BIODays 2014
Post Doc Research Day

Polo Scientifico e Tecnologico “Fabio Ferrari”

June 24th, 2014
Trento (Italy)
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Sacha Genovesi  
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Toma Tebaldi  
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## POST DOC RESEARCH DAY

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Identification of UBC12 as a new cofactor of HIV-1 integrase  
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ABSTRACTS
Kristian Franze
Dept. of Physiology, Development and Neuroscience, University of Cambridge, U.K.

**Mechanical signaling in nervous system development, functioning and disease.**

During the development of the nervous system, neurons migrate and grow over great distances, and also in different disorders neurons and glial cells are highly motile. Currently, our understanding of nervous tissue development and pathologies is, in large part, based on studies of biochemical signaling. Despite the fact that forces are involved in any kind of motion, and that cells thus have to mechanically interact with their environment in order to move, mechanical aspects of CNS functioning have so far rarely been considered. I will introduce experimental techniques that are used to measure cellular forces and tissue stiffness, and to investigate mechanical signaling, including deformable cell culture substrates, traction force microscopy, and atomic force microscopy. I will show how we use these methods to investigate how neurons probe and respond to their mechanical environment. While the growth rate of Xenopus retinal ganglion cell axons was increased on stiffer substrates, their tendency to fasciculate was significantly enhanced on more compliant substrates. If grown on substrates incorporating stiffness gradients, neuronal axons were repelled by stiff substrates. Mechanosensing involved the application of forces driven by the interaction of actin and myosin II, and the activation of stretch-activated ion channels leading to calcium influxes into the cells. In vivo atomic force microscopy revealed stiffness gradients in developing Xenopus brain tissue along which neurons grow. The application of chondroitin sulfate, which is a major extracellular matrix component in the developing brain, changed tissue mechanics and disrupted axonal pathfinding. Ultimately, blockers of mechanosensitive ion channels also disrupted axon guidance in vivo. Furthermore, disorders of the CNS, such as acute traumatic injuries and chronic demyelination, lead to changes in neural tissue stiffness, exposing neurons to different mechanical signals, which might contribute to failing neuronal regeneration in the mammalian CNS. Hence, our data suggest that neuronal growth is not only controlled by chemical signals – as it is currently assumed – but also by the tissue’s mechanical properties.
Corrupted by TNFalpha-induced signaling: a dark side of wild type p53?

Yari Ciribilli¹, Alessandra Bisio¹, Judit Zámborszky¹, Sara Zaccara¹, Mattia Lion¹, Vasundhara Sharma¹, Toma Tebaldi², Alberto Inga¹

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The p53 and NFκB sequence-specific transcription factors play crucial roles in cell proliferation and survival with critical, although mostly opposite, impacts on cancer progression. To investigate cooperative interactions between NFκB and p53, we performed transcriptome analysis in luminal-type breast cancer-derived MCF7 cells upon single or combinatorial treatments with the chemotherapeutic drug doxorubicin (DXR, 1.5nM) and the NFκB inducer TNF-alpha (TNF, 5ng/ml). We also evaluated the impact of 17β-estradiol (E2, 10⁻⁹M) alone or combined to DXR+TNF. The array results revealed 436 up-regulated (log2 FC> 2), and 402 repressed genes (log2 FC< -2) for the DXR+TNF treatment. 185 up-regulated and 292 repressed genes were differentially expressed genes (DEGs) only after double treatment. A more-than-additive effect of the combinatorial treatment was observed for an additional 290 up-regulated and 51 repressed genes. Gene ontology analysis of DXR+TNF DEGs indicated strong enrichment for cell migration, blood vessel morphogenesis and mesenchymal cell development. Addition of E2 to DXR+TNF led to the up-regulation of nearly 100 additional genes including those for secreted chemokines (S100A9, CXCL12, CXCL14 but not CXCL1/2). We focused the validation on 15 up-regulated DEGs using qPCR coupled to p53 activation by DXR, 5-Fluoruracil and Nutlin-3a, or to p53 (using RNA silencing) as well as NFκB (with BAY11-7082) inhibition. We uncovered four genes (LAMP3, ETV7, UNC5B, NTN1) whose expression was more prominently synergistic after DXR+TNF treatment and dependent on both p53 and NFκB activity. Transwell migration as well as wound healing assays consistently showed an increase in migration potential for MCF7 cells upon DXR+TNF compared to mock or single treatments. A 38-gene signature from the array results, considering genes that were strongly synergistic upon DXR+TNF, was able to stratify luminal breast cancer patients that had undergone chemotherapy for the probability of relapse-free survival (111 patients, P value = 0.022; KM plotter tool). We propose that the crosstalk between p53 and NFκB can lead to the activation of specific gene expression programs that may impact on cancer phenotypes, and potentially modify the efficacy of cancer therapy.
PLENARY SESSION

New Paradigms in Autism: one or many signaling pathways are altered in a mouse model of the disease?

Giovanni Provenzano¹§, Paola Sgadò¹*, Sacha Genovesi¹, Giulia Zunino¹, Simona Casarosa²,³ and Yuri Bozzi¹,³

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Keywords: autism spectrum disorders, EN2, FMRP, mGluR5, GABRB3.

Autism spectrum disorders (ASD) are a group of highly heritable neurodevelopmental disorders characterized by a marked genetic heterogeneity. In recent years, mutant mouse models have been extensively used to understand the molecular basis of ASD and identify new therapeutic opportunities. Many evidences indicate that mice lacking the homeobox transcription factor Engrailed 2 (En2/-/- mice) represent a reliable animal model to investigate neurodevelopmental basis and gene expression changes relevant to ASD. Since dysfunctions in both FMRP-mGluR5 signaling pathway and GABAergic system have been proposed as a possible pathogenic mechanism of ASD, in the current study we exploited En2/-/- mice to investigate the hippocampal expression of FMR1, GRM5 and GABRB3 genes. Quantitative RT-PCR was used to assess the expression of FMR1, GRM5 and GABRB3 genes in the hippocampus of En2/-/- and wild-type (WT) adult mice. We observed that all these genes were significantly downregulated in En2/-/- mice, as compared to WT littermates. Western blot and immunohistochemistry confirmed that FMRP and GABRB3 were downregulated also at the protein level, while showing a significant increase of mGluR5 protein in the En2/-/- hippocampus. Our results suggest that the dysregulation of FMRP-mGluR5 signaling pathway, accompanied with a downregulation of GABRB3 expression, may contribute to the “autistic-like” features observed in En2/-/- mice, providing possible molecular targets for future pharmacological studies.
PTRcombiner: mining combinatorial regulation of gene expression from post-transcriptional interaction maps

Gianluca Corrado\textsuperscript{1},†, Toma Tebaldi\textsuperscript{2},†, Giulio Bertamini\textsuperscript{1}, Fabrizio Costa\textsuperscript{3}, Alessandro Quattrone\textsuperscript{2}, Gabriella Viero\textsuperscript{4,2} and Andrea Passerini\textsuperscript{1}

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The progress in mapping RNA-protein and RNA-RNA interactions at the transcriptome-wide level paves the way to decipher possible combinatorial patterns embedded in post-transcriptional regulation of gene expression. Here we propose an innovative computational tool to extract clusters of mRNA trans-acting co-regulators (RNA binding proteins and non-coding RNAs) from pairwise interaction annotations. In addition the tool allows to analyze the binding site similarity of co-regulators belonging to the same cluster, given their positional binding information. The tool has been tested on experimental collections of human and yeast interactions, identifying modules that coordinate functionally related messages. This tool is an original attempt to uncover combinatorial patterns using all the post-transcriptional interaction data available so far. PTRcombiner is available at \url{http://disi.unitn.it/~passerini/software/PTRcombiner/}

Keywords

Post-transcriptional regulation, Boolean matrix factorization, RNA binding protein (RBP), binding site classification, kernel machines, miRNA, translation, CLIP
ONCOLOGY

Monitoring copy number aberration dynamics in sequential plasma samples from castration-resistant prostate cancer (CRPC) patients

Alessandro Romanel1,6, Suzanne Carreira2,6, Jane Goodall2,6, Emily Grist2,3,6, Roberta Ferraldeschi2,3, Susana Miranda3, Davide Prandi1, David Lorente Estelles2,3, Jean Sebastien Frenel2, Carmel Pezaro2,3, Aurelius Omlin2,3, Daniel Nava Rodriguez2, Penelope Flohr2, Nina Tunariu2,3, Johann S De Bono3, Gerhardt Attard2,3,7, Francesca Demichelis1,4,5,7

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2 The Institute of Cancer Research, Sutton, Surrey, UK
3 Royal Marsden NHS Foundation Trust, Sutton, Surrey, UK
4 Institute for Computational Biomedicine, Weill Cornell Medical College, New York
5 Institute for Precision Medicine, Weill Cornell Medical College, New York
6 These authors contributed equally
7 Co-senior authors

Timely evidence of treatment response or resistance in CRPC patients can optimize their treatment course. We tested our ability to quantify tumor content and tumor dynamics during treatment, leveraging the detection of specific somatic copy number aberrations (SCNA) in addition to point mutations (PM) from sequential plasma samples collected after development of castration resistance from patients treated with abiraterone and enzalutamide or cabazitaxel. Clonality of SCNA and PM was also evaluated in patient-matched pre-castration FFPE tumor and CRPC biopsies collected at time of plasma acquisition.

Custom targeted deep sequencing of 367 amplicons using a minimum of 6ng input plasma DNA was performed for 14 individuals. The design included full coverage of the deletion on 21q that results in a TMPRSS2:ERG fusion, PTEN, NNX3-1, FOXA1, SPOP, CYP17A1, TP53 and AR for a total of about 41Kbp. The gene set was selected based upon expected frequent SCNA and PM of interest. 1186 SNPs of which 268 had high minor allele frequency were included in the design to optimize the in house data processing approach. The mean sequencing coverage across the experiments was 1195-1470 (range from 20 to 53,000).

First, by using control healthy volunteer plasma DNA we calculated that the assay’s precision and false discovery rate for single base substitutions at about 1100 coverage were 67% and 0.9% respectively when tumor DNA fraction is 50%. Next, by applying an extended version of CLONET (presented in Baca et al, Cell 2013), we measured tumor fractions in a total of 86 plasma samples that ranged between 9% and 85% (where 49% and 75% of the samples demonstrated > 20% and >15%, respectively). Specifically, plasma cDNA data showed somatic copy number losses for PTEN, 5’ of ERG and NNX3.1 and high level gains of AR in 8/14 patients at progression. Point mutations were detected including D226H in FOXA1 and L702H in AR with allelic fractions ranging from 5% to 40%. By coupling SCNAs data upon quantification of tumor content in plasma samples during
treatment, we were able to detect co-occurring lesions (such as 21q and PTEN deletions) and observed independent dynamics of lesions compatible with over and under-representation of independent tumor clones in the circulation (ERG+/PTEN- clone versus NXK3.1- clone). Changes in fraction of tumor lesion specific circulating DNA are associated with clinical and radiological progression, increases in circulating tumor cell count and in some patients, rises in PSA. Integrating targeted deep sequencing and read based clonality computations can provide timely non-invasive biomarkers for CRPC clinical management. In addition clone specific lesions might suggest response/resistance mechanisms. Our combined approach can therefore lead to the quantification of circulating tumor DNA fractions, the detection of SCNA, and the monitoring of specific clone DNA in the circulation of CRPC patients.
ONCOLOGY

_in-silico_ identification of AR bound enhancer reveals allele dependent activity

Sonia Garritano, Yari Cilibilli, Alessandro Romanel, Alessandra Bisio, Alberto Inga, Francesca Demichelis

**Background:** Knowledge of transcriptional regulators has increased in the last decade improving our understanding of gene expression regulation. Androgen Receptor (AR) and Estrogen Receptors (ERs) are key nuclear receptors and their role has been extensively studied also in the context of cancer development.

**Aim:** To identify enhancer elements responsive to AR or ER that demonstrate differential activity based on overlapping/nearby SNP.

**Material and Methods:** Data from ENCODE were computationally mined to identify genomic loci with the following characteristics i) chromatin signature of enhancer activity (H3K4m1, H3K4me1+H3K4me3) ii) binding by ER and AR iii) presence of a SNP. Selected loci were then validated and characterized _in vitro_ by luciferase assay with or without Dihydrotestosterone treatment in MCF7 cells. Plasmids harboring the alternative alleles of the selected enhancer elements were utilized. Chromatin immunoprecipitation assays (ChIP) with AR antibody, followed by real-time PCR and sequencing analysis was performed.

**Results:** Forty-one loci were identified computationally and two selected for _in vitro_ characterization (on 1q21.3 and 13q34). Both region (~1000nt in length) exhibited enhancer activity regulated by ligand-bound AR (p<0.05). The SNP variant on 1q21.3, rs2242193 (CEU Minor Allele Frequency =0.376), had an impact on a role in the transcription regulation (p=0.028, Student’s t-test) and was enriched in chromatin fragments immunoprecipitated with AR antibody. Sequencing analysis showed that AR was preferentially recruited to the A allele of the (p-value < 0.05) confirming the results of the luciferase assay.

**Conclusion:** Unbiased genome-wide search proved to be an efficient methodology to discover new functional _cis_-elements. The broad coverage of ENCODE annotations allowed us for a robust investigation of the impact that SNPs have in _cis_-regulatory sequences.
ONCOLOGY

Oncogenic amplification of TrkA in malignant melanoma can couple with a fail-safe protective response

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Keywords: TrkA, genomic amplification, malignant melanoma, oncogene-induced growth arrest

The nerve growth factor (NGF) receptor tyrosine kinase (TrkA) is pivotal in determining survival and differentiation of the melanocytic lineage through the induction of MAPK and AKT cascades. Abnormalities in the TrkA pathway are commonly observed in many cancers and few evidences suggest the involvement of TrkA in the progression of malignant melanoma. Here we used array comparative genomic hybridization (aCGH) to detect copy number changes of the TrkA gene in primary melanoma patients and investigate its function in melanoma cells. Our analysis has identified, for the first time, a minimal common amplification of TrkA, where genomic gain was strongly associated with melanoma thickness (P=0.02). When we extended the analysis by genomic quantitative PCR to 64 cases, TrkA amplification emerged as a frequent event in melanoma (50% of patients) associated with metastatic outcome (P=0.04). Interestingly, inducible overexpression of TrkA in two melanoma cell lines resulted in a dramatic suppression of cell proliferation, inhibition of cell division, and S-G2 phase arrest, in a way straightly dependent on NGF stimulation and TrkA activation, resembling a typical cellular phenotype of oncogene-induced growth arrest. Using of pharmacological kinase inhibition suggested that these events might be triggered via MAPK activation but not AKT and involve p21 protein increase. Together, our findings support a model in which TrkA-MAPK pathway may mediate a trade-off between cancer and protective response, envisaging novel therapeutic strategies enhancing this fail-safe mechanism in malignant melanoma.

References:
Circulating miRNAs as Frontotemporal Dementia biomarkers

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Frontotemporal lobar degeneration (FTLD) is the second most common cause of onset dementia after Alzheimer’s Disease (AD)¹, characterized by atrophy of the prefrontal and anterior temporal lobes and representing 5% of all dementia patients². Mutations in progranulin (GRN) gene were found in patients affected by FTD with parkinsonism linked to chromosome 17 (FTDP-17)³⁴. Recent reports suggest that GRN is under the control of some microRNAs (miRNAs) such as miR-29b⁵ and miR-659⁶. It has also been identified a miRNA expression profile in the frontal cortex of FTLD patients population with GRN mutations⁷. Altered expression of miRNAs in many disease states, including neurodegeneration, and increasing relevance of miRNAs in biofluids in different pathologies has prompted us to study a their possible application as FTD plasma biomarkers in order to identify new therapeutic targets. Our hypothesis is that specific miRNAs can be detected in plasma to discriminate patients from healthy controls and to define subtypes of this disease, using a minimally invasive test based on plasma circulating miRNAs analysis obtained with a panel of 750 specific miRNA by single reaction of Real-Time PCR Amplification. A second aim of this project is the sequencing of GRN 3’UTR region of FTD patients to identify possible genetic variants able to influence the binding of miRNAs involved in the regulation of GRN. Hence, this study could open new opportunities to the exploration of circulating miRNAs for FTD diagnostic, prognostic, and therapeutic interventions.

Keywords: microRNA, biomarker, early diagnosis

References:
RNA AND MOLECULAR BIOLOGY

The cytoplasmic role of HuR in response to transcriptional stress

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The RNA Binding Protein HuR is an important player in the process of mRNA availability, and the nature of the transcripts it binds has attract attention for a possible involvement of HuR in cancer development. The most accepted role for HuR in the literature sees the protein bind mRNA in the cytoplasm compartment and prolong the half-life of those transcripts: it protects transcripts from degradation and stimulate translation by loading them on polysomes. The protein is located prevalently into the nucleus and can shuttle between the two compartments. Its nuclear localization points for a role of the protein within these boundaries, as suggested by recent papers where HuR has been described having a role in splicing and alternative polyadenylation. Here we show that productive elongation of Pol-II driven transcription is involved in nuclear retention of the protein. When transcription is blocked the protein is massively relocated into the cytoplasm. Despite its cytoplasmic abundance HuR decreases its presence onto polysomes, while it increases its localization in P-bodies. The majority of HuR target transcripts in analysis follow the exit of HuR from polysomes. Our data show a role for HuR in pausing translation of some genes as a consequence of transcriptional stress, and suggest a possible connection of HuR in the process of transcription and RNA maturation.
miR-375 and miR-192 expression levels distinguish neuroendocrine from non-neuroendocrine tumors. Novel biomarkers from resected samples

Valerio Del Vescovo¹, Margherita Grasso¹, Chiara Cantaloni², Chiara Asensi², Leonardo Ricci³, Mattia Barbareschi² and Michela Alessandra Denti¹

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Pulmonary neuroendocrine (NE) tumors form a distinct group of neoplasms that share characteristic morphologic, immunohistochemical, ultrastructural, and molecular features. The clinical spectrum is various, from low-grade typical carcinoid (TC) and intermediate-grade atypical carcinoid (AC) to high-grade large cell NE carcinoma (LCNEC) and small cell lung carcinomas (SCLC). However, they represent a wide spectrum of phenotypically distinct entities, from which pulmonary NE tumors can sometimes be difficult to differentiate, even for an expert pathologist.

miRNAs, short non coding RNAs (18-21nt), are a promising new class of cancer biomarkers which may potentially affect all aspects of clinical care from early detection, diagnosis, and prognosis. miR-375 was markedly induced by ASH1 in lung cancer cells where it was sufficient to induce NE differentiation. On the other hand miR-192 is known to show an high variability among solid tumors. In the present study we analyze miR-375 and miR-192 expression in a series of surgically resected NE lung tumors, including TC, ATC, LCNEC, SCLC, ADC and SQCC. The two aims of the study are 1. to verify whether there are differences in the expression levels of these putative markers in NE versus non-NE lung tumors, and 2. to verify the miRNAs differential expression within the different subtypes of NE lung tumors.
Dissecting the molecular mechanisms underlying retinal differentiation in mammalian pluripotent stem cells

Tania Incitti\textsuperscript{1}, Andrea Messina\textsuperscript{1}, Angela Bozza\textsuperscript{1} and Simona Casarosa\textsuperscript{1,2}

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Keywords: mESC, neural differentiation, retina

The retina is the multi-layered light-sensitive tissue of the eye. It is surrounded by the retinal pigmented epithelium (RPE), necessary for the survival and function of photoreceptors. Both RPE and photoreceptors can be affected by several degenerative diseases, which are among the major causes of incurable blindness in the world.

Among the possible therapeutic approaches, strong interest is raised by cell replacement protocols based on manipulation of stem cells or photoreceptor progenitors (1). We have evidences showing that the overexpression of high doses of Noggin, a neural inducer molecule and an inhibitor of BMPs, enables Xenopus animal cap cells to differentiate into retinal neurons in vitro and to rescue a complete and functional eye in embryos lacking one of the eye primordia (2). Due to these successful results, we decided to verify whether high doses of Noggin could elicit a similar retinal-inducing activity also in mammalian stem cells. Three lines of mouse embryonic stem cells (ESC) were cultured and differentiated both in suspension and in adhesion and treated with Noggin alone or in combination with a cocktail of growth factors acting on other signalling cascades. In particular, Rx1-GFP KI cells (gift of Prof. Y. Sasai, 3) were a useful tool to monitor the appearance of retinal precursors (GFP positive). Results indicate that retinal precursors are obtained when mESCs are cultured in suspension and Noggin is administered in combination with other secreted molecules. Studies are now ongoing to characterize the Rx1-expressing cells and to verify the feasibility of the protocol for the differentiation of newly generated mouse induced pluripotent stem cells. The elucidation of the pathway through which Noggin and other factors elicit retinal fates will be a first step toward the setup of improved protocols for the differentiation of human stem cells toward retinal neurons.

References:
NEUROBIOLOGY

Expression of inflammation mediators in a mouse model of Temporal Lobe Epilepsy (TLE).

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Purpose: TLE is one of the most represented forms of epilepsy in human. Immune system cells and their mediators have been implied in epileptogenesis and seizure occurrence in TLE. To shed a light on these interplays, we studied changes in inflammatory mediators at gene and protein levels in an experimental model of kainic acid (KA)-injected mice treated with Lipopolysaccharide (LPS).

Method: Mice were unilaterally injected with an intrahippocampal dose of KA. Chronically-epileptic animals were injected with an intraperitoneal dose of LPS to mimic peripheral infection. EEGs were performed to assess frequency and duration of epileptic seizures after LPS administration. Hippocampi were explanted 4 and 24 hours after LPS and used for microarray and RT-qPCR gene expression analysis. Immunofluorescence microscopy was used to observe inflammatory mediators expression in hippocampal tissues, meanwhile multiplex and standard ELISA tests were performed to evaluate their concentrations in sera and hippocampal protein extracts.

Results: EEG showed an increased frequency of recurrent seizures in LPS-treated mice in comparison to controls. RT-qPCR analysis confirmed microarray data, showing that KA+LPS treatment exacerbated the expression of chemokines and interleukins already observed in KA- or LPS-treated hippocampi. Microscopy observations, multiplex and standard ELISA tests showed that inflammatory mediators increase at protein level in several cellular subpopulation in epileptic tissues, sera, and hippocampal protein extracts.

Conclusion: These data suggest that LPS-mediated inflammatory insult may strengthen the expression of inflammatory mediators that occur during chronic epilepsy, and that these molecular changes are associated with an enhanced propensity to spontaneous seizures.
Identification of UBC12 as a new cofactor of HIV-1 integrase

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Keywords: HIV-1, integrase, sumoylation, UBC12.

HIV-1 integrase (IN) mediates the integration of reverse-transcribed viral cDNA into the host cell genome. This activity requires the concerted action of viral and cellular factors and depends on the interaction between IN and multiple proteins. Post-translational modifications of IN, like phosphorylation (1) and acetylation (2,3), modulate its activity and affect its interaction with cellular co-factors. Recently also sumoylation of IN (4) and its interaction with sumoylated proteins (5) have been reported to affect IN activity. Therefore we performed a prediction of IN Sumo Interacting Motifs (SIM), which interact non-covalently with SUMO, and found 4 potential hits (SIM1-4). By mutating each of the potential SIM we found that IN-SIM4(mut) binding to KAP1, a known sumoylated cellular restriction factor that inhibits proviral integration(6), is greatly enhanced. This unexpected result prompted the idea that SIM4 may be responsible for the binding to another sumoylated protein that might act as a KAP-1 antagonist. Immunoprecipitation analysis followed by mass spectrometry allowed the identification of a new cofactor of IN: UBC12, NEDD8 conjugating enzyme. UBC12 accepts the protein NEDD-8 and catalyzes its covalent attachment to other proteins i.e. cullins which play an essential role in targeting proteins for ubiquitin-mediated degradation. Overexpression of UBC12 does not affect HIV-1 integration, while overexpression of its dominant negative form (UBC12-C111S) causes a decrease of integrated proviral DNA. These results suggest an important role for UBC12 during HIV-1 infection.
VIROLOGY & MICROBIOLOGY

Antibody-mediated immunity induced by engineered *Escherichia coli* Outer Membrane Vesicles (OMVs) carrying heterologous antigens

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**Background** – Outer Membrane Vesicles (OMVs) are closed spheroid particles of a heterogeneous size (10-300 nm in diameter) that arise through bulging and pinching off of the outer membrane of Gram negative bacteria. They are composed of outer membrane and periplasmic proteins and have proposed roles in virulence, inflammation and envelope stress response. Recently, OMVs gained increasing attention as vaccine platform for their built-in adjuvanticity and for the possibility of being engineered with heterologous antigens. These two properties offer the opportunity to make highly effective, easy to produce multi-valent vaccines. OMVs can be loaded with foreign antigens by targeting protein expression either to the outer membrane or to the periplasm of the OMV-producing strain.

**Methods** – We have selected a number of proteins from different bacteria, both Gram positive and Gram negative, belonging to different cellular compartments as model systems to demonstrate the possibility to deliver them to the OMV in native conformation. Vesicles expressing heterologous antigens were purified and tested for immunogenicity and protective activities.

**Results** – Functional assays revealed that all the proteins were incorporated into the OMVs in their native conformation. Upon immunization, OMVs induced high functional antibody titers against the recombinant proteins and mice immunized with OMVs were protected against bacterial infection.

**Conclusions** - The efficiency of heterologous antigens expression in OMVs, and the high immunogenicity and protective activity of OMVs carrying recombinant antigens further strengthens the potential of OMVs as vaccine platform.
Engineered *Escherichia coli* outer membrane vesicles as a vaccine against *Staphylococcus aureus* infection

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Outer membrane vesicles (OMVs) are spherical nanoparticles of variable size that are released from all Gram-negative bacteria through a ‘budding out’ of the outer membrane. OMVs mainly contain outer membrane and periplasmic components and have been associated to a variety of biological functions, such as virulence, inflammation and envelope stress response. Since they carry bacterial surface-associated proteins and feature a potent built-in adjuvanticity, native OMVs are currently used as vaccines.

Recently, the immunogenicity and protective activity of heterologous antigens expressed in *Escherichia coli* OMVs have been demonstrated, thus making OMVs a promising platform for recombinant, multivalent vaccines development. Based on this evidence, we have started to design an OMV-based vaccine against *Staphylococcus aureus*, a Gram-positive bacterium commensal of the human skin and nares, which is also responsible for invasive nosocomial as well as community acquired infections. Despite the global medical needs associated with *S. aureus* infections, no licensed vaccines are currently available. Following previous identification and characterization studies, we selected six protein vaccine antigens. Each protein was successfully expressed in an OMV-hyperproducing mutant of *E. coli* and delivered to the OMV compartment. Antigen-containing OMVs were purified and studies in the animal model were started, in order to compare the immunogenicity and protective activity of the OMV vaccine with respect to the recombinant vaccine.
Correction of FTDP17 associated splicing mutations in MAPT gene via antisense oligonucleotides - induced exon skipping.

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**Keywords:** antisense RNA; RNA splicing; Tauopathies

A number of neurodegenerative diseases, including FTDP-17 (a rare autosomal dominant condition), are characterized by intra-neuronal accumulation of tau protein. The cause of about half of the cases of FTDP-17 are mutations affecting the alternative splicing of exon 10 (E10). The aberrant inclusion of E10 in tau mRNA leads to the aggregation of tau as Neurofibrillary Tangles (NFTs) in neurons.

To explore the feasibility of an antisense oligonucleotide (AON)-based gene therapy to correct tau splicing in FTDP-17 we tested whether it was possible to modulate E10 alternative splicing by the use of 2′-O-methyl phosphothioate AONs that mask specific splicing-regulating sequences. The transfection of specific AONs is able to alter the splicing behaviour of tau E10 in the endogenous transcript in a rat pheochromocytoma cell line (PC-12), with variable efficiencies depending on the concentration of the AONs and on the targeted sequence.

Based on these results, to evaluate the effects of AONs on the human tau pre-mRNA, we constructed a minigene reporter system, containing luciferase and that recapitulates to a large extent the behaviour of E10 in the context of the full-length tau gene. We carried out co-transfection into HeLa and SK-N-BE (2) cells and evaluated the induction of E10 skipping by Luciferase Expression Assay, RT-PCR and Western Blotting. We have come up with two AONs which induce extensive or intermediate exon skipping, respectively.

To test in vivo the AONs efficacy and safety further work will be directed to test the therapeutic efficacy of the AONs in the animal model of FTDP-17 (T-279 mouse).
How to identify and explore potential RNA-binding proteins inhibitors

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Keywords: HuR, kinetic, assay, drug screening

RNA binding proteins (RBPs) are key players in the modulation of RNA expression. RBPs usually interact with the untranslated regions (UTRs) of the mRNA. The HuR/ELAVL1 protein is a RBP that binds to AU-rich elements (AREs) promoting the stabilization and the translation of a number of mRNAs into the cytoplasm, dictating their fate.

We applied the sensitive AlphaScreen technology using purified human HuR protein to characterize its binding performance in vitro towards a ssRNA probe corresponding to a ARE consensus. This assay, satisfying criteria of quality, was applied for a high throughput screening looking for small compounds able to disrupt the protein-RNA interaction. We identified two hits, X1 and X2, displaying different destabilizing effects on the interaction and on the RNA probe, respectively. We tested the post-transcriptional influences of X1 in breast cancer MCF-7 cells, providing comprehensive evidences that this compound interferes with HuR binding capacity also in tumor cells with consequences on mRNA target translation. Moreover, we have indications that X1 displays, to some extent, in vitro anti-proliferative effects in HuR-influenced manner and also in vivo tumor growth inhibition of MCF-7 nude xenografted mice. The selective profile of X1 and its efficacy as potential HuR inhibitor remains to be elucidated.

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AC. Pawlyk et al., J Biomol Screen 2010, 9, p. 1099-1106.
TO-DAG cumulative cancer progression model: a new graph-based model of dependencies and timing of mutational events

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Keywords: network inference, probabilistic graphs, cancer progression, prostate cancer melanoma

The order and timing at which the genetic alterations occur during cancer progression reveal important information on the underlying biological process with implications for diagnosis and treatment. High-throughput technologies provide base level resolution data and provide the cancer research community with unprecedented comprehensive datasets of genomic alterations in human cancers. Despite the flourishing of computational models, crucial aspects related to the evolution of the mutation accumulation process still require a mathematical formalization that reflects experimental observations. We present a novel computational method named “Timed Oncogenetic Directed Acyclic Graph” (TO-DAG) that infers the graph of the causal dependencies and the waiting times among mutational events from very large cross-sectional data of genetic alterations in independent human tumor samples. TO-DAG computes the probability of occurrence of each mutation in a pathway as the probability that it occurs when all alterations prior to it have occurred. Therefore, the new probabilistic theory of TO-DAG might allow inferring pathways of causal dependencies among genetic alterations reflecting the real non-memoryless dynamics of the mutation accumulation during cancer formation (as opposed to most methods that work with stochastic memoryless processes and/or are limited to pairwise dependencies). Once the causal structure of the graph is inferred, the waiting times of the mutation events are estimated by TO-DAG as a stochastic function of their conditional probability. We present TO-DAG results on random and controlled synthetic data and discuss the networks of causal relationships inferred from prostate cancer [1] and in melanoma [2] genomic datasets in the light of current literature.

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Noggin mediated Retinal Induction reveals a novel interplay between BMPs, TGFβ and SHH

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Keywords: Noggin, Sonic hedgehog, Xenopus pluripotent cells

Retinal degenerative diseases represent the principal cause of irreversible blindness in the world and regenerative medicine offers one of the principal strategies to cure damaged retina using stem cells-based therapy. We have previously shown that the overexpression of Noggin, a BMP inhibitor, is able to convert animal cap embryonic stem cells (ACES) of *Xenopus laevis* in differentiated retinal neurons. We have analyzed the molecular mechanism by which Noggin acts. Microarray analyses show that the Sonic Hedgehog (SHH) signaling pathway is fundamental in Noggin-mediated retinal fate specification, suggesting a new potential role of SHH in the acquisition of retinal precursors identity. Functional validation experiments show that blocking SHH signaling inhibits the retina-forming properties of high-Noggin expressing ACES cells. On the contrary, activation of SHH signaling allows low Noggin doses-treated ACES cells to become retinal precursors. We also have further results suggesting an involvement of the TGFβ pathway in the ability of Noggin to recruit Sonic Hedgehog. Understanding the mechanism through which Noggin confers a retinal identity to *Xenopus* ACES cells could facilitate the development of differentiation protocols for stem cell-based transplantation therapies for retinal diseases.
Investigation of recurrent alterations of translation factors in glioblastoma multiforme.

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Glioblastoma multiforme (GBM) is the most advanced form (IV grade) of astrocytoma and the most common primary brain tumor among adults. Currently, the therapies available for its treatment give poor results, in part due to the genetic heterogeneity of this disease. The regulation of protein translation is a key point in cancer development and progression, and an increasing number of translation factors is found to be altered at the genomic/expression level or in their activity.

First, a bioinformatic screening was performed in search of translation factors and RNA binding proteins altered in GBM patients. From the resulting list eIF3A, eIF2AK1, eIF4ENIF1 and TSFM were chosen for further investigation. Differences in expression for both mRNA and protein levels were observed in a panel of cell lines and glioblastoma initiating cells (GICs). Some disconcordance between the mRNA and protein levels were also noted, suggesting a potential translational or post-translational regulatory mechanism.

An siRNA-based knock-down system was then successfully employed in a GBM cell line. Morphology, proliferation and cell cycle assays showed no differences after eIF3A, eIF2AK1, eIF4ENIF1 or TSFM knock-down when compared to the control. Neurosphere assay results showed that the knock-down of eIF2AK1 or eIF4ENIF1 both increased the number of neurospheres formed. The knock-down of TSFM or eIF3A both decreased the expression of CDK4 and HuB, and increased SOX2 levels.

In summary, these data suggest that eIF3A and eIF4ENIF1 might be involved in limiting gliomagenesis, while the findings for eIF2AK1 and TSFM remain inconclusive. Further experimental validation will verify these findings.
Total and polysomal RNA-Seq profiling of ALS mice spinal cords

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Keywords: ALS, translational control, polysomal profiling.

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that affects motor neurons and leads to paralysis and respiratory failure, followed by death usually within 2-5 years of diagnosis. Recent advances showed that ALS results in loss or gain of function of factors involved in translational control, especially RBPs, suggesting that the genome-wide polysome profiling could be a useful tool to identify new deregulated targets in the disease.

To evaluate whether a translational impairment would be related to ALS, we processed spinal cords of end stage SOD1 mice, a common model of ALS, by a sucrose gradient fractionation. We then profiled total and polysomal mRNA by RNA-Seq in order to identify genes which display differential expression and/or altered splicing patterns. By a preliminary analysis, we found a small number of transcripts with a significant altered expression in ALS transgenic mice compared with littermate controls (73 genes for polysomal mRNA and 67 genes for total mRNA) and an enrichment for gene ontology terms related to ion channel activity, inflammatory and immune response. Moreover, we observed a high correlation between total and polysomal mRNA profiling, suggesting that, at least in our SOD1 ALS model, the terminal stage of neurodegeneration could be not associated with severe transcriptional and translational defects.
P53 protein evolutionary functional divergence through the lens of a yeast-based transactivation assay

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The tumor suppressor protein p53 is one of the most studied sequence-specific transcription factor (TF). Inactivation of the p53 pathway in cancer frequently occurs through the expression of mutant p53 proteins. Early reports indicated highly conserved p53 DNA binding specificity in vitro, even for evolutionary distant species. Here we used a set of isogenic Saccharomyces cerevisiae reporter strains and the transactivation potential of six p53 proteins derived from the main animal models was evaluated using ten different p53 response elements (REs) integrated at a specific chromosomal location. Hence, yeast was used as an in vivo test tube to examine evolutionary divergence in transactivation specificity. The assay combines the advantage of minimizing the impact of variables such as chromatin state, promoter landscape and cofactors influences, with that of measuring induced transcriptional changes at different levels of p53 protein expression. Thanks to this tool we established, for the first time, that p53 transactivation selectivity has been modified during evolution and differences are apparent even in the comparison between human and mouse p53, the latter protein exhibiting higher relative activity towards lower affinity, non-canonical, or structurally more rigid binding elements. Human p53 showed the widest range of transactivation potentials as a function of RE sequence, while Zebrafish p53 was almost equally active towards different REs. Fruitfly and Xenopus p53s also revealed distinct transactivation specificity, the latter protein being highly temperature sensitive. Our results suggest species-specific adaptations of the p53 function as a sequence-specific transcription factor that should be considered in interpreting results of tumor suppressor pathways and their deregulation in cancer using animal models. In a broader sense, our approach can be employed to investigate transactivation capacity of TFs in evolutionary studies and could be applied to study thermostability as well as utilize chimeric constructs of TFs to enhance the transactivation readout.
Identification and characterization of a slow-cycling stem cell-like subpopulation in primary glioblastoma-initiating cells.

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In glioblastoma multiforme (GBM) therapy inefficacy is due, in part, to the high degree of tumor heterogeneity contributing to both tumor progression and therapeutic resistance. Accordingly with the cancer stem cells paradigm, a small subpopulation of cancer cells with stem cell-like characteristics has been revealed in several central nervous system tumors including malignant gliomas. Therefore, we aimed to discern and characterize a subpopulation of stem cell-like slow-cycling cells in primary glioblastoma-initiating cells (GICs), undifferentiated tumor cells considered to be responsible for tumor initiation, relapse and therapeutic resistance.

To this aim, we explored a recently described label retention approach based on the use of small fluorescent lipophilic molecules Vybrant® DiI, which irreversibly binds the lipid cell bilayer and get proportionally distributed among daughter cells. This allowed us to discriminate between fast (DiI low) and a small subpopulation (< 7%) of slow (DiI bright) dividing cells in primary GICs followed by evaluation of their phenotype/morphology and function.

Our data show that: i) the DiI bright subpopulation exhibited lower proliferation rate as compared to the DiI low subpopulation and showed only a partial overlap with the cancer stem cells markers CD24, CD133, Nestin and SOX2; ii) a cell cycle analysis of the slow-dividing cells revealed a 2-fold increase in G2/M phase cells when compared with the fast-dividing subpopulation suggesting an extended G2/M phase or arrest; iii) under differentiation conditions the slow-cycling cells showed an elongated fibroblast-like cell shape as compared with the fast-cycling cells and were negative for neuronal differentiation markers.

Keywords: GBM, GICs, cancer stem cells, Vybrant® DiI.
PanPhlAn: Pangenome-based strain level metagenomic profiling

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Metagenomics provides the opportunity to explore complex microbial populations in natural and human-associated ecosystems. When sequencing the whole genomic content of a sample (shotgun metagenome sequencing) we aim to obtain a complete picture of the microbial diversity in a specific environment. However, despite the richness of the sequencing dataset, current computational tools are limiting the resolution of the analysis to the species level. To increase the resolution up to strain level and enable metagenomic-based epidemiological studies, we developed a novel Pangenome-based Phylogenomic Analysis (PanPhlAn) approach. Our assembly-free tool detects the presence or absence of each gene of the entire gene set of a species (pangenome) compiled using sequenced reference genomes, thereby capturing the individual gene set of the specific strain of the species of interest present in the sequenced microbiome. This enables both the identification of known organisms and the characterization of new strains of unknown gene composition. When metagenomic and meta-transcriptomic datasets are both available for the same specimen, PanPhlAn also provides gene-specific transcription rates of individual strains in a sample, thus exposing the “in-vivo” transcription activity not available with culture-dependent approaches. We validated PanPhlAn on several synthetic metagenomes obtaining very accurate strain reconstructions and applied it on 4 large metagenomic cohorts (~10 Tb) showing the potentialities of the approach.
A cell-based high-throughput screen addressing 3'UTR-dependent regulation of the MYCN gene.

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Keywords: neuroblastoma, MYCN, screening, post-transcriptional control, luciferase

Both transcriptional and post-transcriptional regulation has a profound impact on genes expression. However, commonly adopted screening assays focus on transcriptional regulation, being essentially aimed at the identification of promoter-targeting molecules. As a result, post-transcriptional mechanisms are largely uncovered. Here we describe the development and validation of a cell-based assay aimed to investigate the role of a mRNA 3' untranslated region (3'UTR) in the modulation of the fate of its mRNA, and to identify compounds able to affect it. Neuroblastoma, the most common extracranial solid tumor of infancy, was used as a biological model and the MYCN oncogene, whose amplification strongly predicts adverse outcome of neuroblastoma, as a target gene.

Luciferase reporter constructs with the MYCN 3'UTR were generated and stably integrated in the CHP134 neuroblastoma cell line. After validation, the developed cell-based reporter assay was used to screen a 2000 compound library including about 1000 FDA-approved drugs. Molecules affecting luciferase activity were checked for reproducibility and counter-screened for promoter effects and cytotoxic activity, resulting in selection of four upregulating molecules as truly dependent on the MYCN 3'UTR. Three of the compounds belong to the anthracycline class, while the forth one is the anti-mycotic compound ciclopirox olamine. The effect of the latter on the endogenous MYCN protein was confirmed, thus validating the approach.

We propose this cell-based reporter gene assay as a valuable tool to screen chemical libraries for compounds modulating posttranscriptional control processes. Identification of such compounds could potentially result in development of clinically relevant therapeutics for various diseases including neuroblastoma.
Supercritical CO₂ induces marked changes in membrane phospholipids composition in *Escherichia coli* K12

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Supercritical Carbon Dioxide (SC-CO₂) treatment is one of the most promising alternative techniques for pasteurization of both liquid and solid food products. The inhibitory effect of SC-CO₂ on bacterial growth has been investigated in different species, but the precise mechanism of action remains unknown. Membrane permeabilization has been proposed to be the first event in SC-CO₂-mediated inactivation. Flow cytometry, high performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) and NMR analyses were performed to investigate the effect of SC-CO₂ treatment on membrane lipid profile and membrane permeability in *Escherichia coli* K12. After 15 min of SC-CO₂ treatment at 120 bar and 35°C the majority of bacterial cells dissipated their membrane potential (95%) and lost membrane integrity, as 81% become partially-permeabilized and 18% fully-permeabilized. Membrane permeabilization was associated to a 20% decrease of bacterial biovolume and to a strong (>50%) reduction of phosphatidylglycerol (PG) membrane lipids, without altering the fatty acid composition and the degree of unsaturation of acyl chains. PGs are thought to play an important role in membrane stability, by reducing motion of phosphatidylethanolamine (PE) along the membrane bilayer, therefore promoting the formation of inter-lipid hydrogen bonds. In addition, the decrease of intracellular pH induced by SC-CO₂ likely alters the chemical properties of phospholipids and the PE/PG ratio. Biophysical effects of SC-CO₂ thus cause a strong perturbation of membrane architecture in *E. coli*, and such alterations are likely associated with its strong inactivation effect.
Understanding the nature of the Nef requirement for HIV-1 infectivity

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Nef is an HIV-1 accessory protein with a fundamental role for virus replication in vivo and for the development of AIDS. Among its several activities, Nef is essential for maintaining HIV-1 maximal infectivity. In cell culture, such activity can account for as much as 98% of the HIV-1 virion infectivity and requires Nef to be expressed in virus producing cells rather than in target cells. However, the cause of the defective infectivity of Nef-negative HIV-1 and the mechanism by which Nef is able to restore such a defect remain elusive.

We analyzed the ability of Nef to enhance HIV infectivity produced in more than 50 cell lines of different histological origin. We found that the requirement of Nef is highly variable. We identified a group of cell lines (Nef-responsive) in which the lentiviral protein is most required (10-50 fold) and a group of cell lines in which Nef is not at all or only weakly required (Nef-unresponsive, max 2-3 fold). All Nef-responsive cell lines belong to the lymphoid lineage, in line with the lymphotropic nature of HIV, while most cell lines belonging to the Nef-unresponsive group include mostly non-lymphoid cells. However, we indentified two Nef-unresponsive cell lines of lymphoid origin, which provide useful tools to investigate the nature of the Nef requirement. To study the dominance of the Nef requirement for infectivity, we have established a heterokaryon assay which allows HIV-1 production only upon fusion of producer cells expressing complementary parts of the virus. Data suggest that the requirement for infectivity is due to a restrictive cellular activity. We are now in the process of analyzing and comparing transcriptomes from cell lines with opposite Nef requirement in order to identify the cellular factor(s) counteracted by Nef.
Mapping hud interactome by cross-linking ligation and sequencing of hybrids (CLASH)

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HuD is a neuronal ELAV-like RNA-binding protein (RBP) that plays important roles in multiple regulatory processes, including pre-mRNA processing, mRNA stability, and translation, governing the fate of a substantial amount of neuronal mRNAs. Furthermore, compelling evidence indicates supplementary roles for HuD in neuronal plasticity, in particular, recovery from axonal injury, learning and memory, and multiple neurological diseases.

We applied CLASH (cross-linking ligation and sequencing of hybrids) technique to identify HuD interactome and accurately mapping of binding sites in mouse motoneuron NSC-34 cell line model. Together with the genome wide analysis of the HuD-RNA binding, CLASH allows also the identification of \textit{in vivo} RNA-RNA interactions (hybrids) located close to the HuD binding site. A preliminary bioinformatics Gene Ontology analysis showed a statistically significant enrichment of distinct terms, such as RNA binding, unfolded protein binding and others and also identified HuD binding to different classes of non-coding RNAs. The bioinformatic study referred to the recognition of the hybrids, is still ongoing as well as the identification of the binding sites.
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