mediated clearance mechanisms in the spleen but the roles of the spleen macrophages in pneumococcal clearance are unknown.
To explore how splenic macrophages respond to *S. pneumoniae*, we utilized an *ex vivo* human spleen perfusion model. To characterize the function of tissue macrophages, biopsies were analyzed through immunohistochemistry for the presence of the activated-caspase3 and lamp1 markers.
The *ex vivo* model of spleen perfusion showed that the organ captures and removes pneumococci from the system, with 90% of bacteria disappearing in the first hour. Immunohistochemistry showed that at 1h post-infection, cleaved-caspase3 increases in macrophages by 10-fold and both the numbers of bacteria and activated caspase3 decrease over time. At the same time, the percentages of lamp1 within tissue-macrophages were not found to increase during the pneumococcal infection, indicating that pneumococcal killing may be complemented by the induction of macrophages cell-death.

Finally, the *ex vivo* human spleen perfusions is allowing us a functional characterization of human tissue macrophages and showed that also in humans, replication in permissive CD169+ macrophages could be the critical point for the start of invasive infections.

Stefania Di Silvestro (38-PNRR, Cappelletti)
Bacterial functionalization of biochar as a sustainable strategy to deal with metal contamination

Bacteria capable of transforming toxic metals have practical applications for the treatment of polluted soils and can be used with porous supports like biochar, which facilitates biofilm production. Biochar, produced from the thermal decomposition of carbon-rich biomass, can support bacterial growth and metabolic activities. This PhD project, funded by Next Generation EU – PNRR D.M. 352/2022, aims to explore the interactions between microorganisms, biochar, and metals, and to investigate the potential use of biochar functionalized with bacterial biofilms for the bioremediation of metal-contaminated sites. Initially, a screening of a collection of microbial strains was carried out to assess their ability to grow in the presence of various toxic metal ions, i.e. arsenite, arsenate, tellurite, thallium. Eight bacterial strains from the genera *Rhodococcus, Gordonia, Pseudomonas, Bacillus*, and *Microbacterium* demonstrated the strongest growth when exposed to all tested metals. These strains were subsequently evaluated for their ability to adsorb metals and their capacity to adhere to biochar for functionalization purposes. To get deeper into the molecular mechanisms behind bacterial interactions with metals, we also analyzed a *Rhodococcus* mutant strain that is hyper-resistant to tellurite. Genetic characterisations and functional assays of the mutant provided first insights into the role of carotenoid biosynthesis in stress response, metal resistance, and transformation.

Ettore Lopo (39, Cappelletti / Sauro)
The biodiversity and biotechnological potential of cave microorganisms and their role in the protection of subterranean ecosystems

Caves are subterranean environments characterized by complete darkness and low nutrient availability. Studying cave microbiomes provides insights into the ecology of subterranean environments, allows the monitoring of groundwater and cave ecosystem health and can lead to the discovery of novel microbes with biotechnological potential. This PhD project (funded by PNRR-DM118/2023) aims at studying the microbial diversity and metabolic potential of microbial communities colonizing both pristine and human-impacted subterranean environments to envision the role of bacteria in the cave ecosystems, their possible exploit in biotechnological applications and use as bioindicators for subterranean environments protection. During the first year of PhD project, 16S rRNA gene sequencing provided information on microbial community structure and composition in different caves, whereas the metabolic potential of bacterial isolates was evaluated through culture-based assays. The analyses of thermal carbonatic caves in Monte Kronio and UNESCO protected gypsum caves in Emilia Romagna showed that microbial communities were mainly represented by members of the phyla *Pseudomonadota* and *Actinobacteria* associated to sulphur and nitrogen cycles and oligotrophic environments, depending on the samples considered. Most of the isolated strains showed specific enzymatic activities involved in the hydrolysis of different compounds and/or antimicrobial activities against common human pathogens. These results provide first insight into the microbial diversity and metabolic potential

of cave-dwelling bacteria across different subterranean ecosystems, setting the stage for future analyses.

Mattia Nicolò Li Volsi (39, Vannini / Roncarati)

Functional and structural study of sRNAs controlled by NikR and Fur in *Helicobacter pylori*

Helicobacter pylori is a bacterium that colonizes the gastric tract, causing serious pathological conditions. Its survival in the hostile gastric environment is supported by a regulatory program governed by both transcriptional and post-transcriptional regulatory networks. The transcriptional regulators NikR and Fur are crucial for maintaining intracellular homeostasis of nickel and iron, which are essential co-factors for enzymes for acid acclimation, energy metabolism, and oxidative stress resistance. NikR and Fur also regulate the expression of other genes involved in different cellular responses and the small non-coding RNAs (sRNAs) Nrr1, Nrr2, and Nc2090.

This project aims to functionally and structurally characterize these sRNAs to understand their role in the post-transcriptional regulation of targeted transcripts. The regulons of each sRNA will be determined by comparing the transcriptome (by RNA-sequencing) and proteome of the knockout mutants to those of the wild type and complementary strains. Furthermore, the in vivo interactome of these sRNAs will be determined adapting the MS2 affinity purification approach coupled with RNA-sequencing (MAPS) protocol to *H. pylori*.

Therefore, this multi-faceted approach will integrate, validate, and strengthen the results, characterizing the sRNA regulatory role and establishing a robust pipeline to study these factors in *H. pylori* and related bacteria.

Ginevra Marie Eloise Peppi (39, Zaffagnini / Trost)

Study of a new class of plant enzymes involved in redox homeostasis with still unknown functional plasticity using biological integrated approaches

In the course of evolution, plants have developed various strategies for survival and response to adverse environmental conditions in which there is an increased production of reactive oxygen and nitrogen species (ROS/RNS).

Our work focused on the enzymatic systems that control the catabolism of nitrosoglutathione (GSNO) - the main intracellular carrier and source of nitric oxide (i.e., the prototype of RNS) – as well as on the analysis of GSNO-mediated redox modulation on selected metabolic enzymes. In plants, GSNO catabolism is mainly controlled by S-nitrosoglutathione reductase (GSNOR), but it has recently been shown that aldo-keto reductases (AKRs) may also be involved in GSNO degradation.

We heterologously expressed and purified GSNOR and AKR4C isoforms (C8-C11) from the model plant *Arabidopsis thaliana*, and conducted a thorough analysis of their biochemical and structural properties.

GSNO is an important signaling molecule that modulate protein function by reacting with cysteine residues yielding nitrosothiols (S-nitrosylation) or mixed disulfide (S-glutathionylation). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an established target of cysteine-based redox modifications, but the molecular mechanisms underlying GSNO-mediated oxidation are not fully elucidated. To fill this gap, we obtained heterologous forms of plant and human GAPDH and analyzed the redox sensitivity to GSNO by means of biochemical and structural analyses. Both enzymes are reversibly inhibited by GSNO via S-nitrosylation of catalytic cysteine, highlighting that

GSNO can modulate GAPDH activity under physiological and pathophysiological conditions in both human and plant cells.

Edoardo Tosato (37, Sparla / Trost)

CYB561-A and CYB561-B are likely involved in intracellular ascorbate redox homeostasis and ROS-mediated signalling

Besides being one of the most important antioxidants, ascorbate (ASC) acts as a substrate for several enzymes, including cytochromes b561 (CYB561s). ASC is biologically active in its reduced state but certain cellular compartments (*e.g.,* vacuoles, ER, apoplast) lack the enzymes that catalyse its reduction from the oxidized forms. CYB561s catalyse the transmembrane transfer of single electrons – reducing monodehydroascorbate on one side of the membrane with electrons from ASC on the other side –, thus they could perform the regeneration of ASC in those organelles. In particular, the genome of *Arabidopsis thaliana* contains 4 CYB561s-encoding genes (*CYB561-A*, *-B*, *-C* and *-D*).
To investigate the physiological role of CYB561-A and -B during the development of *A. thaliana*, we performed the molecular phenotyping of T-DNA mutant plants lacking those genes at two distinct developmental stages. Compared to wild-type plants, mutants show an alteration in the intracellular ASC redox homeostasis: despite having small differences in ASC content, the mutants display an altered activity of several ASC-related enzymes. Additional results suggest the involvement of CYB561s in ROS-mediated signalling: all the mutants show a decreased ROS content, a significant delay in the flowering transition, a lower non-photochemical quenching rate and, under high-light, an increase in anthocyanin accumulation.
Finally, we are investigating the subcellular localization and the electrophysiological characterization of all the CYB561s, while we are producing *CYB561-A/B* double mutants and *CYB561-C* and -*D* antisense mutants.

Rachele Ingrisano (37-PON, Sparla / Trost)

Phytoremediation as a sustainable treatment of dairy wastewater

Microalgae are photosynthetic organisms with great metabolic flexibility. Their trophic habits can vary greatly so many microalgae species can adopt a photoautotrophic or heterotrophic metabolism and combine both through a mixotrophic growth. Their ability to remove unwanted organic compounds through the mixotrophic regime makes them excellent candidates for phycoremediation.
The dairy sector is one of the most impactful agri-food industries because of the high consumption of water resulting in the generation of contaminated dairy wastewater (DWW). Dairy wastewater purification systems based on microalgae and the ability to produce microalgal biomass in mixotrophic cultures enriched with whey as an organic carbon source have received much attention in recent years.
In this study, the use of waste from ricotta cheese processing (Second Cheese Whey, SCW) in a mixotrophic growth medium for the cultivation of the cyanobacterium *A. platensis* and of the microalga *C. zofingiensis* was evaluated. Specifically, we analyzed: the physicochemical properties of SCW, the effect of SCW on biomass production, growth rate, and organic composition of two strains (i.e. pigments, proteins, and carbohydrates), and the removal efficiency of major pollutants present in SCW (i.e. nitrogen, phosphorus, and organic carbon).

Tancredi Bin (37-PON, Ghelli / Francia)
Photosynthetic vesicles for studying the interactions of green chemicals with native membranes

Ionic liquids (ILs) are a promising, *greener* alternative to canonical volatile organic compounds used in many industries. They are salts composed of a combination of both organic and inorganic ions, bearing shielded charges as a result of peculiar structural elements. This feature, while causing them to be liquid below 100°C, might also allow them to permeate the cell membrane and interfere with fundamental bioenergetic processes. While a large amount of ILs has been tested for their overall toxicity against model organisms and for their interactions with liposomes, data about their specific effects on biological membranes are almost completely missing. We have chosen to investigate the effect of some ILs on native membranes using chromatophores, photosynthetic vesicles that can be easily isolated from *Rhodobacter capsulatus*.
Here, by monitoring the absorbance of the carotenoids associated with the light-harvesting complex II, that responds linearly to the amplitude of the membrane electrical potential (◎◎) generated by a single reaction center photoexcitation, we found that some of these compounds cause a rather fast dissipation of the membrane ◎◎ even at low concentrations, and that this behavior is dose-dependent. These ILs do, however, show a milder effect *in vivo.*

Elisa Dell'Anna (38-PNRR, Bernardoni / Perini)
Ribonucleoside diphosphate reductase small subunit (RnrS) downregulation in Drosophila mitotic and post-mitotic neurons and muscles demonstrates the essential role of the enzyme for locomotor activity and viability

Encoded by the nuclear *RRM2B* gene, the human ribonucleotide reductase plays a role in the mitochondrial nucleotide pool salvage pathway and *RRM2B* mutations result in severe Mitochondrial DNA Depletion Syndromes (MDDS). *RnrS* is the *Drosophila* homolog of the human *RRM2B* gene. Here, we present *Rnrs Drosophila RNAi* models reproducing the human disease. We adopted the UAS-GAL4 binary system to express in a tissue-specific manner the silencing RNA. We initially downregulated *RnrS* in proliferating neuroblasts or myoblasts and we observed a decreased locomotor ability and lethality in the late larvae. We then silenced *RnrS* in neurons or muscle fibres that were mostly post-mitotic and we observed that late larvae have strong locomotor problems and few raise the pupal stage with the animals inside the puparium severely affected. These data were confirmed by immunofluorescence analysis on brains in which *RnrS* gene was silenced in mainly post-mitotic neurons. This allowed to observe a strong phenotype consisting in an evident disorganization of both neural cell body and axon bundle patterns. Although we have to confirm these observations with Gal4 lines that activate RNAi only in post-mitotic cells, we analysed the brains of animals expressing Gal4 mainly in differentiated neurons and observed partial mtDNA depletion that could cause mitochondria degeneration and contribute to the observed phenotypes. In conclusion, our *Drosophila* models point to an essential role of RnrS subunit in post-mitotic neurons in preserving the mtDNA content most likely through maintenance of the mitochondrial nucleotide pool and will represent an unvaluable tool to test the efficacy of nucleoside supplementation therapy.

Alena Kaltenbrunner (38-PNRR, Danielli / Costantini)
Nanobiotechnological engineering of the M13 phage for targeted photodynamic cancer therapy

Cancer is one of the leading causes of death worldwide and treatment is often very invasive and not effective. Bacteriophages, the viruses of bacteria, have potential not only as alternative antibiotics but also as nanobiotechnological drug delivery platforms. Biotechnologically engineered M13 phages in particular are promising candidates for targeted cancer treatment. This project explores the development of a phage-based nanovector for targeted, minimally invasive, photodynamic cancer therapy.
The pIII protein, located at the tip of the M13 phage, was genetically engineered to display nanobodies or single-chain fragments that are specific for receptors overexpressed on cancer cell lines. Additionally, photosensitiser (PS) molecules have been conjugated to the major coat protein pVIII of the recombinant phages, enabling photo-induced cytotoxicity through reactive oxygen species (ROS) generation. Novel PS are explored, including innovative approaches in which insoluble PS are attached to human serum albumin (HSA) to enhance their bioavailability and performance.
In-vitro studies have demonstrated the successful synthesis of recombinant phages, conjugated with a high payload of PS while maintaining their integrity. These conjugated, recombinant phages can selectively target cancer cells and eradicate them subsequently in a light-mediated, dose-dependent manner with a significantly increased efficacy when compared to the sole use of photosensitiser.
These findings highlight the potential of engineered phages as innovative tools in the fight against cancer and offer a promising strategy for the development of highly specific and effective anticancer treatments.

Lucia Pappagallo (38, Danielli / Castagnola)
Neurophage: molecular engineering of phage nanoparticles for non-invasive neuronal photostimulation

The M13 phage has emerged as a versatile nanocarrier with a wide range of innovative nanobiotechnology applications. Its distinctive filamentous shape, coupled with the arrangement of different coat proteins along its structure, provides an exceptional cargo capacity for genetically fused or chemically conjugated molecules. In addition, the intriguing and unexplored characteristic of M13 phage to cross the Blood-Brain Barrier (BBB), makes it a promising delivery agent for the treatment of different neurological diseases, overcoming challenges in the biomedical field.
The mechanisms that enable M13 to cross the BBB were investigated *in vitro* on 2D and 3D BBB models. Furthermore, the high cargo capability and ease of genetic handling were exploited to enhance its crossing ability by displaying BBB interacting peptides in fusion with the phage's major coat protein (pVIII).
Additionally, nanobodies can displayed on the phage's minor coat protein (pIII), to enable the retargeting of the nanobiotechnological platform towards specific cell populations. As proof of concept, an anti-ALFA tag nanobody expressed in fusion with the pIII protein allowed the specific targeting of the phage to engineered neurons expressing a synthetic ALFA-transmembrane protein.
After validation of the BBB crossing ability and targeting specificity, further modifications will be introduced to this phage vector platform to carry both moieties at the same time. This will allow to address various central nervous system receptors implicated in pathological pathways.

Claudio Pino (38-PNRR, Musiani / Ghelli)
Precision medicine in cancer: drug repurposing strategies targeting DNA Polymerase ε for cancer therapy

Drug repurposing offers significant advantages over developing new drugs, such as lower failure risk, shorter development times, and reduced costs. This project seeks to establish a virtual screening protocol for drug repurposing using ligand-based and structure-based *in silico* methods. The focus is on molecular targets related to inflammation, cancer, and neuromodulation. In cancer biology, DNA polymerase ε (Polε), a crucial enzyme in DNA replication, has emerged as a key player in tumor progression. Polε ensures high-fidelity DNA replication through its precise polymerase activity and proofreading exonuclease domain. Instability in the hPolε (human DNA polymerase ε) holoenzyme can lead to replication stress, cancer development, and developmental abnormalities. Polε mutations are most frequently found in colorectal cancer and endometrial cancer.
The project initially focused on analyzing existing X-ray crystallographic structures of Polε, creating a complete model using homology modeling, and generating wild-type and mutant models. Molecular dynamics simulations assessed model stability, and these models are under structural and biophysical analysis to identify binding pockets and potential allosteric sites.
Drug repurposing campaigns using DrugBank libraries identified Tegaserod and Terfenadine as potential inhibitors of Polε. Further experimental research will evaluate the effectiveness of these molecules in treating cancers with Polε mutations.

Alberto Rigamonti (37, Perini / Milazzo)
Investigation of PRDM12 locus in colorectal adenocarcinoma cells

PRDM12 is not usually expressed in adult normal tissues; however, a pan-cancer meta-analysis based on The Cancer Genome Atlas reveals that the PRDM12 gene is upregulated in several cancer types. These data may suggest a putative oncogenic role for PRDM12. We decided to investigate the biological and molecular roles of PRDM12 in colorectal adenocarcinoma, where its expression seems more disrupted. First, we investigated its expression in cell line SW620, SW48 and found that the PRDM12 gene is not transcribed from the canonical TSS due to the presence of a highly methylated CpG island before the promoter, that prevents its transcription. PRDM12 transcriptional repression is so tight that neither inhibitors of DNA methylation (5-Azacytidine) PRC2-mediated histone methylation (EED226) nor histone deacetylation (Panobinostat), can restore transcription of the gene. The only way we could succeed in re-expressing the gene was by forcing its transcription through a dCas9-VPR complex positioned nearby the putative TSS.
Notwithstanding that, thorough analyses of the entire PRDM12 locus have, however, revealed the existence of another TSS located in the 3'UTR PRDM12 region. By modulating expression from this new putative promoter (dCas9-Krab-MeCP2 KD-system), we detected and characterized a new transcript in the "canonical" 3'UTR region. Interestingly, the transcript and the epigenetic marks associated with the region suggest that the 3'UTR region carries a new transcribed enhancer. Functional studies suggest that the enhancer up-regulates the expression of the nearby ABL1, a powerful oncogene.
In conclusion our results show that PRDM12 is not per se involved in cancer development. However, the PRDM12 locus harbors a new transcribed enhancer in its 3'UTR that controls expression of the ABL1 gene.

Emanuel Forciniti (38, Sarma / Perini)
Examining neurodevelopmental roles of ADNP through its interactions with HP1 and CHD4 in the ChAHP complex

Activity dependent neuroprotective protein (ADNP) is a zinc finger and homeodomain containing protein critical for normal brain development and its mutations cause ADNP syndrome, a severe autism spectrum disorder. In addition to interacting with nucleic acids via its zinc fingers and its homeodomain, ADNP also interacts with the chromatin remodeler CHD4 and with HP1, a heterochromatin protein that recognizes H3K9me3, to form the ChAHP complex. Whether ADNP interactions with CHD4 and HP1 contribute to regulate distinct targets remains unclear. The goal of this project is to determine the distinct contribution of ADNP-HP1 and ADNP-CHD4 interactions in neurodifferentiation.
We established mouse embryonic stem cell (ESC) models which contain ADNP mutations that abolish HP1 or CHD4 interactions. We found that mESCs where ADNP-HP1 interactions are disrupted cannot differentiate into neuro-progenitor cells (NPCs), while mutations in ADNP that affect CHD4 binding show no defects in neurodifferentiation. To elucidate the mechanisms responsible for these differences, we performed CUT&RUN analysis for ADNP binding and RNAseq in both mutants. Our findings reveal that ADNP interactions with HP1 and CHD4 regulate distinct classes of repetitive elements, including long terminal repeats (LTRs), long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), and Major Satellite Repeats (MSRs). Specifically, ADNP-HP1 interaction appears critical for the repression of MSR transcripts at pericentromeric regions.

Luca Grillini (37, Gardini / Perini)
Recurring mutations of PP2A impair neurogenesis

RNA Polymerase II activity in higher eukaryotes is characterized by a "pause-release” mechanism, which is deemed essential for the spatio-temporal control of gene expression. This mechanism is controlled by the antagonistic activity of Cyclin-dependent Kinases (CDKs), in particular pTEFb, and the Integrator-PP2A complex. pTEFb-mediated phosphorylation promotes release of RNAPII and elongation while PP2A, through de-phosphorylation, restrains RNAPII in its paused conformation. The PP2A phosphatase complex is minimally composed by a scaffold subunit (PPP2R1A or PPP2R1B), and a catalytic subunit forming a heterodimer. In cells, PP2A dimers associate with a set of regulatory subunits to form functional trimeric complexes (canonical PP2A) or can associate with the Integrator complex, a multi-subunit modulator of transcription and RNA-processing. We study recurring point mutations (R182W) of the PPP2R1A scaffold that are linked to complex neurodevelopmental disorders (characterized by severe impairments of the central and peripheral nervous systems). We generated an Induced Pluripotent Stem Cells (iPSCs) model to analyze the R182W mutation and its impact on human neuronal differentiation using a broad array of genomic and proteomic approaches. Our data show that PPP2R1A mutations primarily impair the function of Integrator-PP2A and derail neurogenesis, suggesting that RNAPII pausing is essential for neuronal development.

Sara Aloisi (37, Perini / Milazzo)
RCoR2 sustains super-enhancer-driven oncogenic transcription in ADR neuroblastoma

CoREST complexes, composed of LSD1, HDAC1/2, and RCoR1/2/3, are pivotal in neurodevelopment and have long been recognized as transcriptional repressors across various cancers. However, the distinct roles of the RCoR factors remain poorly understood. Here, we unveil non-canonical functions of RCoR2 in MYCN-amplified neuroblastoma (NB), highlighting its unique features compared to its paralogues.

NB cell growth and tumorigenesis critically depend on RCoR1 and RCoR2, with the RCOR2 gene exhibiting high histone acetylation levels and selective expression in NB. In contrast to the repressive function of RCoR1, RNA-seq and ATAC-seq analyses unexpectedly show that RCoR2 acts as a positive regulator of gene expression and facilitates chromatin accessibility. RCoR2 predominantly occupies regions of open chromatin marked by H3K27ac and Pol2, typical of active promoters, while RCoR1 primarily targets enhancers.
Importantly, CoREST complexes co-occupy chromatin along with NB core regulatory transcription factors (CRTFs), which positively drive NB-specific signatures. More specifically, RCoR2, and not RCoR1, physically interacts with CRTFs and regulates their expression, implicating it as a new component of the adrenergic NB core regulatory circuitry (CRC). HiChIP data reveal that RCoR2 mediates chromatin looping, facilitating enhancer-promoter interactions and maintaining 3D chromatin architecture to sustain oncogenic transcriptional programs.
Taken together, our data underscore RCoR2 as a key determinant of the NB chromatin landscape. Based on these findings, we propose a model in which RCoR2 facilitates interactions between CRTF-bound enhancers and their associated transcription start sites, thereby sustaining the expression of genes essential for neuroblastoma survival. Consequently, RCoR2 represents a critical vulnerability in high-risk neuroblastoma and a promising target for cancer therapeutics.

Federico Guerra (38, Capranico / Marinello)
Role of RNA polymerase II backtracking in genome instability in human cancer cells

During the first part of my Ph.D., I focused on characterizing how the Topoisomerase I poison, camptothecin (CPT), induces genome instability in human cancer cells. We investigated the consequences of the topoisomerase I-DNA cleavage complex (Top1cc), a transient intermediate in Top1's catalytic activity. This research, published in *Science Advances* (PMID: 38787953), showed that CPT treatment increases R-loop levels and induces double-strand breaks (DSBs) in highly transcribed genes within early replication zones. These DSBs coincide with hybrids downstream of RNA polymerase II (RNAPII) accumulation at the 5'-ends of genes. We also discovered that TFIIS reduces CPT-induced genome instability by attenuating R-loop formation, micronuclei accumulation, and transcription-replication conflicts (TRCs). I am currently generating a cell line engineered to express a degron system for the rapid degradation of endogenous TFIIS following dTAG treatment. This cell line will be used for LORAX-seq during my time abroad in Professor. Nudler's lab. Additionally, to explore RNAPII backtracking's role in TRCs and genome instability, I am investigating two POLR2B mutations (H1060Q and H502P) that resist backtracking in bacteria. Preliminary data indicate these mutations significantly reduce Top1cc-induced micronuclei in HeLa cells, providing an encouraging starting point to confirm the involvement of backtracking in the occurrence of genome instability in human cancer cells.

Monica Procacci (38, Capranico / Miglietta)
G4-mediated genome instability and its role in promoting an innate immune response in cancer cells

G-quadruplexes (G4s) are non-canonical DNA structures transiently occurs under physiological conditions at functionally relevant genomic sites. Due to their role in genomic stability and gene regulation, G4s have become promising targets in cancer therapy[1]. Various G4 binders have been synthesized and studied as potential anticancer agents because of their ability to induce genomic instability, telomeric imbalance, and alter gene expression profiles. Our laboratory has demonstrat-

ed that G4 binders can activate an innate immune response, suggesting their potential use in cancer treatment[2,3]. I analyzed several G4 binders, including PDS, RHPS4, BRACO-19, and CX5461, as well as newly synthesized hydrazone derivatives from R. Morigi's group[4]. Using ELISA assays and immunofluorescence in murine fibrosarcoma cell lines, we assessed their ability to induce micronuclei (MNi) formation and stimulate Interferon-B (IFNB) production. Our findings show that while all G4 binders tested promote MNi accumulation, their ability to induce IFNB synthesis varies significantly[5]. The IFNB production was confirmed also in spheroid culture. To comprehend the differences, we performed a proteomic analysis of micronuclei induced by RHPS4 or PDS in collaboration with CNIO, Madrid. The multiomic data will allow to define the specific mechanism that leads to IFNB-dependent response or to its suppression and its impact on therapeutic potential.

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Emma Coschina (38-PNRR, Angelini / Capranico / Ravegnini)
Characterization of 3D bioprinted human endometrial cancer models

Endometrial carcinoma (EC) is the most common gynaecological cancer in Western countries. While advances in screening, detection and treatment have been made, mechanisms underlying recurrence and chemotherapy resistance are not fully understood.
In this scenario, employing adequate preclinical models is of major importance. The two-dimensional (2D) cell model, widely used to study cancer, does not faithfully reproduce the *in vivo* pathophysiology. Indeed, tumours are highly complex structures where cancer cells closely interact with other elements of the tumor microenvironment. In this context, 3D bioprinting technology offers a more relevant approach by generating in vitro models where cells are encapsulated within naturally derived hydrogels.
We were able to generate EC 3D models and to investigate the differences between the 2D and 3D, by evaluating cell viability and miRNAs expression of two EC cell lines, RL-95 and VOA-1066. Significant differences between 2D and 3D models were observed, particularly in miRNA expression profiles and pathway activations. In the 3D models, pathways associated with cell migration, proliferation, apoptosis, and tumor progression were upregulated at both day 7 and day 14, reflecting an altered behaviour of the cells in a 3D environment.
In conclusion, we established long-term 3D culture models that have the potential to advance knowledge in endometrial cancer biology research, a first step toward new and more complex EC 3D in vitro models.

Iacopo Gherardi (38, Porcelli / Ghelli)
TMEM65 controls mitochondrial activity through respiratory complex I assembly

Transmembrane protein 65 (TMEM65) is a protein localized in the inner mitochondrial membrane. A previous work identified a homozygous loss-of-function mutation in *TMEM65* gene in a patient with a clinical presentation resembling a mitochondrial disorder [1]. Knock down of TMEM65 expression in human fibroblasts was shown to severely affect mitochondrial content and respiration [1], but the exact mechanism remains unknown. Interestingly, by performing a quantitative proteomic screening, we observed an accumulation of TMEM65 protein in osteosarcoma cells lacking respiratory complex I (CI).
Thus, to get a deeper understanding of the function of this protein we obtained a TMEM65 KO human cell line and analysed the effects on mitochondrial function of the absence of the protein. Ablation of TMEM65 resulted in a mild reduction of mitochondrial OXPHOS capacity, associated with a reduction of fully assembled functional CI and abnormal accumulation of CI subassemblies. Moreover, TMEM65 KO cells showed impaired CI biogenesis kinetics, suggesting the presence of an assembly defect. Overexpression of full-length TMEM65 was able to partially rescue this defect, confirming the role of the protein in promoting correct CI assembly.
Taken together, the data suggest TMEM65 plays a role in mitochondrial respiratory chain function by assisting CI assembly. Gaining deeper knowledge on the molecular function of this protein will help understanding the mitochondrial disorders caused by mutations in TMEM65 gene.

References:
[1] A. Nazli, A. Safdar, A. Saleem et al, A mutation in the TMEM65 gene results in mitochondrial myopathy with severe neurological manifestations, Eur J Hum Genet 25 (2017) 744–751

Laura Sandoni (37, Maestrini / Bacchelli)
Genomic analysis of autism spectrum disorder and functional characterization of rare variants

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by a distinct set of social and communication deficits, repetitive behaviors and restricted interests and often characterized by various degrees of intellectual disability (ID). The genetic architecture of ASD is complex, with multiple types of risk variants involved and different models of inheritance.
To further understand this genetic complexity, the first part of my Ph.D. project focused on SNP-array and whole exome/ whole genome sequencing (WES/WGS) data analysis of a family-based cohort of 116 ASD families, including 435 total individuals and 144 ASD individuals.
This allowed the integrated analysis of different classes of variants, both inherited and arising *de novo* in the probands, and ultimately led to the identification of 37 rare *de novo* potentially damaging single nucleotide variants (pdSNVs), impacting both known ASD risk genes and promising new candidates.
Interestingly one of these new candidates, *RAB11B*, had been previously implicated in intellectual disability (ID) but was never associated with ASD specifically. Given the well-known genetic correlation between ID and ASD, the second part of my Ph.D. project focused on the functional characterization of the *de novo* missense pdSNV in *RAB11B* discovered in our cohort, along with two additional missense variants in the same gene reported in literature but still not functionally described.

To this end, immunofluorescence analyses were carried out on ARPE-19 cells, a retinal pigment epithelium cell line in which primary ciliogenesis, a cellular mechanism co-regulated by RAB11B, can be induced in vitro. In this context, we were able to study the co-localization of the three RAB11B mutants with key cellular compartments involved in ciliogenesis and assess morphological differences in the primary cilium in cells overexpressing the recombinant proteins.
Finally in the very last part of my Ph.D., thanks to an international collaboration with Neurolentech, a neurodevelopmental drug discovery start-up in Klosterneuburg (Austria), I was able to learn how to genetically edit patient-derived induced pluripotent stem cells (iPSCs), in order to obtain patient-specific neuronal cell cultures. These cell models will be used to test new drug targets for epilepsy and ASD.

Martina Santulli (38-PNRR, Perini / Monti)
Role of the PRR12 factor in Neurodevelopmental Visual Disorders (NDVDs)

Neuroocular syndrome (NOC) is a newly identified hereditary disorder, reported in up to 30 cases. It is characterized by deficits in learning ability, typically coupled with structural eye defects. All patients share mutations in the PRR12 gene, which encodes for the PRR12 protein, a putative DNA binding protein densely packed with proline-rich regions. PRR12 is categorized as a T-dark gene as nothing is known about it, and only recent findings show that PRR12 is involved in the regulation of the cohesin complex and genome integrity.
As a potential nuclear cofactor with still unknown functions, the aim of this project is to thoroughly investigate the PRR12's molecular mechanism and to understand how PRR12 mutations disarray such mechanism.
Immunofluorescence staining on medulloblastoma and neuroblastoma cells show that PRR12 is both nuclear and cytosolic. Neuronal cell lines are the most suitable model for studying PRR12, as it is expressed during the early stages of brain development. Moreover, immunoblot analyses revealed different patterns of bands that are probably linked to different isoforms of the protein. Interestingly, it seems that medulloblastoma and neuroblastoma cells express a unique set of PRR12 isoforms.
To study the effect of PRR12 mutations, CRISPR/Cas9 technology has been used to introduce random frameshift at different levels in the PRR12 gene. As a first attempt, four gRNAs were designed across the fourth exon, that is the longest exon where most patient mutations occur. Then, gRNA sequences were cloned into the Cas9 expressing-px459 plasmid construct. Finally, transient transfection in HEK-293t cells was performed to evaluate their capability to generate genomic cuts. After DNA genomic extraction, four different gPCR were performed and sequenced (Sanger sequencing) to validate their ability to generate genomic mutation. All gRNAs successfully cut and introduced indels with high efficiency rates (60%). The best one(s) will then applied to WT human iPS cells to generate cellular models of the disease.

Chiara D'Silva (39, Massenzio / Monti)
Investigating neuroinflammation influence on adult neurogenesis in in vitro models of Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia, characterized by a progressive decline in memory and cognitive functions. Its incidence is increasing with age, while its prevalence is expected to triple by 2050. Neuroinflammation, a major physiopathological feature of AD,

seems to occur before amyloid-β deposition; it plays a key role in AD onset and progression, as well as in adult neurogenesis dysfunction.
Since neural stem cells (NSCs) are multipotential self-renewing cells able to differentiate into distinct brain cells, including neurons, they could play a role in AD-related brain injuries repair. In this framework, neurospheres are common in vitro models to study progenies originated each from single NSCs. Furthermore, NSCs-derived extracellular vesicles emerge as a useful way to modulate the nervous system's local microenvironment and distant neuronal activities. EVs, membrane-bound organelles, mirror their cells of origin's properties and can selectively target cells with similar phenotypes.
My PhD project aims to observe adult neurogenic impairment through the in vitro model of neurospheres from the subventricular zone of 8 month-old 5xFAD transgenic mice model, which recapitulates various AD-related phenotypes. We will characterize neurospheres-derived EVs under proliferative and spontaneous differentiation conditions to demonstrate whether their content replicate NSCs abilities and influence neuroinflammation and adult neurogenesis. We will also assess gender-associated differences.

Cecilia Franciosi (39, Monti / Alberini)
Angelman syndrome: a study on neuronal cultures derived from patients' iPS cells

Angelman Syndrome (AS) is a rare neurodevelopmental disorder characterized by developmental delay, intellectual impairment, ataxia, and seizures, caused by a deficiency in the ubiquitin ligase E3A (UBE3A) gene product. Although ubiquitously expressed, the UBE3A gene is paternally imprinted in mature neurons. Consequently, deletions, uniparental disomy, imprinting defects and gene mutations of the maternal chromosome 15q11-13 result in UBE3A targets accumulation in neurons. Current AS pathophysiology insights mainly come from AS murine model, therefore alternative models based on patient-derived stem cells are required to better understand the molecular basis of the pathology in humans. To this aim, five hiPSC lines, derived from children with various AS- associated genotypes, were provided by the FAST ITALIA biobank. After confirming stemness, proliferation analysis showed no differences between patient and control iPSC lines. These iPSCs were differentiated into Neural Progenitor Cells and then into cortical neurons, currently being analysed for proliferation, morphology and autophagic flow, since a block of autophagy has been observed in AS mouse brain. Moreover, UBE3A expression is being evaluated at each differentiation stage. With no effective therapies for AS deficits, the insulin-like Growth Factor II (IGF-II) receptor is emerging as a promising therapeutic target due to its role in regulating protein metabolism. Our goal is to establish a reliable human cellular model for testing IGF- II receptor activation as therapeutic approach.

Vanessa Croatti (37, Vitali / Parolin)
Exopolysaccharides of vaginal *Lactobacillus crispatus* as mediators of cellular interactions

Understanding the dynamics of vaginal microbiota balance is essential for improving women's health. Unlike other body areas, the vaginal microbiota in a healthy state shows low microbial diversity being primarily dominated by *Lactobacillus* species, which provide a first line defense against pathogenic infections. Lactobacilli exopolysaccharides (EPS) are extracellular polymers, either released (r-EPS) or attached to the bacterial cell wall (capsular, c-EPS), playing essential roles in cell protection and recognition, while also contributing to the beneficial effects of probiotics.

In this study, we analyzed the role of c-EPS from vaginal *L. crispatus* in cellular interactions. To achieve this, we constructed by double homologous recombination a knock-out mutant strain lacking the *epsE* gene, which encodes a key enzyme in EPS biosynthesis. The impact of the *epsE* deletion on c-EPS production was assessed using various methodologies, including transmission electron microscopy, crude c-EPS isolation, and mass spectrometry. Furthermore, we evaluated the impact of *epsE* deletion on *L. crispatus* adhesion and ability to modulate host immune responses.
We showed that deleting *epsE* reduced c-EPS production and impaired biofilm formation. However, it also increased adhesion to vaginal cells and triggered a stronger immune response in human monocyte and vaginal cells compared to the wild-type strain.
These findings highlight the essential role of c-EPS in maintaining *L. crispatus* biofilm structure and in regulating bacteria-host cells interactions within the vaginal environment.

Tommaso Rossi (37-PON, Danielli / Trevisi)
Natural and modified bacteriophages and protein-based nanoscaffold in the sustainable control of porcine post-weaning diarrhea

Post-weaning diarrhea (PWD) can cause severe losses in industrial pig farming. It is frequently caused by enterotoxigenic Escherichia coli (ETEC), expressing F4 or F18 fimbriae that bind to the intestinal microvilli of the piglets after weaning. Concerns about the emergence of antimicrobial resistance and environmental contamination have been raised globally due to the excessive use of antibiotics and zinc oxide to treat PWD. Therefore, alternative approaches to treat ETEC and preventing PWD are urgently needed.
This work explores the use of self-assembling nanoparticles as decoys to counter intestinal adhesion of ETEC F4+ to microvilli. mi3, an icosahedral self-assembling protein, is bioengineered to exhibit anti-ETEC F4 single domain antibody (sdAb, nanobody) fusions at the N-terminus of each monomer. The mi3 scaffold is made up of 60 monomers. Expression and purification of the bioengineered protein was optimized to obtain significant amounts of soluble protein. The modularity of the platform was examined by expressing different types of nanobodies on its to increase the adhesion capacity to the fimbriae. The recombinant protein's expression was refined and immunoblotting and atomic force microscopy (AFM) were used to confirm the protein's purification by Size Exclusion Chromatography (SEC).
In vitro tests showed that the anti-ETEC F4-mi3 nanoparticles bind ETEC bacteria and trigger its accumulation, leading to their incapability of binding intestinal microvilli. These promising results were confirmed in independent microscopy assays and through western blot analysis and will be further tested and optimized. Currently, the same methodology is being used to evaluate other nanobodies that recognize distinct F4 fimbrial epitopes. Furthermore, strategies to increase the yields of anti-ETEC F4-mi3 nanoparticles are explored; a particular approach is the use of a SpyTag/SpyCatcher system to conjugate recombinant anti-ETEC sdAbs irreversibly in order to purify the mi3-SpyC scaffolds.

Mattia Longanesi (38-PNRR, Cavaliere)
***Drosophila melanogaster* as a model to identify new bioinsecticides molecules for pest insect control**

Polydnaviruses are obligate symbionts integrated as proviruses in the genome of some ichneumonoid wasps that parasitize lepidopteran larvae. Virion particles, injected into the host at oviposition, express virulence factors that alter immunity and development.
Using *Drosophila melanogaster* model system, my project focuses on the functional analysis of virulence factors with the aim to identify molecules that can be used to develop new bioinsecticides environmentally sustainable.
My work focuses on the study of *Tn*BVANK1, a member of the ankyrin gene family of the bracovirus associated with *Toxoneuron nigriceps*, a wasp that parasitizes the noctuid moth *Heliothis virescens*. I used molecular genetics approaches based on the UAS/Gal4 system and its derivative TARGET system, to induce respectively the specific expression of *TnBVank1* in tissues of interest or its ubiquitous expression at different stages of development.
Previous results showed that flies expressing ubiquitously *Tn*BVANK1 have a significantly lowered survival compared to control flies.
Furthermore, we observed that the prolonged ubiquitous expression of *Tn*BVANK1 leads to an increased expression level of some antimicrobial peptides belonging to the Toll and IMD signaling pathways.

Manon Elise C. Libotte (38, Zuccheri / Monti)

Self-assembled DNA nanostructure applied on immunomodulation of microglia in neuroinflammation

Alzheimer's disease is one of the most common cognitive disorders, affecting approximately 35 million people worldwide. This disease is partly driven by chronic neuroinflammation in the brain, leading to the degeneration of neurons. A major contributor to this neuroinflammation is the accumulation of amyloid-beta (Aβ) protein, which activates microglia and shifts them from a resting (M0) state to a pro-inflammatory (M1) phenotype. Research has demonstrated that this neuroinflammation further promotes Aβ accumulation, creating a self-pernicious cycle of inflammation and protein buildup. This ongoing activation of microglia results in the release of cytokines, signalling proteins, DNA, and particularly, microRNAs (miRNAs).
DNAzymes are catalytic DNA molecules capable of cleaving RNA, and can be specifically designed to target and degrade a variety of RNA sequences. Our research focuses on developing a DNA nanostructure (DNS) containing a DNAzyme that targets a miRNA associated with pro-inflammation. Based on prior studies, we designed a tetrahedral DNS that is capable of self-assembly, autonomous cell uptake, and is biocompatible, non-toxic, and biodegradable. The DNS targets miRNA-34a, which is known to decrease the expression of TREM2, a protein involved in the clearance of Aβ aggregates.
Using fluorescence microscopy and polymerase chain reaction (PCR), we confirmed the uptake of the DNS by the cells. Furthermore, PCR and western blot analyses showed a reduction in the levels of miR-34a in activated microglia treated with the DNS, as well as a decrease in the expression of inflammation markers such as inducible nitric oxide synthase (iNOS). These promising results indicate that DNS could potentially enhance therapeutic strategies against neurodegenerative diseases.

Noemi Carosella (39, Zambelli / Ciurli)

Phosphorylation and lipid binding of the intrinsically disordered region of NDRG1, a possible target for lung cancer therapy

NDRG1 (N-myc downstream regulated gene) is a human protein involved in cell growth and differentiation, lipid biosynthesis, stress responses, cancer development among other functions. It contains a N-terminal α/β hydrolase domain and a C-terminal, 83 residues long, intrinsically disordered region (IDR, NDRG1*C).1 The latter is characterized by a three-times repeated sequence of ten residues, binds nickel2 and lipidsand is functionally regulated by phosphorylation. The effects of NDRG1*C phosphorylation and lipid binding on protein folding and interactions are investigated through biophysical techniques. The polypeptide was expressed and purified from *Escherichia coli* both in the unmodified and phosphorylated form. Experiments of isothermal titration calorimetry, light scattering and circular dichroism were carried out to establish the impact of post-translational modification on its metal-binding activity, as well as secondary and quaternary structure. Preliminary NMR data on the phosphorylated protein indicated the position of the phosphorylated residues and confirmed that the protein remains disordered in the phosphorylated form. The interaction of NRG1*C with lipids was followed by FT-IR spectroscopy, circular dichroism, isothermal titration calorimetry, co-sedimentation assay, electron paramagnetic spectroscopy. These techniques suggest that there is an interaction between the C-terminal domain of the protein and the negative charged lipid DMPG (1,2-Dimyristoyl-sn-slycero-3-phosphoglycerol) and these data suggest a changing in the secondary structure of the disordered region.

1 Mustonen V. et al., FEBS J. 2021, doi: 10.1111/febs.15660.
2 Beniamino Y. et al., Biomolecules 2023, doi: 10.3390/biom12091272

Tânia Raquel Cunha Alves (39, Monti / Musilek)
Unraveling the connection between circadian rhythms and neural stem cells in Alzheimer's disease

Alzheimer's disease (AD) is a complex progressive neurodegenerative disease characterized by a deterioration of cognitive abilities. Disturbances in the sleep-wake cycle and circadian rhythms are recognized as early symptoms in AD patients. Emerging evidence suggests a bidirectional relationship between AD and circadian clock (CC) disruption with conceivably circadian disruptions influencing the onset and progression of AD while, a proper circadian system provides greater resilience to AD pathogenesis. Circadian rhythms are controlled by internal molecular feedback loops that regulate various physiological processes. Neural stem cells (NSCs) play a crucial role in brain homeostasis, neurogenesis, and repair, and their dysregulation is implicated in AD pathology. However, the relationship between circadian rhythms, their regulation, and NSC function in AD remains poorly understood. This study aims to examine the effects of circadian rhythm disruption on NSC proliferation, differentiation, and survival in Alzheimer's disease and to elucidate the molecular mechanisms underlying circadian clock dysregulation in NSCs of Alzheimer's disease. Simultaneously, to clarify the circadian clock's molecular basis in AD, this project aims to establish a genetically modified iPSC cell line for BMAL1-knockout and AD-linked mutations, APP and PSEN1, by CRISPR/Cas9 genome editing technology.

Matteo Bergonzoni (39, Lieberman / Perini)
Investigating the role of PAX5 in Epstein-Barr virus latency and disease progression

Epstein-Barr virus (EBV) is a human herpesvirus that infects the majority of the global population. While EBV typically establishes a lifelong latent infection that remains asymptomatic, it is the most common causal agent of mononucleosis and has been associated with the etiology of a spectrum of

malignancies such as gastric carcinomas and lymphomas. EBV is also associated with autoimmune disease, particularly multiple sclerosis. The mechanisms by which EBV transitions from a benign to pathogenic infection in at-risk individuals remain, however, poorly understood.
B-cell specific transcription factor PAX5 is known to regulate immunoglobulin (IgG) gene chromosome structure, and has been shown to bind to the Terminal Repeats (TR) of the EBV genome. PAX5 binding to the TR is thought to regulate transcription of the viral immediate early gene BZLF1 (Zta). PAX5 has also been found to interact with the EBV nuclear antigen 1 (EBNA1) to promote EBNA1/*oriP*-dependent binding and transcription, enabling latency establishment and viral-episome maintenance. However, the potential function of PAX5 in regulating the chromatin structure of the viral episome, similar to how it regulates the cellular IgG locus has not yet been examined.
To further investigate the role of PAX5, we are performing both a shRNA-mediated knockdown of PAX5 in EBV infected B-cells at different stages of latency, specifically MUTU I and LCL352, and a PAX5 overexpression in YCCEL1 gastric cancer cells, which do not express PAX5 in normal conditions. With this study, we aim to elucidate the interplay between cellular and viral factors involved in regulating viral latency, chromosome structure and disease progression.

Martina Tomirotti (39, Kulp / Perini)
Precision epitope engineering for influenza B Hemagglutinin: leveraging ai and structure-guided approaches

The hemagglutinin (HA) protein of influenza virus B (IVB) is a critical target for vaccine design due to its role in viral entry and its antigenic variability. This study investigates the epitope landscape of HA of IVB over time, employing structural-guided design and advanced computational tools to develop novel immunogens focused on highly conserved epitopes. A comprehensive analysis of HA evolution helped us define conserved regions as viable targets for the development of a universal vaccine. Our approach leverages AI and computational strategies developed in our laboratory, including the Cloaking with Glycans pipeline. This predicts optimal glycan addition sites within the HA structure, shielding non-protective epitopes and focusing the immune response on selected epitopes. We also employed de novo protein design to create minimal antigens mimicking the immunogenic and conserved regions of the protein. These designs are then validated to refine and select the most promising candidates for fast and resource aware bench testing. In vitro assays, including antibody binding and neutralization tests, are underway to validate and further refine the designs. In summary, this research demonstrates the potential of integrating AI-driven tools with structural biology to innovate vaccine development for Influenza by focusing on conserved epitopes.

Mario Marino (39, Lieberman / Perini)
Utilizing Oxford nanopore technologies for comprehensive analysis of TERRA and the cellular transcriptome

During my first year as a PhD student in the lab of Dr. Paul Lieberman, I explored various experimental techniques to delve deep into the intricate interactions between telomeric repeat–containing RNA (TERRA) and human telomeres. Initially, I employed a combination of traditional and modern methodologies, including RT-qPCR, RNA dot blot with p-32, and near-infrared fluorescent RNA dot blot. However, considering preliminary results and project requirements, I deci-

ded to focus on sequencing using Oxford Nanopore Technologies (ONT). The use of long-read sequencing (LRS) technologies such as ONT is particularly suited for this study, as TERRA is transcribed by RNA polymerase II, producing transcripts that vary in length from 100 bases to at least 9 kilobases. This technology allows for the sequencing of long DNA/RNA molecules. Moreover, the ability to directly sequence RNA through Nanopore direct RNA sequencing (DRS), which sequences native RNA without the need for reverse transcription (RT) or amplification, opens opportunities to identify RNA modifications (e.g., m6A) and RNA editing (e.g., inosine), which are crucial for many biological functions. This technology is not only promising for my specific focus on long non-coding RNAs but also has the potential to provide insights into the entire cellular transcriptome. I am currently analyzing the generated data to gain insights into both specific long non-coding RNAs and the broader cellular transcriptome, including potential viral RNAs. This analysis will provide significant data on gene expression dynamics and RNA modifications.

Giulia Pantella (39, Murphy / Perini)
PADI4 citrullinates p53 and affects its transcriptional activity

TP53 is a critical tumor suppressor gene and the most frequently mutated gene across human cancers. It encodes the protein p53, a transcription factor that is activated by genotoxic and cytotoxic stress to regulate over 3,000 genes involved in cell cycle arrest, apoptosis, and other cellular processes. p53 undergoes various post-translational modifications that impact its activity, stability, localization, and interactions with other proteins. In this study, we identify citrullination, the irreversible conversion of arginine to citrulline, as a novel post-translational modification of p53. Our research highlights peptidyl arginine deiminase type 4 (PADI4), a p53 target gene, as the enzyme responsible for this modification. Published data from our lab indicate that PADI4 is not transactivated in tumor-associated hypomorphic p53 variants and that PADI4 overexpression induces a tumor-suppressive phenotype in both *in vitro* and *in vivo* models. Using an inducible PADI4 expression system in a p53-stabilized HCT116 cell line, we demonstrate that p53 can be citrullinated by PADI4, mostly in the C-terminus. Our findings suggest that citrullination affects p53 oligomerization and its DNA binding ability. Chromatin immunoprecipitation sequencing (ChIP-seq) analysis reveals colocalization of PADI4 and p53 at non-canonical p53 target genes. These results establish PADI4 as a key regulator of p53's transcriptional response. Further research is warranted to elucidate the impact of p53 citrullination by PADI4 on its functional activity.

Lorenzo Serra (39, Tian / Perini)
CHERP modulates cells resistance to splicing and elongation inhibitors through regulation of intronic polyadenylation

Alternative polyadenylation (APA) is an RNA processing mechanism that alters the 3' end of transcripts, affecting over 70-80% of human genes. While most APA sites reside in the last exon, a substantial number of APA sites are in introns, commonly referred to as intronic polyA (IPA) sites. A growing body of evidence suggests that the usage of these sites depends not only on the cleavage and polyadenylation activity, but also on splicing efficiency and transcriptional elongation.
CHERP is a splicing factor associated with the U2 snRNP complex, previously found to be upregulated in colorectal cancer and mutated in other cancer types. This protein was recently shown to interact with the RNA polymerase II C-terminal region, suggesting its involvement in coordinating splicing with elongation.

Using 3'READS+, a specialized sequencing technique to capture the 3' end region of polyadenylated transcripts, we analyzed the effects of knockdown (KD) and overexpression of CHERP on mRNA abundance and APA isoform expression. We observed that CHERP KD significantly activates IPA, while its overexpression displays an opposite effect, strongly suppressing IPA. We decided to further study the effects of suppression or activation of IPA on metabolism and drug resistance using an *in vitro* assay based on the competition of cells transduced with inducible CHERP. Thus far, our data indicates that CHERP may be responsible for an increase in resistance to these inhibitors, suggesting that CHERP upregulation may increase cancer cells tolerance to oncogenic splicing alterations.

Zainul Abe Din (39, Escolano / Perini)
Tailored DNA / RNA Suits for Immunogens

Despite decades of research, no universal vaccines exist against highly mutating viruses like HIV-1 and Influenza. These viruses exhibit significant diversity, characterized by numerous circulating variants with unique antigenic and infectivity properties. Developing efficacious vaccines that provide broad protection against all the variants requires the induction of antibodies targeting conserved viral epitopes. However, the immune response typically focuses on non-conserved immunodominant epitopes, which do not confer broad protection. To overcome this challenge, we are designing Novel RNA Suits that leverage the aptamer technology to mask mutating epitopes directing the immune response toward conserved targets. These Tailored RNA Suits, being generated through Selex (Systematic Evolution of Ligands by exponential Enrichment) are engineered to silence non-conserved epitopes. We are assessing their ability to bind non-conserved regions, using Bio-Layer Interferometry (BLI) and evaluating their capacity to block these epitopes through Enzyme-Linked Immunosorbent Assay (ELISA). Following these in vitro validations, we will proceed to test their efficacy in vivo to determine their potential in enhancing vaccine efficacy by guiding the immune system to recognize and target the most critical viral components.

Leonardo Cimadom (39, Perini / Chemello)
Investigation of synthetic polyamines effects on Neuroblastoma cells

This research project focuses on evaluating the antitumor potential of newly synthesized polyamines, specifically PF1 and C12, against Neuroblastoma, a common and aggressive pediatric cancer. Neuroblastoma's resistance to conventional therapies necessitates the development of novel treatment strategies. Our study investigates the efficacy of PF1 and C12, which have shown a significant reduction in the proliferation of Neuroblastoma cells and induced cellular death at micromolar concentrations. To better understand the molecular mechanisms behind these antitumor effects, we performed RNA sequencing, revealing the most deregulated genes in response to treatment. Notably, HMOX1, a key player in the ferroptosis pathway, was upregulated. HMOX1 is involved in the degradation of heme into free iron, which promotes lipid peroxidation, a hallmark of ferroptosis. Given this finding, we aim to verify whether the cell death mechanism is related to ferroptosis, apoptosis, or necrosis. Additionally, targeted gene knockdowns using CRISPR technology are being conducted to investigate the specific roles of these deregulated genes in mediating the observed effects of PF1 and C12. The outcomes of this research could lead to the identification of novel therapeutic targets and the development of PF1 and C12 as promising candidates for neuroblastoma treatment, offering new hope for improving outcomes in patients with this challenging malignancy.

Fabrizio Bertolazzi (38, Schug / Perini)
Identifying acetate transporters through a CRISPR dropout screen

The connection between cancer progression and deregulated cell metabolism is well established, as metabolites act as biomass sources and modulators of the tumor microenvironment (Pavlova et al., 2022). Acetate, a major alternative nutrient source of carbon for both cancer and immune cells, can supply acetyl-CoA and consequently also influence histone acetylation and epigenetics. While many studies have examined acetate-metabolizing enzymes and their roles in cancer, how acetate crosses the plasma and inner mitochondrial membranes to contribute to cell metabolism remains underinvestigated (Bose et al., 2019). We hypothesize that by altering both the culture conditions and the metabolic capabilities of cells we can apply selective pressure for acetate utilization, such that loss of acetate transporters will be detrimental for cell proliferation or survival.
Based on this hypothesis, we designed and validated two *in-vitro* screening methodologies to identify acetate transporter proteins in both human (HEK-293) and murine (hepatocellular carcinoma-derived) cells: one untargeted and one targeted. For the untargeted approach, we culture cells in a medium supplemented with lipid-depleted serum and acetate while blocking the activity of the enzyme ATP-citrate lyase (ACLY) by either chemical or genetic intervention. We show that acetate boosts cell proliferation in these conditions by supplying cytosolic acetyl-CoA for *de novo* fatty acid biosynthesis and histone acetylation. Using this assay, we are performing a genome-wide CRISPR dropout screen to identify known and novel acetate transporters. For the targeted approach, we use our observation that acetate supplementation extends the survival of cells fed with galactose in place of glucose by lowering their rate of glutamine uptake for anaplerosis to the TCA cycle. Both assays, as well as acetate uptake flux measurements through the plasma membrane, will be used to validate candidate hits.

Kelvin Maduabuchi Okpokpo (38, Sarma / Perini)
Analysis of the connection of ATRX with ADNP and CTCF

Mutations in the ATRX chromatin remodeler cause ATRX syndrome, a rare developmental disorder characterized by intellectual disability. Many studies examining the molecular mechanisms of ATRX have discovered its critical function in regulation of gene expression and maintenance of telomere integrity. In addition, recent studies show that ATRX regulates the localization of the architectural protein CTCF at imprinted genes, but how ATRX regulates CTCF is an open question. To understand the relationship between CTCF and ATRX, we used a model where we knocked out or knocked down ATRX (ATRX KO or ATRX KD respectively). We found that ATRX KO leads to a decrease in repressive histone modifications as well as DNA methylation, which may explain new CTCF gaining sites. However, this does not explain the vast majority of CTCF sites that are gained when ATRX is not present, suggesting other mechanisms through which ATRX regulates CTCF independent of methylation. We recently identified an interaction between ATRX and the ADNP protein which is known to regulate CTCF localization at specific genomic sites. We perfomed Cut&Run and ChIPseq on ADNP and ATRX respectively to identify sites where the two proteins colocalize. The results show that ATRX and ADNP colocalize mainly on repetitive elements (LINE and ERKVS). Furthermore, after IPs of different truncated forms of ATRX we were able to find the ADNP-binding domain. With this information, we will develop a separation-of-function model where we will abolish the interaction between ADNP – ATRX (CRISPR/Cas9) and see the role of the complex in neuro differentiation.

Federica Severi (38, Claiborne / Perini)
Multivalent CAR T cell therapy shows superior potency in controlling HIV escape and replication in BLT humanized mice

Chimeric antigen receptor (CAR) T cell therapy has shown potential in enhancing the natural T cell response against HIV. We explored the use of CAR T cells based on broadly neutralizing antibodies (bNAbs) to create highly effective therapies targeting diverse HIV strains.
We generated 19 bNAb-based CAR T cell variants targeting six conserved HIV envelope (Env) epitopes. Through *in vitro* assays, we assessed the efficacy of these CARs, finding that those targeting more distal epitopes exhibited superior killing potency. In investigating into the mechanisms that dictates these differences, we found out that epitope accessibility and binding avidity influenced potency between and within epitope specificities, respectively.
In vivo studies using HIV-infected humanized mice treated with CAR T cells revealed that CAR monotherapy led to escape mutations at well-known bNAb contact residues. Mapping these escape mutations allowed us to identify effective CAR product combinations with complementary escape paQerns. Combinayion CAR therapy, consisting of 3 monospecific CAR products, restricted the kinetics of HIV escape, and resulted in a significant and durable reduction in plasma viremia in humanized mice. We next developed tri-valent CAR products, simultaneously expressing the 3 most potent bNAb-CARs, which demonstrate greater potency and suggest the potential for more durable viral control *in vivo*.
Overall, our findings highlight the superior potency of certain bNAb CAR classes and the importance of combining CARs targeting different epitopes to limit HIV escape and achieve long-term viral control. Trispecific CAR products represent a promising advancement in HIV cellular therapy and offer a potential pathway towards an HIV cure.

Sara Morelli (37, Capranico / Russo)
Analysis of mutational signatures of Topoisomerase I poisons in colorectal cancer

DNA topoisomerase 1 (Top1) alleviates torsional stress from cellular processes like replication and transcription by forming a temporary Top1-DNA complex (Top1cc). Top1 inhibitors (Top1i), such as camptothecin (CPT), stabilize Top1ccs, leading to DNA double-strand breaks, genome instability, and apoptosis in rapidly dividing cells, such as tumors. Despite their use, the full molecular mechanisms of Top1i anticancer effects remain unclear. In our laboratory, we recently demonstrated that high Top1cc levels favour conflicts between transcription and replication in colorectal cancer cell lines triggering DSBs at highly transcribed genes, mainly during S phase, leading to micronuclei formation. So, Top1i may favour rearrangements and incorporation of mutations at DNA damage loci. As the specific mutational consequences of Top1i activity are not yet defined in cells and tumours, the aim of my project is to establish the specific mutational signatures and genomic consequences of Top1i-induced DNA damage in human cancers. I analyzed mutational signature contributions of Indels (IDs), single/double-base substitution (SBSs, DBSs) in three metastatic colorectal patient groups from the Hartwig Medical Foundation datasets: 1) those treated with Irinotecan (a Top1 inhibitor), 2) those treated with non-Top1i drugs, and 3) those who were not treated. Pair-wise comparisons revealed that Top1i-treated patients showed enrichment in two novel mutational signatures (ID denovo 5, SBS denovo 4), along with ID14 and DBS5, already catalogued in COSMIC. After an additional filter to separate the contributions of other drugs administered together with Top1i, ID denovo 5, appeared to be more specifically linked to Top1i. However, fur-

ther analyses are necessary to fully define the mutational impacts of Top1i in metastatic colorectal cancer.

Ilan Kirkel (37, Gardini / Perini)
Investigating the role of INTS3 in transcriptional regulation

Integrator (INT) is an RNA polymerase II-associated transcription complex, comprising fifteen subunits clustered into distinct functional modules. Each module individually contributes to Integrator's various regulatory roles in transcription which include modulation of RNA Pol II (RNAPII) pause-release transition, endonucleolytic cleavage and processing of nascent RNA species, targeted dephosphorylation of basal transcriptional machinery, and DNA repair. In particular, Integrator subunit 3 (INTS3) has been implicated in DNA damage repair in conjunction with the Sensor of ssDNA (SOSS) complex, alongside a role in preventing reassociation of RNAPII following premature transcriptional termination.
However, a satisfactory model of the mechanism by which INTS3 contributes to overall transcriptional regulation still remains elusive. In this light, a dTAG targeted protein degradation (TPD) system for INTS3 was generated in OVCAR8 cells to probe the rapid effects of INTS3 ablation on transcriptional regulation. The degradation of INTS3 is observed to upregulate the expression of various INT subunits and phosphostates of Ser2, Ser5 and Ser7 of the carboxyterminal domain (CTD) of RNAPII, and additionally may have an effect on INTAC (INT conjugated with PP2A) complex formation. Furthermore, INTS3 ablation is correlated with changes to the transcriptome with respect to the upregulation of immediate early response genes (IERGs). In a wider context, SOSS complex constituents were found to colocalize with INTS3 across the genome, predominantly at the AP-1 promoter element which is responsible for IERG expression. Moreover, it was observed that splicing factor 3 (SF3) subunits are differentially pulled down dependent on INTS3 ablation. Lastly, INTS3 degradation results in the reduction of RNAPII across the genome alongside an increase in pSer2 and pSer5 states, as well as a more efficient release of RNAPII into the gene body indicated by a decreased traveling ratio for RNAPII. Overall, these results implicate INTS3 in a variety of transcriptional regulatory events from affecting the INT complex directly, to changing RNAPII prevalence throughout the genome, to broader changes to the transcriptome.