BtBs day 2012

University of Milano-Bicocca

Department of Biotechnology and Biosciences

December 3rd 2012

Supported by:

Program

(Room U3-05)

09h45 - 10h00 **Opening**

-***Morning session*** *(chairmen Andrea Galimberti – Paola Sperandeo)*

10h00 - 10h50 **G. B.** *Müller (Department of Theoretical Biology, University of Vienna)***:** "Novelty in Limb Development"

10h50 - 11h15 **M. Casiraghi:** "Next Generation Sequencing Techniques for Biodiversity Investigation"

11h15 -11h40 **A. Galimberti:** "DNA-based Approaches for The Monitoring and Implementation of Biodiversity and Habitat Connectivity"

11h40 - 12h05 **G. Strona:** "Ecological Frameworks of Host-Parasite Networks"

12h05 - 13h35 **Lunch break and Poster session**

-***Afternoon session*** *(chairmen Laura Dato – Alessandra Martorana)*

13h35 - 14h00 **R. Ambrosini:** "Ecology of Glacial Environments"

14h00 - 14h25 **G. Adamo:** "Evolution of Copper Tolerance in Yeast Cells"

14h25 - 14h50 **A. Broggi:** "Migratory, and Not Lymphoid-resident, Dendritic Cells Maintain Peripheral Self-tolerance and Prevent Autoimmunity Via Induction of iTreg Cells"

14h50 - 15h05 **Coffee break**

15h05 - 15h30 **R. Rizzetto:** "Pathophysiology of the Late Sodium Current: From Cardiac to Pancreatic B Cells"

15h30 - 15h45 **M. Lecchi - S. Tavazzi:** "Biocompatible Materials for Applications in Contact Lenses"

15h45 - 16h00 **P. Galli:** "MALDIVES: NOT JUST A PARADISE. Hot Topics in Sharing with Mario Negri Institute, MIT and Politecnico di Milano"

16h00 - end **Poster prize and Closing**

Abstract

Evolution of copper tolerance in yeast cells

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In living organisms, copper (Cu) contributes to essential functions but at high concentration it may elicit toxic effects. Cu-tolerant yeast strains are of relevance both for biotechnological application and for studying physiological and molecular mechanisms involved in stress resistance. One way to obtain tolerant strains is to exploit an experimental approach called *evolutionary engineering* that uses the evolution principles to direct the selection of organisms with a desired set of phenotypes, allowing for the improvement of microbial properties and for the development of complex phenotypic traits. However, in most cases the molecular and physiological bases of the phenotypic changes produced have not yet been unraveled. In our work we described an evolutionary engineering strategy to obtain *Saccharomyces cerevisiae* cells endowed with tolerance toward high concentration of Cu ions. Natural Cu-sensitive yeast cells (*non-evolved*) were step-wise evolved through continuous cultivation in presence of increasing Cu concentrations. As a result, cells stably improved their Cu tolerance up to 2.5 g/L $CuSO_4$ and after prolonged cultivation they were able to accumulate high amounts of metal ions. Diminished cell mortality and ROS production resulted associated with Cu tolerance of *evolved* cells

We then investigated the determinants of Cu tolerance in *evolved* cells. We found that the expression level of several genes encoding proteins involved in Cu metabolism and oxidative stress response were similar in the Cu-tolerant (*evolved*) and in the Cu-sensitive (*non-evolved*) strain. The major difference detected in the two strains was the copy number of the gene *CUP1* that encodes a metallothionein. In *evolved* cells, a 7-fold amplification of *CUP1* was observed, accounting for its strongly and steadily increased expression.

Our results implicate *CUP1* in protection of the *evolved S. cerevisiae* cells against Cu toxicity. In these cells, robustness towards Cu is stably inheritable and can be reproducibly selected by controlling environmental conditions. This finding corroborates the effectiveness of laboratory evolution of whole cells as a tool to develop microbial strains for biotechnological application.

Ecology of glacial environments

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Ice and snow environments represent large ecosystems as they cover more than 13% of Earth surface. They have been studied extensively by geologists and glaciologists, but their biological and ecological investigation has lagged behind. Provided that liquid water is present, even the most extreme glacial environment hosts communities of functional organisms dominated by microorganisms and supports ecological processes. The study of the ecological processes of these extreme environments therefore requires a multi-disciplinary approach. Two years ago the authors (an ecologist, a microbiologist and a glaciologist) started working together in a multi-disciplinary team to investigate the ecological processes of glacial environments. In my contribute I will present this team and show the first results we obtained on the investigation of bacterial communities in the debris-cover of two debris-covered (black) glaciers (i.e. glaciers whose ablation area is covered mostly by debris) in the Alps. To the best of my knowledge, this is the first study that investigates biological communities of debris covered glaciers. The study of high-elevation cold environments also allows investigating controversial hypotheses on microbial biogeography, particularly the so called “Everything is everywhere, the habitat selects” (EisE) paradigm, stating that microbes, by virtue of their ability to survive long transports, typically have a worldwide distribution. Indeed, these environments are considered ideal model systems for microbial biogeographical studies, as they are isolated and ‘extreme’ ecosystems, are geographically widespread, usually contain low microbial diversity, and are linked to one another by the movements of cold dry air masses. The results we obtained provide further evidences challenging the EisE paradigm.

Migratory, and not lymphoid-resident, dendritic cells maintain peripheral self-tolerance and prevent autoimmunity via induction of iTreg cells.

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There is evidence that dendritic cells (DCs) induce peripheral tolerance. Nevertheless, it is not known whether immature DCs in general are able to tolerize CD4(+) T cells or if this is a prerogative of specialized subtypes. Here we show that, when autoantigen presentation is extended to all conventional mouse DCs, immature lymphoid tissue resident DCs are unable to induce autoantigen-specific regulatory T (iTreg) cell conversion. In contrast, this is an exclusive prerogative of steady-state migratory DCs. Because only lymph nodes host migratory DCs, iTreg cells develop and are retained solely in lymph nodes, and not in the spleen. Mechanistically, in cutaneous lymph nodes, DC-derived CCL22 contributes to the retention of iTreg cells. The importance of the local generation of iTreg cells is emphasized by their essential role in preventing autoimmunity.

Next Generation Sequencing techniques for biodiversity investigation

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The genomic approaches of last ten years have been characterised by high throughput sequencing techniques, collectively named as Next Generation Sequencing (NGS) techniques.
NGS is a heterogeneous set of methods, grouped on the common basis of the generation of an incredible number of sequences produced by each run in an impressive short range of time. NGS techniques have been used widely in genome resequencing, in metagenomics researches and in wide biological screening. Resequencing, using a genome as a reference guide, was claimed as the opportunity to reach the "1000 US dollar" genome (in the case of our species), the starting point of the "personal medicine". In metagenomics NGS allows the molecular investigation of environments in order to recover most of the physiological reactions independently by the organism hosting the biological activity. In biological screening NGS is very useful to generate wide, accurate and fast patterns of mutations, SNPs for single organisms (putatively unhealthy) for those species in which genomes have been sequenced and accurately annotated.
In spite of many useful applications, NGS techniques are not easy to be used. Pipelines are available and the technical issues can be overcome, but to recover all the data from studies involving NGS researchers skilled at least in molecular biology, ecology, statistics and general biology are needed. This is particularly true when NGS is applied to the study of biodiversity, a field in which the ZooPlantLab has been involved in recent years. In the study of biodiversity NGS is apllied not at the "whole genomic level", but only on the investigation of few markers useful for (hopefully) species discrimination. We used this approach both for eukaryotic characterization of soil biodiversity and for gut microbiomes. These two fields shared similarities, but also relevant differences. First of all the level of discrimination: bacteria are the main biomass of gut microbiomes, and the species discrimination is not reachable for technical and biological issues ("what is a species in bacteria?").
On the whole, the results are very encouraging, but caution is required for the use of NGS in this field.

Glucose deprivation induces cell death in glycolytic K-ras transformed cells by activating Unfolded Protein Response

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Several cancer cells are reported to use more glucose and to accumulate extracellular lactate even under normoxic conditions (Warburg Effect). Accordingly, glucose deprivation in these cells results in activation of cell death mechanisms that are often not observed in non-transformed ones. Since may be relevant to exploit these mechanisms to achieve new tumor therapy, in this paper we sought to identify them by using high-throughput transcriptome and proteome analysis applied to a well established glucose-addicted cellular model of transformation, namely murine NIH-3T3 fibroblasts harboring an oncogenic k-ras gene as compared to parental cells, NIH-3T3. Noteworthy, our transcriptional analysis showed that glucose deprivation modulates the expression, in both cell lines, of several genes considered hallmark of the Unfolded Protein Response (UPR). Importantly, we show that this response was strictly associated to transformed cell death, given that its attenuation, by means of cycloheximide or 4-Phenyl butyrate (4-PBA) treatments, rescued specifically Transformed cells survival. Glucose deprivation-mediated transformed cell death was also prevented by inhibition of an UPR downstream pro-apoptotic kinase, JNK. Strikingly, UPR activation and transformed cell death were completely prevented by addition of a Hexosamine Biosynthetic Pathway (HBP) substrate, namely N-Acetylglucosamine, suggesting a strict relation between the two processes. Interestingly, the human MDA-MB-231 cell line, characterized by oncogenic k-ras expression and glucose addiction, showed similar effects after UPR modulating treatments. Thus we show that glucose deprivation can induce an UPR-dependent transformed cell death mechanism, which is activated by harmful accumulation of unfolded proteins, probably as consequence of N- and O-glycosylation protein reduction. The full elucidation of this response could be relevant to design new synthetic lethal therapeutic strategy.

DNA-based approaches for the monitoring and implementation of biodiversity and habitat connectivity

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Environmental surveys are often based on the characterization of biodiversity and habitat fragmentation to evaluate the conservation status of natural or protected areas and/or to produce reliable guidelines for a correct management or restoration activities. Such studies were generally conducted directly in the field with classical approaches (i.e. population counts, vegetation plots, radiotracking, ecc…) and required the involvement of many operators with different knowledge (e.g. botanists, zoologists, ecologists, ornithologist, entomologists and so on). This condition can result in a consistent investment of time and resources, especially when rare or cryptic taxa occur. Funding problems can determine an insufficient coverage of study areas and a poor sampling of target taxa with obvious implications on the final results of the monitoring. Molecular tools such as DNA barcoding and in general, the modern use of molecular markers, represent a valid alternative to overcome these limits and permit to produce informative data at a multilevel scale (e.g. from the characterization of soil meiofauna to the investigation of gene-flow of target species among different study areas). In this context, ZooPlantLab is part of a network of different specialists, regional and national protected areas and other institutions that work on environmental monitoring and conservation. In the last years we have started several studies using DNA-based techniques to assess the degree of ecological connectivity and the levels of biodiversity at different levels and on different taxa (animals, plants, etc.). In this way we obtained useful guidelines for the conservation status of the study areas and on the efficiency of the extant ecological corridors. The integration of these results with available resources is the core for an innovative management of natural resources.

Pathophysiology of the late sodium current: from cardiac to pancreatic β cells.

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The late sodium current (I_{NaL}) has been recently highlighted as a feature shared in different pathologic conditions, ie heart failure and epilepsy. In cardiac cells it has been proposed that an increase in I_{NaL} exerts its toxic action by causing chronic Ca^{2+} overload in the cytosol. Recently it has been discovered that ranolazine, a well-known anti-angina drug and selective blocker of I_{NaL}, improves glycemic control in type II diabetic patients. Moreover, it has been demonstrated that ranolazine is able to prevent apoptosis of pancreatic β-cells and restore the physiological response to high glucose in terms of insulin secretion. We investigated the hypothesis that I_{NaL} is involved in the physiology of pancreatic β-cells and if its increase may play a role in the development of diabetes. For this purpose we tested the effect of common I_{NaL} blockers, ranolazine and TTX, on the electrical activity of a pure β-cell population, represented by the INS1-E cell line, and on β-cells derived from human islets. We used the perforated patch clamp technique in the I-clamp configuration to measure the effect of I_{NaL} modulators on electrical activity induced by the secretagogue tolbutamide (50 μM). The blockade of the sodium current by TTX abolished action potential firing, while ranolazine was able to slightly depolarize the minimum interspike potential. To simulate a potential pathologic increase of I_{NaL} we used the sodium ionophore veratridine (40 μM). As expected, veratridine strongly depolarized the membrane potential and its effect was inhibited by both TTX and RAN, suggesting that the increase of I_{NaL} may become relevant to the membrane potential homeostasis.
To assess the ionic currents underlying membrane potential changes, we used slow voltage ramps by the ruptured patch clamp technique. We found a complex pattern in the RAN and TTX sensitive currents, suggesting the presence of a K conductance sensitive to intracellular Na concentration (I_{KNa}). To assess the impact of these ionic conductances on the Ca^{2+} handling, we used the Ca-sensitive dye Fluo4-AM to monitor calcium changes by confocal microscopy. Despite of the absence of direct effects of TTX in basal conditions, we observed a large increase in intracellular Ca^{2+} induced by veratridine, which was sensitive to TTX blockade. Since RAN and TTX effects on tolbutamide-induced electrical activity were not referable to direct I_{NaL} blockade we hypothesize that in β-cells there is a balance between I_{NaL} and I_{KNa}, which may act as a protective buffer. A sudden increase of I_{NaL} may disrupt this balance and produce the calcium overload already known in cardiac myocytes, which in turn can lead to chronic dysfunction in β-cells.

Funding: Gilead Sciences Inc., Fremont (CA)
Active collaborations: Ospedale Niguarda Ca' granda (MI), Gilead Sciences Inc. (CA)

Ecological frameworks of host-parasite networks.

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Investigating how host features affect the structure of host-parasite bipartite networks may enlighten several ecological, biogeographical and (co)evolutionary issues. However, the potential of this approach can be limited by data availability, especially when researchers' efforts are directed towards understanding general patterns. Thus we created a large database of fish parasite records (the largest currently available) by merging several checklists coming from scientific literature, Internet databases and museum collections. Then we combined these data with ecological, phylogenetic and biogeographical information about fish hosts using FishBase and the Ocean Biogeographic Information System (OBIS). Finally we developed a suite of mathematical tools (Fish Parasite Ecology Software Tool – FishPEST, freely available online) that makes it possible to use these data to model the shape of host-parasite networks using different approaches, and to test the relative influence of host ecology, biogeography and phylogeny on parasite distribution. Besides having been validated using Catalogue of Life and the World Register of Marine Species (WoRMS), all the data included in FishPEST database have been externally peer reviewed. This enables researchers to confidently perform broad scale analyses by limiting the risk of introducing errors due the inaccuracies that often accompany large checklists. Here we describe the functioning and the potentialities of the above mentioned tools, reporting the outcomes of several analyses performed using FishPEST. In general, our results demonstrate that any approach aimed at estimating parasite biodiversity should take into account the fine structure of host-parasite networks.

Development of new strategies for the study of microbiomes and bacterial communities

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An estimated 1,000,000 bacterial species exist on this planet, according to the Global Biodiversity Assessment, yet fewer than 4500 have been described. Most of them, live in close association with microbial organisms and have evolved in the context of complex communities. Moreover, organism's complements of microbial inhabitants are even called "forgotten organs" and the totality of microbes, their genetic elements (genomes) and environmental interactions in a particular environment is called a microbiome.

Even if until recently the interaction between organisms and the microbial world has been defined mostly in the context of disease states, understanding differences in the composition of the microbial communities is of major importance, not only in microbial ecology, but also in the ecology of the host species and environment. It is only recently, however, that the advances in sequencing technologies allowed many microbial communities to be characterized using gene sequences amplified directly from environmental samples.

High-throughput sequencing technologies and computational techniques allow to address large-scale questions about evolution that have never before been accessible. ZooPlantLab research combines novel computational approaches with large-scale next-generation sequencing techniques to ask questions about the evolution of the composition of biomolecules, genomes and communities. Moreover ZooPlantLab research concerns the investigation of the functioning and variability of microbiome from various organisms or bacterial communities in different environments and the study of its alteration in structural composition.

In particular, ZooPlantLab focuses on the biological interpretation of the issues coming from the analysis of microbial structures and their interaction. Indeed ZooPlantLab is developing computational methods for the analysis of next-generation sequence data and the investigation of community properties from 16S rRNA gene sequences and metagenomics data, which are applied in a wide range of environments.

ScSAM2 and weak organic acid tolerance

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Weak organic acids are known to cause stress to the cell: in their undissociated form they can diffuse through the cell membrane into the cytoplasm where they dissociate releasing the proton and the corresponding anion. Differential proteomic analyses previously performed in our laboratory on plasma membrane enriched fraction (PMEF) of yeast grown in the presence or absence of lactic acid highlighted a difference in the levels of the enzyme S-Adenosylmethionine synthetase, isoform 2. S-Adenosylmethionine synthetase is the only enzyme known to catalyse the biosynthesis of S-Adenosylmethionine (SAM or AdoMet) from L-methionine and ATP. A *SAM2GFP* strain was created by substituting the *SAM2* endogenous ORF with the *SAM2GFP* construct to evaluate the Sam2p levels and its localization (still unknown) in the absence or presence of different concentrations of lactic acid. The exposure to lactic acid correlates with an increase in the Sam2p levels, evaluated by western blot, while no relevant differences were found in the localization in the conditions analyzed. In particular, the signal of Sam2GFP appears diffused through the whole cell. Considering the increase in Sam2p levels in presence of lactic acid, the effect of PERSISTENT and PULSED STRESS caused by lactic acid on wild type, *ΔSAM2* and *SAM2* overexpressing strains was evaluated.
*SAM*2 deletion determines a considerable increase in lactic acid tolerance in different *S. cerevisiae* laboratory strains. Moreover, *SAM*2 deletion increases lactic acid production in an industrial strain engineered for said production. The obtained data together with future perspectives are presented and discussed.

The localization of active Ras is regulated by cAMP-dependent protein kinase A (PKA) and stress in the yeast *Saccharomyces cerevisiae*

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In the yeast *Saccharomyces cerevisiae* the Ras/cAMP/PKA pathway plays an important role in the control of metabolism, stress resistance and proliferation. PKA, a central component of this pathway, is a tetramer consisting of two regulatory subunits (encoded by *BCY1*) and two catalytic subunits (encoded by *TPK1*, *TPK2* and *TPK3*). PKA activity depends on cAMP synthesized by adenylate cyclase (encoded by *CYR1*), which is activated by the Ras proteins and the GPCR system. Binding of cAMP to the PKA regulatory subunit results in dissociation and thereby activation of the catalytic subunits, which phosphorylate a variety of proteins. Mutants with attenuated PKA activity display hyperaccumulation of cAMP, whereas mutants with an hyperactivated PKA pathway display reduced cAMP level. Data in literature show that PKA regulates the localization of certain cAMP/PKA signaling pathway components, like the *PDE2*-encoded high-affinity cAMP phosphodiesterase, Cdc25 and the Ras2 protein. With the aim to investigate whether PKA activity plays a role in the localization of active Ras, we used a probe consisting of a eGFP fusion with a trimeric Ras Binding Domain of Raf1 (eGFP-RBD3), which binds Ras-GTP with much higher affinity than Ras-GDP. Our results show that the localization of the probe is dependent on PKA activity. In particular, in a strain with high PKA activity no active Ras is localized at the plasma membrane, while in a strain with either absent or reduced PKA activity more then 60% of the cells show a strong plasma membrane localization of active Ras. Recently it has been published that alkaline pH stress causes a transient decrease in cAMP. Our results show that alkalinization causes a delocalization of the probe almost exclusively to the plasma membrane, reinforcing the hypothesis that the localization of this GTPase is actually regulated by PKA activity. The localization of Ras-GTP in response to other stress conditions was also investigated. Our results show that H_2O_2 and especially acetic acid treatment cause a delocalization of the probe to the mitochondria. Since *S. cerevisiae* commits to a programmed cell death process in response to these compounds, the investigation of the functional involvement of Ras proteins in mitochondrial function may improve understanding of apoptosis.

A new role for protein kinase Snf1/AMPK as regulator of SBF/MBF dependent genes

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The Serine/Threonine protein kinase Snf1 of *Saccharomyces cerevisiae* is a member of the SNF1/AMPK (Sucrose Non-Fermenting 1/AMP-activated protein kinase) family, highly conserved in all eukaryotes.
In budding yeast, Snf1 is required for adaptation to glucose limitation and for growth on non-fermentable carbon sources. In those conditions Snf1 controls the expression of 400 genes regulating different transcription factors or influencing chromatin remodeling.
Apart from carbon metabolism, Snf1 regulates response to different environmental stresses (osmotic and alkaline stresses) and controls various cellular processes such as sporulation, aging, filamentous and invasive growth.
Our group previously proposed that Snf1 positively regulates yeast cell cycle progression by promoting the expression of *CLB5* mRNA. We also demonstrated the existence of an interaction between Snf1 and Swi6, the regulatory subunit of the transcriptional complexes SBF (Swi4-Swi6) and MBF (Mbp1-Swi6), which promote the expression of G1-specific genes.
Here we focus on the role of Snf1 as a transcriptional regulator. In particular we define the function of Snf1 and of its kinase activity for the expression of SBF- and MBF-dependent genes.

Dma2 ubiquitin ligase regulates cytokinesis in *S. cerevisiae*

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Cytokinesis is the last step of the cell cycle that leads to the physical separation of the mother from the daughter cell. The budding yeast *S. cerevisiae* divides aymmetrically, with each division cycle producing two cells of distinct sizes and fates. The newly born daughter cell (the bud) is approximately two-thirds of its mother in size. Cytokinesis can be viewed as an integrative process of several spatiotemporally coordinated events: (1) division site specification; (2) actomyosin ring (AMR) assembly, contraction, and disassembly; (3) targeted membrane deposition, primary septum (PS) and secondary septum (SS) formation; and (4) cell cycle control of the division machinery. While the mechanism for specifying the division site in different organisms varies widely, the core components and mechanisms involved in other aspects of cytokinesis are largely conserved. In *S. cerevisiae* efficent cytokinesis depends on the interplay between the AMR and the PS formation and dynamics. Problems in the AMR formation and/or contraction often cause an asymmetric PS deposition suggesting that AMR may guide PS formation. Conversely, problems in the PS deposition cause an asymmetric AMR contraction suggesting that PS deposition may stabilize the AMR during its contraction. Thus, the AMR and the PS appear to be functionally interdependent during cytokinesis. While the mechanism of deposition and regulation of the PS are enough know, the regulation of the AMR contraction need to be elucidated. It is known that the positive signal for AMR contraction is provided by the interaction between two proteins localized at the division site: Iqg1 and Tem1. Notwithstanding this, the regulation of this interaction is still unknown.

The budding yeast Dma2 is an ubiquitin ligase functional redundant to Dma1 and both belong to the same FHA-RING ubiquitin ligase family. Currently it has been demostrated that these two proteins appear to control different aspects of the mitotic cell cycle: the positioning of the mitotic spindle and the checkpoint that controls this process (SPOC) (Fraschini et al., 2004), the septins dynamics (Merlini et al., 2012), and also the cell morphogenesis and nuclear division in response to DNA replication stress (Raspelli et al., 2011). Moreover, Dma1 and Dma2 ubiquitin ligase activity *in vitro* has been recently described (Loring et al.,2008), but their molecular targets *in vivo* are still unknown. Our data suggest a role of Dma 1 and Dma2 in the regulation of cytokinesis, in particular they may act as a negative regulators of both actomyosin ring contraction and primary septum formation. In fact, Dma2 overproduction inhibits actomyosin ring contraction and impairs the interaction between the Tem1 and Iqg1 proteins, which is required for this event. In addition, it causes asymmetric primary septum deposition and mislocalization of the Cyk3 positive regulator of this process. We have also discovered that unperturbed cycling cells undergo Tem1 ubiquitylation that increases in response to Dma2 overproduction. Interestingly, ubiquitylated Tem1 levels fluctuate during cell cycle and decrease concomitantly with actomyosin ring contraction.

Fluorescent ligands for Lipolysaccharide receptors

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Mammalians have an extremely efficient endotoxin sensing system capable of a fast and effective response to very small amounts of Gram-negative lipopolysaccharide or to portions of its structure (e.g. Lipid A). This extreme sensibility is due to the signal cascade that involves the coordinated and sequential action of at least four proteins: LPS binding protein (LBP), CD14 (both in the soluble and GPI-anchored form), Toll-Like Receptor 4 (TLR4) and MD-2 (both soluble and TLR4-associated). The activated complex $(TLR4{\cdot}MD\text{-}2{\cdot}LPS)_2$ triggers an intracellular signal cascade that leads to the production of pro-inflammatory cytokines and immunomodulators that induce an adaptive immune response.
It has been recently discovered that TLR4 and its pathway are involved in the response to cellular damages and neurological pathologies (e.g. neuropathic pain and neuroinflammation).
With the aim of studying more in detail the action mechanisms of these receptors, our research group developed and tested several synthetic small-molecules that modulate the activity of endotoxin receptors, that besides basic research could also become useful to develop a treatment for hyper-inflammatory syndromes (e.g. acute sepsis or septic shock) or immunostimulants (e.g. adjuvants for vaccines).
Imaging using fluorescently labelled TLR4 and CD14 could be useful in order to clarify the molecular aspects of a wide array of infective and neurological diseases. The most common techniques that have been used for labelling TLR4 and CD14 in cells involve the fusion with fluorescent proteins (like GFP) or the use of fluorescently labelled anti-CD14 and anti-TLR4 monoclonal antibodies, but these approaches have both some drawbacks, such as altering the localization/function of the target protein or cross-linking/oligomerization of their target. These limitations can be override by small-molecule fluorescent tags that will label proteins *in vivo* with high affinity and selectivity.
In our laboratory we synthesized several fluorescein-labelled glycolipidic probes and tested them on innate immunity cells. These molecules derive from a compound developed in our laboratory that proved to selectively bind CD14 both *in vitro* and *in vivo*. Their interaction with cells was investigated by confocal microscopy and flow cytometry. These molecules proved to be active in inhibiting the inflammatory action of *E. coli* LPS in a dose-dependent manner, and their molecular target is under investigation. We also prepared a fluorescein-labelled lipooligosaccharide, obtaining significantly higher fluorescein:LPS ratios (1:5 or higher) than the commercially available product (1:20).

CD14 and NFAT mediate lipopolysaccharide-induced skin edema formation in mice

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Inflammation is a multistep process triggered when innate immune cells - for example, DCs - sense a pathogen or injured cell or tissue. Edema formation is one of the first steps in the inflammatory response; it is fundamental for the local accumulation of inflammatory mediators. Injection of LPS into the skin provides a model for studying the mechanisms of inflammation and edema formation. While it is known that innate immune recognition of LPS leads to activation of numerous transcriptional activators, including nuclear factor of activated T cells (NFAT) isoforms, the molecular pathways that lead to edema formation have not been determined. As PGE2 regulates many proinflammatory processes, including swelling and pain, and it is induced by LPS, we hypothesized that PGE2 mediates the local generation of edema following LPS exposure. Here, we show that tissue-resident DCs are the main source of PGE2 and the main controllers of tissue edema formation in a mouse model of LPS-induced inflammation. LPS exposure induced expression of microsomal PGE synthase-1 (mPGES-1), a key enzyme in PGE2 biosynthesis. mPGES-1 activation, PGE2 production, and edema formation required CD14 (a component of the LPS receptor) and NFAT. Therefore, tissue edema formation induced by LPS is DC and CD14/NFAT dependent. Moreover, DCs can regulate free antigen arrival at the draining lymph nodes by controlling edema formation and interstitial fluid pressure in the presence of LPS. We therefore suggest that the CD14/NFAT/mPGES-1 pathway represents a possible target for antiinflammatory therapies.

Genome mining of *rhodococcus opacus* R7 provides insights into a catabolic powerhouse

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Naphthalene, methylbenzenes and *n*-alkanes are examples of common hydrocarbons used in industrial processes and are thus widespread environmental contaminants. This has led to extent studies on the metabolism of these compounds either in Gram-negative bacteria and in Gram-positive bacteria that has not been investigated to the same extent. In this prospective the *Rhodococcus opacus* R7 strain was characterized for its ability to degrade naphthalene, *o*-xylene and some *n*-alkanes. The metabolic pathway for naphthalene, *o*-xylene degradation has been previously described in R7 strain. We focused our research on the isolation and identification of genes involved in these catabolic pathways. By PCR using degenerated primers a 2.0 kb fragment from genomic DNA of *R. opacus* R7 was amplified. Sequence analysis of the region allowed to identify two genes encoding for the two components of a naphthalene dioxygenase. From these sequences we designed primers to perform inverse PCR identifying two *gene clusters* containing genes involved in naphthalene degradation and, on another region, genes involved in salycilate catabolism. The presence of an activation system for salycilate degradation by CoAligase suggested that R7 strain could degrade this compound separately from the naphthalene pathway. Moreover, the presence of *orf7* gene encoding for a monooxygenase homologous to the one of *R. opacus* TKN14 strain involved in the *o*-xylene degradation, indicated that also in R7 it can be used for the same catabolism. Reverse Transcription PCR experiments, after RNA extraction from culture grown in different cultural conditions, were performed. These results indicated that naphthalene is the only substrate able to induce the transcription of all the found genes.
Concerning *n*-alkanes metabolism, a chromosomal region which includes the *alk gene cluster* encoding for a non-heme di-iron monooxygenase, two rubredoxins and one rubredoxin reductase, was identified from the R7 genome. Moreover, the activity of the *alkB* gene promoter was examinated in the presence of some *n*-alkanes along with the intermediates of the putative *n*-alkanes degradation pathway. Cloning of the different *gene clusters* were performed by *E. coli-Rhodococcus* shuttle vector and expression studies are in progress.

Anti-inflammatory and antinociceptive efficacy of palmitoylethanolamide in a rat model of osteoarthritis

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Osteoarthritis (OA), the most common form of arthritis, is characterized by extensive remodelling of subchondral bone and permanent destruction of articular cartilage leading to joint pain. The most commonly used drugs are non-steroidal anti-inflammatory drugs (NSAIDs). However, their prolonged use induces serious side effects. For this reason the identification of alternative drugs is crucial for the OA pathology. For this purpose, animal models are useful to effectively mimic the human pathology, such as the model obtained by a single intra-articular injection of sodium mono-iodoacetate (MIA) (2mg/25µl) in the intra-patellar ligament of the knee of male Wistar rats. Local injection of MIA, an inhibitor of glycolysis, disrupts chondrocytes metabolism and produces cartilage degeneration. The aim of this study was to investigate, in such an animal model, the anti-inflammatory and antinociceptive efficacy of palmitoylethanolamide (PEA), an endogenous lipid analogous of the endocannabinoid anandamide, which displays, when exogenously administered, anti-inflammatory and antinociceptive efficacy, as demonstrated by our research group to in mouse model of neuropathy associated with chronic constriction injury of the sciatic nerve (Costa et al., Pain, 2008). As expected, MIA-treated rats developed knee swelling, mechanical allodynia, thermal hyperalgesia, motor impairment (calculated as sciatic functional index) and a significant cartilage erosion, compared with non-OA rats. The oral administration of PEA 50 mg/kg once a day for 15 days reduced such symptoms and slowed the degradation of cartilage. PEA efficacy was superimposable and in some cases greater than that evoked by 10 mg/kg nimesulide, one of the most employed NSAIDs for OA treatment, so suggesting a therapeutic use of PEA in clinic. Furthermore, PEA repeated treatment didn't evoke adverse effects while the repeated treatment with nimesulide, led to the development of duodenal ulcers, one of the major adverse effects limiting NSAIDs use in humans. Since OA patients showed elevated levels of pro-inflammatory and pro-algogen mediators such as TNF-α and NGF in their synovial fluid, and since PEA is able to inhibit *in vitro* mast cell degranulation so reducing TNF-α and NGF release, experiments were performed to determinate the levels of such markers, in order to postulate PEA mechanism of action. As expected, NGF and TNF-α levels were increased in the synovial fluid of OA rats. A significant reduction in NGF levels was detected in the synovial fluid of animals treated with PEA 50 mg/kg, while the same PEA treatment did not affect TNF-α increase. Further experiments will be performed in order to measure this mediator at an early stage of the disease, characterized by a greater inflammatory component. Basing on these data, an hypothesis of the mechanism of action of PEA can be formulated. Locally, PEA could interact with cannabinoid-like receptors expressed by mast cells by inhibiting their degranulation and the release of TNF-α and NGF, so protecting from the cartilage damage. Concomitantly, at spinal cord level, PEA could interact with cannabinoid-like receptors expressed by microglial cells thus inhibiting the release of cytokines and the recruitment of inflammatory cells. In view of these results we can propose that the efficacy of PEA in OA should be further characterize since it could represent a viable alternative for the osteoarthritis treatment than the classical NSAIDs.

SGLT1-mediated anti-inflammatory and protective effect of dansyl-glycoderivatives. Synthesis, biological evaluation and studies on the mechanism of action

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SGLT1 (Sodium Glucose Co-Transporter 1) is a transport protein mainly expressed on the surface of intestinal epithelial cells (IECs), which constitute the so called "brush border membrane", which is devoted to the absorption of nutrients in the intestine. SGLT1 acts as a glucose/galactose co-transporter, but recently a new immunological role, of a significance similar to its physiological role, has been associated to this protein. Several works[1-3] outline a protective effect of SGLT1 with high D-glucose doses on damages induced by TLRs ligand in IECs, both *in vitro* and *in vivo* models of septic shock and inflammation, liver injury induced by LPS or acetaminophen overdose[4]. Recently, in order to overcome the drawbacks associated to the high concentration of glucose requested to obtain the protective effect, a C-glucoside, able to block the inflammatory response at pharmacological concentration, has been synthesized[5]. Experimental data indicate an involvement of SGLT1 for the protective role with this compound. At present, our work is focused on the synthesis and biological evaluation of the C-glycoside analogues, in order to develop structure-activity relationship studies. Furthermore, we are setting up NMR studies in order to achieve an epitop map of our biological active molecules, in order to understand the nature of the interaction of these glycosides with SGLT1. For the same purpose, radioactivity, mass-imaging and fluorescence experiments are in progress, to elucidate the interaction of our compounds with the SGLT1 target. Moreover, we are studying the protective role exerted by the C-glycoside on *in vitro* and *in vivo* models of Doxorubicin-induced intestinal mucositis, Ovalbumin-induced allergic asthma and colangiopathies, since SGLT1 is expressed also in pulmonary tissue and cholangiocytes.

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The chromatin-remodelling factor Brahma modulates the choice of alternative terminal exons

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The human protein Brahma (BRM) is one of the two ATP-ase subunits of the mammalian SWI/SNF chromatin-remodelling complex. BRM-containing SWI/SNF complexes are enriched in neurons, where they regulate the expression of genes involved in differentiation. Moreover, it has been reported that BRM regulates the alternative splicing (AS) of internal exons. While investigating with splicing-sensitive microarrays the gene expression changes triggered by mitochondrial stress, we found that BRM is strongly downregulated in human neuroblastoma cells expressing the SOD1(G93A) protein, one of the genetic causes of Amyotrophic Lateral Sclerosis. SOD1(G93A) expression also induces changes in the AS of genes involved in axon growth and guidance. Based on these observations and using the SOD1(G93A) cells as a model, we investigated the link between BRM and the AS of these genes. We identified several genes that are regulated by alternative terminal exon usage in a BRM-dependent manner. In five out of six genes that were analyzed, we found that the overexpression of Brm promotes skipping of their proximal terminal exons, while knock-down of Brm promotes their inclusion. By ChIP and Re-ChIP experiments, we monitored the distribution of several proteins along the constitutive and alternative exons of *RPRD1A*, one of the "BRM-responders" genes. When BRM is expressed, it localizes on the preferentially skipped proximal last exon of *RPRD1A*, concomitantly with the cleavage stimulating factor (CstF) complex and BARD1, a protein involved in the repression of pre-mRNA 3' end processing. These localizations are abrogated in the BRM-depleted cells, in which we observed, in the same genomic region, an accumulation of the "slow processive" RNA Polymerase II (pSer5), which has been linked to exon inclusion. Our data suggest a novel mechanism by which BRM negatively regulates the AS of proximal terminal exons, possibly by recruiting a complex which inhibits 3' end processing of the pre-mRNA.

DNA barcoding as an applicative tool for science and society

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DNA barcoding is a molecular and bioinformatic tool that aims primary to identify biological species through the study of the variability in a single (or few) standard molecular marker(s). In the last ten years this approach gained a role of primary importance in different fields of biology, becoming a popular and widespread tool in the world of modern taxonomy. Since its foundation, ZooPlantLab applied DNA barcoding to discriminate a wide range of organisms, especially plants and animals. Parallel to theoretical and taxonomical studies we went further, starting to use DNA barcoding in different contexts with an environmental and social impact. Such a technique revealed to be extremely useful in the monitoring of biodiversity at both single organisms and environmental matrices level, allowing to detect the occurrence of rare and/or cryptic taxa that can be adopted as bioindicators. Thanks to its rapidity ,accuracy and versatility (if a proper reference barcode dataset is available), we use DNA barcoding as a diagnostic tool for the rapid detection of ingested poisonous plants or to reveal the presence of parasites of both medical and veterinary interest. We also used this approach on different case studies with a relevant social impact such as food traceability, forensic analyses on illegally traded animals and to achieve a pharmacognostic identification of some last-generation “smart drugs”, present on the market as room scents.

Metallothionein: small protein for a big effect?

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Metallothioneins (MTs) are a class of small, cysteine-rich metal-binding proteins that are present in both prokaryotic and eukaryotic organisms. A unique property of this class of proteins is their inducibility in response to the treatment of cells with appropriate metals. MT gene transcription is induced by a wide range of metal ions as well as chemicals that generate oxidative stress (1, 2). Furthermore MTs role in the scavenging of Reactive Oxygen Species (ROS) generated during an oxidative stress phenomenon is reported.
The large diversity of inducing factors for MTs biosynthesis implicates these proteins in a variety of cellular functions, from metal tolerance and homeostasis, due to their ability to bind metal ions through the thiol groups of their cysteine residues, to protective role against apoptosis (3).
At the moment, the interest in obtaining robust cell factories for different production processes has enormous relevance in scientific community. Because oxidative stress can result not only from pollutants, but also from chemical or physical stressors (such as some alcohol and organic acid) (4), we were interested in investigating MTs defensive role against cell damage caused by the different stressors acting during an industrial process of production.
One of the major limiting steps for high production and productivity for biofuel production, no matter if ethanol, butanol or other biofuel compounds are produced, is the tolerance of the cell factory to the final product.
Can we use the MTs versatility to increase robustness during alcohol production in yeast?

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Study of defense response in plants, homology or analogy with animals?

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In recent years, studies about the molecular principles involved in the defense response to diseases in plants have revealed that the mechanisms implicated in the process of defense to pathogens are very similar to those used by mammals and insects in their innate immune response. Therefore we could assert that the immune response in animals and in plants has a common origin. In many cases, the plant response is initiated by a "gene for gene" interaction that involves a dominant R gene in plants and a corresponding avirulence (avr) gene in the pathogen. However, in addition to the R gene mediated and highly specific mechanisms, plants have acquired the ability to recognize basal and more general defense elicitors. In fact the plants, such as animals and humans, have evolved specific receptors, localized on the cell surface, able to recognize pathogenic microorganisms, part of them or molecules produced by the pathogens themselves. These elicitors are indicated with the abbreviation PAMPs (pathogen-associated molecular patters) and include lipopolysaccharides (LPS) from gram-negative bacteria, bacterial flagellin, lipoteichoic acid from gram-positive bacteria, peptidoglycans and bacterial nucleic acids. The defense system mediated by the PAMP perception in plants is similar to the innate immunity in animals. Discovery of FLS2 protein confirmed the hypothesis about the existence of cell membrane receptors, able to recognize and bind molecules released or present on pathogenic microorganisms in plant. In this project we are interested to understand the mechanisms involved in the interaction between plants and gram-negative bacteria and to define which of these mechanisms have been conserved during evolution, showing similarity to those of animals. It is known that LPS triggers an immune response in animals and in plants such as *Arabidopsis thaliana* and *Nicotiana tabacum*. The main purpose of our project is to identify and isolate the cell membrane receptor in plant: "TLR4 (Toll-like receptor) is the receptor of our interest, known in insects and mammals". Our study is conducted on *Nicotiana tabacum* suspension culture (BY-2) and protoplasts in presence of *E. coli* LPS. Confocal fluorescence microscopy analysis shows a binding of LPS molecules at the outer surface of tobacco cells (wall and membrane). Moreover in BY-2 cells a rapid process of endocytosis has been observed, through which the LPS molecules are conveyed into cell cytoplasm, probably mediated by a protein membrane receptor. These observations were confirmed by FACS analysis, in order to obtain an estimate of cell population.
Finally the perception of lipopolysaccharides (LPS) by plant cells can lead to nitric oxide (NO) production and defense gene induction. The NO release and the PR1 expression (gene for pathogenesis-related protein) confirm the pathogenicity of the LPS of *E. coli* in tobacco cells (BY-2).

Detoxification of ROS caused by acetic acid stress occurs differently in wild type and ascorbic acid producing yeast cells

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Biotechnological processes are of increasing significance for industrial production of fine and bulk chemicals, including biofuels. Unfortunately, under operative conditions microorganisms meet multiple stresses such as non optimal pH, temperature, oxygenation and osmotic stress. Moreover, they have to face inhibitory compounds released during the pre-treatment of lignocellulosic biomasses, which constitute the preferential substrate for second generation production processes. Inhibitors of yeast performance in lignocellulosic hydrolysates include weak organic acids, furan derivatives and phenolic compounds: their presence impairs cellular metabolism and growth and, as a consequence, reduces the productivity of the process.
Therefore, the development of robust cell factories with improved production rates and resistance is of crucial importance.
Yeast strains engineered to endogenously produce vitamin C exhibit a strong robustness and increased tolerance compared to the parental strains when exposed to acetic acid at moderately-toxic concentration, suggesting that ascorbic acid can counteract the toxicity caused by the organic acid. Acetic acid, which is one of the most toxic compounds commonly deriving from biomass pre-treatments, may lead to oxidative stress in yeast cells, resulting in reactive oxygen species (ROS) accumulation.
In this work we focus our attention on the effect of intracellular ascorbic acid production on the generation of ROS and on the activation of enzymes that act directly as ROS detoxifiers, such as superoxide dismutase and catalase, during acetic acid stress. Results are shown and discussed, together with future perspectives.

Study of the role of nfat signaling in innate immune during chronic inflammatory diseases

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Innate immunity is the most ancient form of response to pathogens and it relies on evolutionary conserved signaling pathways. Increasing evidence suggests that factors that have appeared more recently in evolution, such as the Nuclear Factor of Activated T cell transcription factor family (NFATc), also contribute to innate immune-response regulation in vertebrates. Exposure to inflammatory stimuli induces the activation of NFATc factors in innate immune cells including conventional dendritic cells (DCs). NFAT has pro- and anti-inflammatory action but the study of the role of the NFAT signaling pathway in vivo presents several difficulties due to the redundancy of the system. The generation of new tools allowing the inhibition of all NFAT isoforms will be necessary for the comprehension of the role of this signaling pathway in this most ancient arm of the immune system. Cyclosporin A (CsA) and FK506 are the most commonly used drug in the treatment of acute transplant rejection and some chronic inflammatory diseases to block IL-2 and other NFATc-dependent cytokine production. Although highly successful, CsA and FK506 have severe side effects due to their general inhibition of the enzymatic activity of Cn, which plays other physiological roles besides NFAT activation, and they are not specific for DCs. Herein we describe a new NFAT inhibitor specific for innate myeloid cells that can be used for treatment of rheumatoid arthritis and acute transplant rejection.

Evidence of human sialidase Neu3 involvement in colorectal carcinogenesis

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Human sialidase NEU3, an enzyme of the glycosidase family, catalyzes the hydrolytic cleavage of nonreducing terminal sialic acid residues linked to glycoconjugates oligosaccharidic chains, regulating the lipid bilayer sialic acid content and the modulation of gangliosides content. It was shown that HsNEU3 is involved in colorectal cancer (CRC).
NEU3 seemed to inhibit apoptosis, but also to activate the epidermal growth factor receptor (EGFR) pathway; in literature, a correlation was established in HeLa cells between NEU3 overexpression and EGFR phosphorylated protein expression, whereas silencing resulted in the opposite. EGFR activation affected KRAS and MAP kinase pathway, resulting in cell differentiation and proliferation.
In collaboration with Istituto Cantonale di Patologia (Locarno), tumor tissues from CRC patients surgically resected were analyzed by Real-time PCR and a frequent correlation was observed between NEU3 and EGFR mRNA levels. These data were confirmed on human colon cancer cell lines.
We studied the effect of NEU3 transfection of different human colorectal cancer cell lines with pcDNA3.X-HsNEU3-HA construct. Protein expression was investigated by Western blot analysis. Cells transfected showed increased EGFR and MAP kinase (MEK 1/2 and ERK 1/2) phosphorylation and Akt activation. In addition, it was observed that NEU3 overexpression causes lipid phosphatase PTEN reduction that inhibits Akt, but not MAP kinase pathway.
Since NEU3 is involved in EGFR pathway, it might be considered a good molecular target for CRC patients resistant to Panitumumab and Cetuximab, drugs that target epidermal receptor and cause suppression of its activation.

Snf1/AMPK and PKA pathways physically and functionally interact in budding yeast

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Snf1 is a serine/threonine kinase required by the yeast *Saccharomyces cerevisiae* to adapt to nutrient limitation and to utilize alternative carbon sources, such as sucrose and ethanol. Activation of Snf1 upon nutrient limitation determines its translocation to the nucleus and the phosphorylation of the transcription inhibitor Mig1, thus causing the expression of over 400 genes. The cAMP-dependent protein kinase A (PKA) is a serine/threonine kinase which controls a great number of cellular processes, such as fermentative growth, nutrient sensing and stress responses. PKA controls the expression of a large subset of genes. For example, it negatively regulates the expression of the STRE genes through the inhibition of the nuclear import of the transcription activators Msn2 and Msn4 and inhibits the transcription activator Adr1, which controls the expression of glucose-repressed genes.

Several elements of crosstalk between the Snf1 and PKA pathways have been reported. PKA negatively regulates the recruitment of Snf1-Sip1 to the vacuole and phosphorylates the upstream activating kinase Sak1 influencing the activation of Snf1. The two pathways also functionally interact on downstream targets controlled by both pathways, such as the transcription activators Msn2 and Msn4 and Adr1. Moreover it has been reported that in mammalian cells PKA directly phosphorylates and inactivates the Snf1 horthologue AMPK.

Here we present the identification of the physical interaction between Snf1 and adenylate cyclase (Cyr1), a key regulator of the PKA pathway. This interaction is stable in cells growing with different glucose concentrations, while appears to be regulated after glucose depletion.

To investigate the functional interaction between the two pathways we validated the PKA-dependence of the expression of *HXT1*, *HXT7* and *ATR1* genes upon glucose depletion and repletion. Then, we assessed the expression of these genes upon glucose depletion in the presence of mutants of Snf1 such as the catalytic deficient Snf1-K84R and Snf1-G53R, which shows a constitutive activation. We found that the Snf1-G53R mutant shows a strong de-repression of *HXT7*, a PKA-repressed gene, in cells growing in 2% glucose.

These data confirm the functional interaction between the Snf1 and PKA pathways and suggest that the crosstalk between the two pathways could exist also on upstream elements such as adenylate cyclase.

Monitoring fatty acid accumulation by FTIR in oleaginous yeasts under nutrient limitation

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Microbial lipids can represent a valuable alternative feedstock for biodiesel production and a potential solution for a biobased economy.
In order to find an alternative way to produce biodiesel without competing with food crops, microbes could be the answer. Microbial oils, also called *single cell oils*, are produced by some oleaginous microorganism such as yeast, fungi, bacteria and microalgae. Some of these microbes, such as *Rhodosporidium toruloides*, *Cryptococcus curvatus* or *Rhodotorula glutinis*, can accumulate intracellular lipids as high as 80% of their biomass dry weight. The majority of those lipids are triacylglycerol (TAG) containing long-chain fatty acids that are comparable to conventional vegetable oils. Remarkably, some of those oleaginous species show the ability to metabolize pentoses, demonstrating the potential to produce TAG from lignocellulosic biomasses and other cheap materials.
To estimate and optimize the industrial process of TAG production, we tried to find an easy, fast and reliable protocol for monitoring the fatty acids accumulation, using FTIR (Fourier Transform InfraRed) technique. The FTIR method involves passing infrared radiation through a sample and measuring the amount of radiation transmitted or reflected by the sample. This spectrum is unique to the molecular structure of a material, making this technique convenient both for its speed and sensitivity.
Three different species of oleaginous yeast (*R.toruloides*, *C.curvatus* and *R.glutinis*) and a *S. cerevisiae* strain (used as a control) have been cultivated in a batch process under nitrogen limitation, monitoring growth and lipid accumulation, both via FTIR and fluorescence microscopy, during time.
Our results showed a correlation between lipid accumulation and FITR measurements. All the spectra obtained showed similar bands, typical of lipids but with consistent differences between the three oleaginous species, while the *S. cerevisiae* used as control showed different bands, especially in wavelength typical of TAG. Fluorescence microscopy confirmed this trend, showing the presence and accumulation of typical oil droplets inside the cells of oleaginous yeasts.
Current data and future necessary experiments will be presented and discussed, to demonstrate the reliability of this faster and simpler tool for future studies of these cell factories.

Search for new targets of the protein kinase Swe1 in spindle elongation in *S. cerevisiae*

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Swe1 is the effector kinase of the morphogenesis checkpoint that, in budding yeast, provides a link between cell morphology and entry into mitosis. Although there are some differences due to the particular kind of cell division established by the budding, Swe1 functions and regulators are evolutionarily conserved, indicating that this is an ancient cell cycle control strategy that has been adapted to respond to cytoskeletal signals in vertebrate as well as in *S. cerevisiae* cells. Swe1 blocks entry into mitosis through inhibitory phosphorylation of the catalytic subunit of the cyclin-dependent kinase Cdk1, Cdc28; Cdc28 activity is required both for entry into mitosis and for the switch from polar to isotropic bud growth, so when Cdc28 is phosphorylated on Tyr19 (Y19) both these events are inhibited. Swe1 stabilization prevents mitotic entry in response to different problems, such as abnormal cell morphology, actin cytoskeleton or septins perturbations. In addition, elevated Swe1 levels inhibits mitotic spindle formation and elongation and several data indicate that, apart Cdk1, other Swe1 targets are likely involved in this process. In fact, the introduction of Cdc28 alleles that could escape from Swe1 inhibition is not sufficient to restore proper spindle elongation nor progression through mitosis of cells that overexpress Swe1. We tried to identify new Swe1 targets acting in mitotic spindle dynamics and progression through mitosis by analyzing putative candidates among factors known to be involved in these processes and by performing a genetic screen. The most recent results of this research will be presented.

Identification of a novel glucose-responsive calcium transporter

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Calcium is one of the most important second messengers in eukaryotic cells, where it plays essential roles in the regulation of several cellular functions, such as nutrients sensing and the response to many cellular stresses in yeast cells and the response to many growth factors in mammalian cells. Many extracellular stimuli, such as pheromone exposure, glucose addition to derepressed cells or osmotic shocks, cause a rapid and transient increase in cytosolic Ca^{2+} concentration in yeast cells, consisting both in an influx of Ca^{2+} from the extracellular environment and in its release from internal stores.Glucose addition to glucose-starved *Saccharomyces cerevisiae* cells triggers a quick and transient influx of calcium from the extracellular environment, which was reported to be mediated by the High Affinity Calcium System (HACS), involving Mid1/Cch1 subunits. The Low Affinity Calcium System (LACS) doesn't seem to be involved in glucose-induced Ca^{2+} signal.
A high affinity glucose-responsive calcium transport system exists in yeast, not yet identified, that can substitute the known systems when they are inactivated. This unknown channel, called GIC (for Glucose Induced Calcium) channel, is regulated by the action of Calcineurine, an eukaryotic Ca^{2+}- and calmodulin-dependent serine/threonine type 2B phosphatase (PP2B), representing the major player in Ca^{2+}-dependent signal transduction pathways. Calcineurin control does not involve the transcriptional factor Crz1, suggesting a post-translational control.

An interaction, an application LptC binds lipopolysaccharide

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Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria and it is essential for their viability. LPS is a complex glycolipid that can be structurally divided in: Lipid A, the hydrophobic moiety that anchors LPS to the outer membrane, the oligosaccharide region named core, and the O-antigen polysaccharide chain. Since LPS is highly toxic and biologically active even at a concentration as low as pg/ml, the specific and sensitive detection of endotoxin contamination is mandatory in pharmaceutical production and highly relevant in life science and medical research. Currently, enzymatic Limulus Amebocyte Lysate (LAL) assay is clinically used for the determination of endotoxin via the gel formation between LAL and endotoxin. However, the LAL assay has many drawbacks. Recently, another assay has been introduced on the market, EndoLISA®, in which LPS is detected by its intrinsic capacity to activate the zymogen form of factor C producing a fluorescence signal. This assay solves some troubles of LAL assay, but it is not indicated for human body fluids.
Our aim is to develop a simple assay for the detection of LPS in samples of different origin using the specific interaction between labeled-LPS and His-tagged LptC from *E. coli*, a protein involved in LPS transport across the periplasmic space. In particular, we investigated two different methods, one in which nanoparticles are used for the detection and one based on fluorescence.

Oxidative stress response of *Kluyveromyces marxianus* during C-5 and C-6 batch fermentations in different aeration conditions

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The yeast *Kluyveromyces marxianus* has a great biotechnological potential because of some of its traits, such as growth on a broad variety of substrates, high thermotolerance and high specific growth rates.
K. marxianus is classified as facultative fermentative and Crabtree-negative (Fonseca D.D. *et al*. 2008), despite some reports underlined significant differences which appear to be strain-dependent (Lane M.M. *et al*. 2011).
Compared with *Saccharomyces cerevisiae*, very little is known about the oxidative stress response in *K. marxianus*: no data are currently available either about reactive oxygen species (ROS) accumulation at single cell level or about the activity of specific detoxifying enzymes during a batch fermentation process.
With the aim of studying the metabolism of microorganisms that can naturally ferment pentoses, batch fermentations of *K. marxianus* (strain CBS-712) were performed on glucose and xylose containing media, under different aeration conditions (1,75% and 11% O_2). During these fermentations, samples were collected for enzymatic and cytofluorimetric analyses. More in detail, the analysis at single cell level revealed that during the fermentation process the intracellular levels of ROS are significantly high, independently from the aeration conditions. The cellular viability is also very high under any tested condition.
Further analysis including enzymatic assays will be presented and discussed in the view of a deeper understanding of the *K. marxianus* CBS-712 metabolic response during a simulated industrial process.

Natural compounds against Alzheimer's Disease: NMR investigation on the interaction between rosmarinic acid and amyloid beta peptides

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The amyloid peptides Aβ1-40 and Aβ1-42 represent one of the main molecular targets for the development of potential drugs for the treatment of Alzheimer's Disease (AD). Oligomeric and fibrillar aggregates generated by these peptides are among the principal components of amyloid plaques found *post-mortem* in patients suffering from AD [1]. Synthetic or natural compounds effective in preventing the aggregation of amyloid peptides *in vitro* and to delay the progression of the disease in animal models are already known [2]. However, little information is available about the molecular mechanism by which these compounds exert their effect. Among the molecules known for their anti-aggregating activity against Aβ peptide, but for which the mechanism of action is not known, there is rosmarinic acid [3]. To elucidate rosmarinic acid mechanism of action at molecular level, STD-NMR and trNOESY experiments were performed. They confirmed the interaction between Aβ1-42 oligomers and rosmarinic acid in physiological conditions and allowed the identification of its binding epitope. NOESY spectra revealed also some differences in rosmarinic acid conformation after the binding to Aβ peptides. Moreover, some rosmarinic acid derivatives were synthesized and tested to further clarify the role of the two aromatic rings in the interaction. [4]

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Acknowledgements
The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no.212043 (NAD) and from Regione Lombardia, Fondo per la promozionedi accordi istituzionali, Progetto no. 4779 ''Network Enabled Drug Design (NEDD)''. The authors also thank FCT-Portugal for a post-doc research grant (SFRH/BPD/65462/2009) and for the financial support of CQB Strategic Project PEst-OE/QUI/UI0612/2011. The group at Madrid thanks MINECO of Spain, (grant CTQ2012-32025), Comunidad de Madrid (MHit project), and EU (GlycoHit and Glycopharm projects, and COST actions CM1102 and BM1003).

Analysis of the action mechanism of small compounds displaying Ras inhibitory properties.

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Ras GTPases cycle between inactive GDP-bound state and active GTP-bound state to modulate a diverse array of processes involved in cellular growth control. Guanine nucleotide exchange factors (GEFs) activate Ras proteins by stimulating the exchange of GTP for GDP in a multistep mechanism which involves binary and ternary complexes between Ras, guanine nucleotide, and GEF. Recently water-soluble Ras inhibitors were synthesized and *in vivo* experiments show that the addition of compounds in the culture medium of normal and *kras*-transformed fibroblasts induces a dose-dependent decrease in proliferative potential.
The compounds inhibit in a dose-dependent manner GEF-catalyzed dissociation of the guanine nucleotide from Ras and the entrance of the new nucleotide.
The action mechanism of compounds is being analyzed in detail on both wild type and mutant Ras using both computational (docking and molecular modelling) and biochemical and biophysical data including calorimetric assays (ITC), SPR and mass spectrometry experiments.
By using a computer model describing the possible interaction of the compounds with the Ras/GEF cycle (based on a model of the Ras/GEF cycle published by Lenzen et al., 1998) we are evaluating alternative models for the action of the compounds. The choice of unknown parameters for the simulations is constrained by above described experiments. This integrated computational and experimental system-level approach should prove valuable in fine characterization of drug mechanism and in the development of novel drugs with improved selectivity and /or efficacy.

1,4-diaryl-2-azetidinones as specific anticancer agents: activation of AMPK and induction of apoptosis

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Natural compounds are often used in traditional medicine for their different and multiple therapeutic effects. Among them Combretastatins, a group of polyhydroxylated stilbenes isolated from the South African tree *Combretum caffrum*, were shown to inhibit the formation of the mitotic spindle by blocking tubulin polymeriztion, and to have anti-vascular properties *in vivo*. In particular Combretastatin A-4 (CA-4) exhibits potent anticancer activity against a panel of human cancer cells including multi-drug resistant ones. Two problems, however, have limited the use of Combretastatins as therapeutic agents for a long time: their low water-solubility and *cis*/*trans* isomerization which may occur during storage and administration, causing a dramatic loss of activity. The first problem has been overcome by water-soluble phosphate prodrug (CA-4P, Zybrestat, Fosbretabulin), which is currently under investigation in human clinical trials (phase III) as an anticancer drug. The second problem has been approached by designing a variety of conformationally restricted *cis*-locked analogues, such as β-lactam derivatives, which have low toxicity but high anticancer activity.

Here we present the preparation of a small library of 1,4-diaryl-2-azetidinones bearing a hydroxyl or an amino group in position 3 of the four-membered ring, to improve water solubility of the final products. We have found that the new 1,4-diaryl-2-azetidinones show specific antiproliferative activity against duodenal and colon cancer cells. Strong cytotoxicity was observed with the best compounds (±)-trans-20, (±)-trans-21, as well as enantiomers (+)-trans-20 and (+)-trans-21, which exhibited IC_{50} values of 3-13 nM against duodenal adenocarcinoma cells. They induced inhibition of tubulin polymerization and subsequent G2/M arrest. This effect was accompanied by activation of AMP-activated protein kinase (AMPK), activation of caspase-3 and induction of apoptosis. Additionally, the most potent compounds displayed antiproliferative activity against different colon cancer cell lines, opening the route to a new class of potential therapeutic agents against colon cancer.

Going toward the development of nanoparticles for Alzheimer's disease's diagnosis and therapy.

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Nanoparticles (NPs) are attractive tools in biomedical applications thanks to their biocompatibility, non-immunogenicity, non-toxicity, biodegradability, high physical stability, possibility of drug loading and releasing, and high surface functionalization possibilities; in particular, liposomes are being extensively explored for their potentialities in the medical field. This work deals with the synthesis of different type of nanoparticles functionalized with amyloid beta ligands (Aβ-ligands), imaging tools and/or blood brain barrier-transporters (BBB-transporters) for the therapy and the diagnosis of Alzheimer's disease (AD).
Amyloid β (Aβ) aggregates are considered possible targets in the war against AD. It has been previously shown that some small molecules target Aβ plaques and, in particular, curcumin interacts with their precursors, suggesting a potential role for the prevention of AD. Herein, a chemoselective ligation procedure was used to generate nanoliposomes decorated with new potential Aβ peptide ligands, designed to maintain all the features required for interaction with Aβ. Our approach starts from the molecular design and synthesis of functionalized phosholipid analogues monomers suitable for the assembly of new functionalized NPs. The monomers and the Abeta ligands present functional groups suitable for the chemoselective decoration of the NPs surface by covalent conjugation. In particular, the synthesized compounds show triple bond or azide group that can be reacted exploiting the chemoselective click chemistry. This work describes the preparation and characterization of novel curcumin decorated nanotools with improved affinity (KD = 1.7 nM) for Aβ peptide and ability to pass the BBB. They could be exploited as ligands and/or vectors for the targeted delivery of new diagnostic and therapeutic molecules for AD (theragnosis).
The NPs preparations and the biological results were obtained in collaboration with scientists involved in a joint European project: NAD - Nanoparticles for therapy and diagnosis of Alzheimer's Disease.

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The committee is very grateful to Marina Lotti for giving us this gratifying opportunity.

The organization of this event would not have been possible without the essential aid of Carla Smeraldi, Francesca Loreto and Anastasia Sguera.