

**Venetian Institute of Molecular Medicine**

**7<sup>th</sup> Annual Retreat**

**21 - 22 November, 2008**

**Bassano del Grappa**

**Programme and Book of Abstracts**



**Città di Bassano  
del Grappa**



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# WELCOME

Dear Friends,

As in the last annual meeting of the VIMM, three prizes will be awarded to recognize the work of three young Ph.D. students or postdocs working at our Institute.

The awards have been generously offered by Mrs Manzin, as a tribute to her father and her husband.

Welcome to Bassano del Grappa to the 7<sup>th</sup> annual VIMM retreat.

*Tullio Pozzan*

*VIMM Scientific Director*



# **PROGRAMME**



**November 21<sup>st</sup>: Friday morning**

- 07:30 Departure from Padova
- 09:30 Arrival and refreshment at Teatro Astra
- 10:00 – 10:15 Official Opening
- 10:15 – 11:00 **Lecture Rosario Rizzuto**  
*Mitochondria, calcium and cell death by apoptosis or necrosis*
- 11:00 – 11:30 **Dmitry Lim (Carafoli)**  
*Interplay of DREAM with Presenilin in neuronal Ca<sup>2+</sup> signaling: possible role in Alzheimer's disease*
- 11:30 – 12:00 **Laura Cendron (Zanotti)**  
*The intriguing transmembrane type-four secretion system of *H. pylori*: molecular and functional characterization of the CagD component*
- 12:00 – 12:30 **Elena Papinutto (Pinna)**  
*Role of structural analysis in the development of CK2 inhibitors*
- 12:30 – 14:30 Lunch

**November 21<sup>st</sup>: Friday afternoon**

- 14:30 – 15:00 **Elena Serena (Elvassore)**  
*Micro-engineered substrates for in vitro culture of human cardiac and skeletal muscle cells*
- 15:00 – 15:30 **Rosa Aiello (Battistutta)**  
*Production and characterization of different variants of the SulP transporters STAS domain*
- 15:30 – 16:00 **Fabio Anselmi (Mammano)**  
*Connexin hemichannels, gap junctions and calcium signaling in the inner ear*
- 16:00 – 17:30 Assembly of VIMM members
- 17:30 – 19:30 Poster mounting and poster session at Teatro Astra
- 20:30 Dinner & Party at the restaurant “Alla Favorita”

**November 22<sup>nd</sup>: Saturday morning**

- 09:15 – 10:00 **Lecture Marco Mongillo**  
*Defective control of cardiac Ca<sup>2+</sup> release channels in familial arrhythmias and heart failure*
- 10:00 – 10:30 **Anna Raffaello (Sandri)**  
*JunB Transcription Factor promotes muscle growth and blocks atrophy*
- 10:30 – 11:00 **Bert Blaauw (Schiaffino)**  
*Akt activation prevents the force drop induced by eccentric contractions in dystrophin-deficient skeletal muscle*
- 11:00 – 11:30 Coffee Break
- 11:30 – 12:00 **Mariangela Mancini (Pagano)**  
*The vegetable extract *Serenoa repens* activates the intrinsic apoptotic pathway in prostate cancer*
- 12:00 – 12:30 **Elena Palma (Bernardi)**  
*Rescue of myopathic collagen VI null mice by genetic inactivation of mitochondrial cyclophilin D*
- 12:30 – 13:00 **Federica Calore (Scorrano)**  
*Bax and Bak regulate endosomal hijack on mitochondria during apoptosis induced by *H. pylori* VacA*
- 13:00 – 15:00 Lunch

**November 22<sup>nd</sup>: Saturday afternoon**

- 15:00 – 15:30 **Lisa Franceschini (Alberti)**  
*Insulin resistance in chronic hepatitis C: causes and consequences*
- 15:30 – 16:00 **Gaia Codolo (de Bernard)**  
*NapA of *Borrelia burgdorferi* drives Th17 inflammation in Lyme Arthritis*
- 16:00 – 16:30 **Sabrina Manni (Semenzato)**  
*Role of Glycogen Synthase Kinase 3 in multiple myeloma*
- 16:30 – 17:00 **Barbara Molon (Bronte/Viola)**  
*RNOS-induced chemokine inactivation in cancer*
- 17:00 – 17:30 Coffee Break
- 17:30 – 17:45 “Best presentation” prize sponsored by Mrs Manzin
- 18:00 Departure





# **LECTURE ABSTRACTS**

## **Invited speakers**



**Defective control of cardiac Ca<sup>2+</sup> release channels in familial arrhythmias and heart failure**

Marco Mongillo

The ryanodine receptor 2 (RyR2) intracellular Ca<sup>2+</sup> release channel is required for excitation-contraction coupling in the heart. Mutations in RyR2 causing a congenital 'leaky' channel phenotype have been linked to inherited exercise-induced sudden cardiac death. Similarly, chronic PKA phosphorylation of the channel leading to SR Ca<sup>2+</sup> leak occurs in acquired conditions associated with sudden death as heart failure. Altered control of SR Ca<sup>2+</sup> release is thus a common finding in cardiac diseases leading to pro-arrhythmogenic conditions. Targeting RyR2 might therefore be a promising therapeutic strategy to reduce the burden of sudden cardiac death.

**Mitochondria, calcium and cell death by apoptosis or necrosis**

Rosario Rizzuto

Mitochondria rapidly accumulate Ca<sup>2+</sup> through a low-affinity uptake system (the mitochondrial Ca<sup>2+</sup> uniporter, MCU) because they are exposed to high [Ca<sup>2+</sup>] microdomains generated by the opening of ER Ca<sup>2+</sup> channels. These rapid [Ca<sup>2+</sup>] changes stimulate Ca<sup>2+</sup>-sensitive dehydrogenases of the mitochondrial matrix, and hence rapidly upregulate ATP production in stimulated cells. Ca<sup>2+</sup> also sensitizes to cell death mediators, e.g. ceramide. Accordingly, we demonstrated that Bcl-2 reduces the state of filling of ER Ca<sup>2+</sup> stores, and this alteration is effective in reducing the sensitivity to apoptotic challenges. I will here review our latest data focusing on: 1) The effect on mitochondrial Ca<sup>2+</sup> homeostasis of other signalling pathways involved in autophagy and apoptosis (Akt, FHIT). 2) The signalling route that links oxidative stress to the activation of p66shc, an isoform of a growth factor adapter acting as apoptotic inducer. PKCβ, activated by the oxidative challenge, induces p66shc phosphorylation, with ensuing alteration of mitochondrial structure and function. We also showed that this route is involved also in adipose differentiation of muscle-derived precursors, highlighting a novel process of utmost interest in pathophysiological conditions. 3) The molecular elements of the mitochondria-ER Ca<sup>2+</sup> connection. I will discuss the role of VDAC in rapidly channelling Ca<sup>2+</sup> through the outer mitochondrial membrane and the specific functions of VDAC isoforms in autophagy and apoptosis.



# ORAL PRESENTATION ABSTRACTS

(Underlined: speaker, **Bold**: Principal Investigator)



**Interplay of DREAM with Presenilin in neuronal Ca<sup>2+</sup> signaling: possible role in Alzheimer's disease**Laura Fedrizzi <sup>2,3</sup>, Dmitry Lim <sup>1,2</sup>, **Ernesto Carafoli** <sup>1,2</sup>, Marisa Brini <sup>2,3</sup><sup>1</sup> Venetian Institute of Molecular Medicine (VIMM), Padua, Italy<sup>2</sup> Dept. of Biochemistry, University of Padua, Padua, Italy<sup>3</sup> Dept. of Experimental Veterinary Sciences, University of Padua, Padua, Italy

DREAM is an EF hand protein that acts as a Ca<sup>2+</sup>-dependent transcriptional repressor of target genes. Also, DREAM interacts with Presenilin (PS). We have investigated the role of DREAM on neuronal Ca<sup>2+</sup> homeostasis. We have generated stable clones of SH-SY5Y cells overexpressing DREAM wt or a Ca<sup>2+</sup>-insensitive DREAM mutant (EFmDREAM) which represses gene transcription irreversibly. We have monitored intracellular Ca<sup>2+</sup> concentrations using aequorin and we have found that DREAM overexpression reduced the endoplasmic reticulum (ER) Ca<sup>2+</sup> levels. Recent findings indicate a correlation between the deregulation of ER Ca<sup>2+</sup> homeostasis and AD. In particular, it was shown that PS forms a Ca<sup>2+</sup> leak channel in the ER membrane. Importantly, the AD-linked PS mutations disrupt the putative PS-mediated channel. To investigate the possible role of DREAM as a modulator of the Ca<sup>2+</sup> channel function of PS we have analyzed the effects of DREAM and PS co-expression on ER Ca<sup>2+</sup> content. EFmDREAM and PS co-expression further reduced the ER Ca<sup>2+</sup> content in SH-SY5Y cells. These effects are likely to be due to the silencing of the PS gene by DREAM. The GST pull-down experiments showed direct interaction between DREAM and presenilins, supporting the suggestion of a post-transcriptional role of DREAM on Ca<sup>2+</sup> homeostasis. This could occur through a modulation of the proposed PS leak channel in a Ca<sup>2+</sup> independent manner, since the interaction is independent of the presence of Ca<sup>2+</sup>.

**The intriguing transmembrane type-four secretion system of *H. pylori*: molecular and functional characterization of the CagD component**Laura Cendron <sup>1,2</sup>, Marc Couturier <sup>3</sup>, Alessandro Angelini <sup>1,2</sup>, Nicola Barison <sup>1,2</sup>, Lorenza Sisinni <sup>1,2</sup>, Markus Stein <sup>3</sup>, **Giuseppe Zanotti** <sup>1,2</sup><sup>1</sup> Venetian Institute of Molecular Medicine (VIMM), Padua, Italy<sup>2</sup> Dept. of Chemistry, University of Padua, Padua, Italy<sup>3</sup> Dept. of Medical Microbiology and Immunology, University of Alberta, Alberta, Canada

*Helicobacter pylori*, the pathogen that infects the stomach of about half the world's population, is associated with a spectrum of gastric diseases, ranging from gastritis to peptic ulcers and gastric cancer. The most pathogenic strains of the bacterium code for a so-called "type four secretion system" (T4SS), a multi-subunit transport apparatus composed of about 30 genes. Several different techniques have been used to understand the localization and function played by each individual member of the Cag-PAI. While 10 of the Cag-PAI components show some homologies with the *A. tumefaciens* VirB/D proteins, others are unique to the *H. pylori* Cag-PAI and their roles remain elusive. In the framework of a project aimed at the structural and functional characterization of the cagPAI proteins, the crystal structures of 2 components of T4SS, CagZ and CagS, were previously determined by our group, and we present here the structure of a third one, CagD (HP0545). We show that the protein is a covalent dimer with intriguing structural similarities with the SycT chaperone of *Yersinia enterocolitica* typeIII secretion system. Our data indicate that CagD is strictly required for CagA translocation into the host epithelial cells, but it does not seem absolutely necessary for pilus assembly. Significant amounts of CagD have also been identified in the culture supernatants, but not as a result of general bacterial lysis, indicating that CagD is released into the supernatant during host cell infection and then binds to the host cell surface. Overall, our results suggest that CagD may serve as a unique multifunctional component of the T4SS which is strictly related to CagA secretion at the inner membrane and may be released to promote additional effects on the host cell.

**Role of structural analysis in the development of CK2 inhibitors**

Elena Papinutto<sup>1</sup>, Marco Mazzorana<sup>2</sup>, Giorgio Cozza<sup>3</sup>, Stefania Sarno<sup>1,3</sup>, Roberto Battistutta<sup>1,4</sup>,  
**Lorenzo A. Pinna**<sup>1,3</sup>

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<sup>4</sup> Dept. of Chemical Sciences, University of Padua, Padua, Italy

In order to be a “druggable target”, a protein must have the ability to bind small molecules with the appropriate chemical properties and sufficiently high binding affinity, while being related to a disease or to a cell dysfunction. Protein Kinases, whose deregulation is associated to various diseases, account for more than 20% of the whole “druggable genome”. CK2 is probably the most pleiotropic Ser/Thr kinase, highly conserved and ubiquitous, implicated in a number of global functions, including cell survival, prevention of apoptosis and tumorigenesis. The recent analysis of several ATP-competitive inhibitors co-crystallized with the catalytic subunit of CK2 has provided a thorough insight into the active site of this enzyme. In particular, from the mode of binding of most selective inhibitors, we can now evaluate the structural elements which are critical for their recognition by CK2. The extensive study of ATP site-directed ligands resulted in the identification of promising classes of selective inhibitors with  $K_i$  values in the sub-micromolar range. A remarkable example is provided by the anthraquinone derivative Quinalizarin, a potential ligand identified through virtual screening of a library of hydroxyl-antraquinones based on CK2 crystal structure in complex with emodin. Quinalizarin turned out to be a much more potent and selective inhibitor than emodin with respect to CK2. The crystal structure of the complex reveals a peculiar way of binding that might be unique of CK2 and account for the unusually narrow selectivity of Quinalizarin.

**Micro-engineered substrates for *in vitro* culture of human cardiac and skeletal muscle cells**

Elena Serena<sup>1,2</sup>, Elisa Cimetta<sup>1,2</sup>, Susi Zatti<sup>1,2</sup>, **Nicola Elvassore**<sup>1,2</sup>

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<sup>2</sup> Venetian Institute of Molecular Medicine (VIMM), Padua, Italy

The *in vivo* cell microenvironment has a major role in regulating cell fate. Our aim is coupling micro-engineering tools and cell biology principles for the development of innovative *in vitro* culture systems reproducing the proper *in vivo* physiological conditions. Two case studies are presented. A) Development of human functional myotubes. We used a biomimetic approach on human myoblast culture derived from both healthy and Duchenne Muscular Dystrophy biopsies (7 patients). The substrate was designed with tissue-like mechanical properties (elastic modulus,  $E$ , from 12 to 21kPa) and topological organization (micropatterning of collagen, laminin, fibronectin and matrigel in parallel lanes, 75x5000 $\mu$ m) to promote cell alignment and differentiation. The optimum resulted in  $E \approx 12$ kPa and matrigel patterning: in 7 days of culture 65% of myotubes showed sarcomeric striation of myosin heavy chain and  $\alpha$ -actinin. Myotubes showed calcium release after electrical stimulation. Healthy myotubes expressed dystrophin on membrane after 11 days. B) Array of human beating cardiomyocytes. Human embryonic stem cells (HES2) were differentiated to cardiomyocytes (hES-CM) with a specific temporal exposition to Activin, BMP4, FGF and hypoxia. Cardiomyocytes were obtained with an efficiency of 40% and troponinT showed sarcomeric organization. A cell topology of arrayed circular dots was imposed. Ongoing studies are aimed at evaluating the behavior of hES-CM under ischemic conditions for a potential use in cell therapy.

**Production and characterization of different variants of the SulP transporters STAS domain**

Rosa Aiello<sup>1,2</sup>, Elisa Pasqualetto<sup>1,2</sup>, Sandra Quarantini<sup>1,2</sup>, **Roberto Battistutta**<sup>1,2</sup>

<sup>1</sup>Dept. of Chemical Sciences, University of Padua, Padua, Italy

<sup>2</sup>Venetian Institute of Molecular Medicine (VIMM), Padua, Italy

The Sulfate Permease, SulP, is a large and ubiquitous family of anion transporters. In mammals, the members of the SulP family, known as SLC26, have important roles in normal physiology and human pathophysiology. The clinical relevance of the SLC26 gene family has been highlighted with the identification of pathogenetic mutations in four genes, which have been shown to lead to human disorders. SulP proteins show a similar structural organisation: a hydrophobic core with a variable membrane topology and a C-terminal cytoplasmic portion that includes a STAS domain, which plays a fundamental role in the proteins function. The STAS domain of prestin is essential for the plasma membrane targeting and function. In *A. thaliana*, the STAS domain of SULTR1.2 is crucial for the whole sulfate transport activity. In order to shed light into their role in the context of the SulP transporters, we have cloned and produced more than ten recombinant STAS variants, in milligrams amounts of purified material, enough to perform structural studies with both X-ray crystallography and solution NMR. We are currently working on STAS domains from distance-related species: mammalian prestin and pendrin, plant SULTR1.2 and bacterial Rv1739c. These proteins were characterised in solution by SEC, DLS and CD, and important information on the aggregation propensities, relevant for both NMR and crystallography, have been obtained. Most of these constructs are currently submitted to crystallization trials.

**Connexin hemichannels, gap junctions and calcium signaling in the inner ear**

Fabio Anselmi<sup>1</sup>, Victor H. Hernandez<sup>1</sup>, Giulia Crispino<sup>1</sup>, Anke Seydel<sup>1</sup>, Saida Ortolano<sup>1,2</sup>, Stephen D. Roper<sup>3</sup>, Nicoletta Kessaris<sup>4</sup>, William Richardson<sup>4</sup>, Gesa Rickheite<sup>5</sup>, Mikhail A. Filippov<sup>6</sup>, Hannah Monyer<sup>6</sup>, **Fabio Mammano**<sup>1,2</sup>

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<sup>6</sup>Dept. of Clinical Neurobiology, University Hospital of Neurology, Heidelberg, Germany

Extracellular ATP controls various signaling systems including propagation of intercellular Ca<sup>2+</sup> signals (ICS). Connexin hemichannels, P2x7 receptors (P2x7Rs), pannexin channels, anion channels, vesicles, and transporters are putative conduits for ATP release, but their involvement in ICS remains controversial. We investigated ICS in cochlear organotypic cultures, in which ATP acts as an IP3-generating agonist and evokes Ca<sup>2+</sup> responses that have been linked to noise-induced hearing loss and development of hair cell-afferent synapses. Focal delivery of ATP or photostimulation with caged IP3 elicited Ca<sup>2+</sup> responses that spread to several orders of unstimulated cells. Furthermore, we recorded calcium signals from an ATP biosensor apposed to supporting cells outside the photostimulated area in WT cultures. ICS propagated normally in cultures lacking either P2x7R or pannexin-1 (Px1), as well as in WT cultures exposed to blockers of anion channels. By contrast, Ca<sup>2+</sup> responses failed to propagate in cultures with defective expression of connexin 26 (Cx26) or Cx30. Lanthanum, a connexin hemichannel blocker that does not affect gap junction (GJ) channels when applied extracellularly, limited the propagation of Ca<sup>2+</sup> responses to cells adjacent to the photostimulated area.

Our results demonstrate that these connexins play a dual role in inner ear Ca<sup>2+</sup> signaling: as hemichannels, they promote ATP release, sustaining long-range ICS propagation; as GJ channels, they allow diffusion of Ca<sup>2+</sup> mobilizing second messengers across coupled cells.

**JunB Transcription Factor promotes muscle growth and blocks atrophy**

Anna Raffaello<sup>1,5</sup>, Giulia Milan<sup>2,3,4</sup>, Gerolamo Lanfranchi<sup>1</sup>, Alfred L. Goldberg<sup>5</sup>, **Marco Sandri**<sup>2,3,4</sup>

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<sup>5</sup> Dept. of Cell Biology, Harvard Medical School, Boston, MA, USA

The size of skeletal muscle, like that of all cells, is precisely regulated by intracellular signaling networks that determine the balance between overall rates of protein synthesis and degradation. Muscle fiber growth and protein synthesis are stimulated by the IGF1-Akt-mTOR pathway 1, and muscle wasting, as occurs with disuse, denervation, and various systemic diseases (e.g. cancer, sepsis) results from excessive protein breakdown in muscle and induction of a set of atrophy-related genes by FoxO transcription factors. Here we show that the transcription factor, JunB, a proto-oncogene that stimulates cell proliferation is also a major regulator of growth and atrophy of adult (post mitotic) muscle cells. We found that JunB mRNA decreases in various types of atrophying muscles, and that in atrophying myotubes, JunB protein was excluded from the nucleus. However, when JunB's expression was transfected into denervated muscles to maintain its level high, fiber atrophy was prevented, and the induction of the critical atrophy-associated ubiquitin-ligases, atrogin-1 and MuRF-1, was completely blocked. JunB inhibited their induction by associating with FoxO3 and preventing its binding to their promoters and thus blocked the stimulation of protein breakdown. Furthermore, in normal muscles of adult mice JunB over-expression increased fiber diameter dramatically (up to 40% in 7 days), and like IGF-1 or insulin, stimulated protein synthesis but without activating the Akt/mTOR pathway. On the other hand, decreasing JunB expression by RNAi in muscles of adults reduced fiber size. Thus JunB is important, not only in dividing populations, but also in mature skeletal muscle where it is required for the maintenance of muscle size and can induce rapid hypertrophy and block atrophy.

**Akt activation prevents the force drop induced by eccentric contractions in dystrophin-deficient skeletal muscle**

Bert Blaauw<sup>1,2</sup>, Cristina Mammucari<sup>1,2</sup>, Luana Toniolo<sup>4</sup>, Lisa Agatea<sup>1,2</sup>, Reimar Abraham<sup>2</sup>, Marco Sandri<sup>1,2,3</sup>, Carlo Reggiani<sup>4</sup>, **Stefano Schiaffino**<sup>1,2</sup>

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Skeletal muscles of the mdx mouse, a model of Duchenne Muscular Dystrophy, show an excessive reduction in the maximal tetanic force following eccentric contractions. This specific sign of the susceptibility of dystrophin-deficient muscles to mechanical stress can be used as a quantitative test to measure the efficacy of therapeutic interventions. Using inducible transgenesis in mice, we show that when Akt activity is increased the force drop induced by eccentric contractions in mdx mice becomes similar to that of wild type mice. This effect is not correlated with muscle hypertrophy and is not blocked by rapamycin treatment. The force drop induced by eccentric contractions is similar in skinned muscle fibers from mdx and Akt-mdx mice when stretch is applied directly to skinned fibers. However, skinned fibers isolated from mdx muscles exposed to eccentric contractions in vivo develop less isometric force than wild type fibers and this force depression is completely prevented by Akt activation. These experiments indicate that the myofibrillar-cytoskeletal system of dystrophin-deficient muscle is highly susceptible to a damage caused by eccentric contraction when elongation is applied in vivo, and this damage can be prevented by Akt activation. Microarray and PCR analyses indicate that Akt activation induces up-regulation of genes coding for proteins associated with Z-disks and costameres, and for proteins with anti-oxidant or chaperone function. The protein levels of utrophin and dysferlin are also increased by Akt activation.

**The vegetable extract *Serenoa repens* activates the intrinsic apoptotic pathway in prostate cancer**Antonella Baron <sup>1</sup>, Mariangela Mancini <sup>1,2</sup>, Paolo Bernardi <sup>3</sup>, **Francesco Pagano** <sup>1</sup><sup>1</sup>Urological Malignancies Lab., Venetian Institute of Molecular Medicine (VIMM), Padua, Italy<sup>2</sup>Urological Clinic, University of Padua, Padua, Italy<sup>3</sup>Dept. of Biomedical Sciences, University of Padua, Padua, Italy

The ability of *Serenoa repens* (Sr), a natural extract used for treatment of benign prostate hyperplasia, to induce apoptosis in prostate cancer is controversial. We investigated the effect of Sr in PC-3 and LNCaP prostate cancer cells, with attention to the mitochondria as mediators of apoptosis in this model. Cells were treated with Sr extract at different concentrations (10, 50, 80 100 mg/ml) and analyzed by light and electron microscopy. With treatment the cells underwent massive vacuolization, increase in cellular complexity and cytosolic condensation followed by cell death. High numbers of punctuated mitochondria, seen by electron microscopy and mitotraker red staining, and a progressive decrease of the membrane potential, evaluated by TMRM and JC-1 specific dyes, are detectable within 2 hrs and before the apoptotic morphology develops. Translocation of Bax to the mitochondria and release of cytochrome C and SMAC/Diablo to the cytosol, evaluated by western blot, are seen at 4 hours. Caspase 9 activation and PARP cleavage are seen at 16 hours. At 24 hours we detected a subG1 peak and a 55% of TdT positive cells. These results indicate the ability of Sr-extract to induce an apoptotic cell death in prostate cells via the mitochondrial pathway. The ability of Sr to target the mitochondria is a novel finding. There is a rising interest in natural phytochemical-based compounds with anti-cancer activity, because they are relatively non-toxic, inexpensive and available as oral form. Our results prove the legitimacy of this natural compound, with very few clinical side effects, to be further investigated for possible use in prostate cancer prevention strategies or as therapy in advanced cancers irresponsive to traditional therapies.

**Rescue of myopathic collagen VI null mice by genetic inactivation of mitochondrial cyclophilin D**Elena Palma <sup>1</sup>, Emy Basso <sup>1</sup>, Alessia Angelin <sup>1</sup>, Tania Tiepolo <sup>2</sup>, Paola Braghetta <sup>2</sup>, Patrizia Sabatelli <sup>3</sup>, Nadir M. Maraldi <sup>3</sup>, Mike Forte <sup>4</sup>, Paolo Bonaldo <sup>2</sup>, **Paolo Bernardi** <sup>1</sup><sup>1</sup>Dept. of Biomedical Sciences, University of Padua, Padua, Italy<sup>2</sup>Dept. of Histology, Microbiology and Medical Bioechnologies, University of Padua, Padua, Italy<sup>3</sup>IGM-CNR at the Istituto Ortopedico Rizzoli and Dept. of Anatomical Sciences, University of Bologna, Bologna, Italy<sup>4</sup>Vollum Institute, Oregon Health and Sciences University, Portland, OR, USA

Considerable progress in understanding the pathogenesis of collagen VI diseases has been made in mice with targeted disruption of the Col6a1 gene, which display an early-onset myopathic syndrome due to lack of collagen VI. Mitochondria in skeletal muscle fibers and in myoblasts from Col6a1<sup>-/-</sup> mice depolarize in response to oligomycin, an anomalous response that can be corrected by cyclosporin (CsA). This finding suggests that in collagen VI myopathies flickering of the permeability transition pore (PTP, an inner membrane high-conductance channel) is increased and causes depletion of pyridine nucleotides, progressive impairment of respiration, and switch of the F1FO ATP synthase into an ATP hydrolase maintaining the membrane potential at the expense of glycolytic ATP. This interpretation is consistent with the therapeutic effect of treatment of Col6a1<sup>-/-</sup> mice with CsA, which desensitizes the PTP in vivo. To further test the role of the PTP in the pathogenesis of collagen VI myopathies, we have generated Col6a1<sup>-/-</sup>Ppif<sup>-/-</sup> mice (Ppif is the unique mouse gene encoding for mitochondrial cyclophilin D, whose inactivation desensitizes the PTP). We will report the striking rescue of Col6a1<sup>-/-</sup>Ppif<sup>-/-</sup> mice from the myopathy despite their total lack of collagen VI.

**Bax and Bak regulate endosomal hijack on mitochondria during apoptosis induced by *H. pylori* VacA**

Federica Calore<sup>1,2</sup>, Christophe Genisset<sup>2</sup>, Marzia Rossato<sup>3</sup>, Mauro Degli Esposti<sup>4</sup>, Luca Scorrano<sup>1,5</sup>, Marina de Bernard<sup>1,6</sup>

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The vacuolating cytotoxin VacA is one of the most important virulence factors of *Helicobacter pylori*, the bacterium causing severe gastro-duodenal diseases such as ulcer and cancer. Multiple effects on mammalian cells have been ascribed to VacA, including the ability to trigger epithelial cell apoptosis.

Here we demonstrate that VacA requires the mitochondrial but not the ER gateway of apoptosis controlled by the multidomain proapoptotics Bax and Bak to induce apoptosis. Following internalization, VacA recruits Bax on endosomal membranes which are then rapidly hijacked on mitochondria in a process that requires the channel activity of the toxin and occurs before the activation of the mitochondrial death cascade. Cells lacking both Bax and Bak are resistant not only to apoptosis, but also to endosomes-mitochondria intermixing induced by VacA.

Thus, trafficking of VacA to mitochondria unveiled an unprecedented mechanism of cell death that involves recruitment of Bax to endosomes and their Bax, Bak dependent accumulation on mitochondria.

**Insulin resistance in chronic hepatitis C: causes and consequences**

Martina Gerotto<sup>1</sup>, Lisa Franceschini<sup>1</sup>, Gladis Bortoletto<sup>1</sup>, Moira Marcolongo<sup>1</sup>, Davide Campagnolo<sup>1</sup>, Silvia Mirandola<sup>1</sup>, Alfredo Alberti<sup>1,2</sup>

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Insulin resistance (IR) is often seen in hepatitis C and it's associated to reduced response to interferon (IFN). We have investigated the pathogenic mechanisms underlying this association by 1) analysing the correlation between the early kinetic of viremia in patients treated with IFN and levels of IR and insulinemia 2) by developing an in vitro model to assess whether insulin has a direct effect on IFN induced cellular response. 1) In 62 patients, HCV RNA levels after 24 h from first IFN dose revealed an inverse correlation between insulinemia and HCV-RNA decay. Patients with HOMA-IR >3 showed a significant reduction in virus decay compared to HOMA-IR <3. 2) HepG2 cells were treated with IFN or IFN+insulin. Expression of IFN-related (PKR, MxA, 2'-5'OAS) and insulin-related (IRS-1, IRS-2 and SOCS3) genes/proteins, and ser473AKT phosphorylation were measured. IFN alone induces over-expression of PKR, MxA, 2'-5'OAS and, surprisingly, of IRS-1 gene. In co-treated cells, reduction in PKR, MxA, 2'-5'OAS is observed while IRS-1 levels are comparable to controls. Insulin alone had no effects on these genes. IFN reduced insulin-induced pAKT while, no pAKT is observed in cells with IFN alone. We, for the first time, describe IFN induction of IRS-1 in hepatocytes. The evidences that insulin can modulate IFN inducible genes and that IFN affects the insulin signal underlie the relevance of a correct cross talk between the two pathways to ensure their efficacy in hepatocytes.

**NapA of *Borrelia burgdorferi* drives Th17 inflammation in Lyme Arthritis**

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Human Lyme arthritis caused by *Borrelia burgdorferi* is characterized by an inflammatory infiltrate, consisting mainly of neutrophils and T cells. The Neutrophil Activating Protein A (NapA) was found to be essential for *B. burgdorferi* persistence within ticks, but its structure and role in immune response in Lyme arthritis were unknown. Here, we report that this virulence factor is a major antigen of the humoral response in patients with Lyme arthritis. We show that T cells from synovial fluid of patients with Lyme arthritis produce interleukin (IL)-17 in response to NapA. NapA is a Toll-Like Receptor-2 agonist able to induce the expression of IL-23 in neutrophils and monocytes, and IL-6, IL-1 $\beta$  and transforming growth factor- $\beta$  in monocytes. We conclude that NapA of *B. burgdorferi* represents an important driver of T helper (Th) 17 immune responses, and elicits a synovial Th17 response that might play an important role in the pathogenesis of Lyme arthritis.

**Role of Glycogen Synthase Kinase 3 in multiple myeloma**

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We investigated the function of Glycogen Synthase Kinase 3, GSK3, in multiple myeloma (MM) cells. The expression and localization of GSK3 and its inactive phosphorylated form varied between MM cell lines, peripheral blood mononuclear cells, and normal *in vitro* generated plasma cells. While GSK3 localized mostly in the cytoplasm in MM cell lines and other cell types, patient derived MM cells pools of GSK3 colocalized with Wnt pathway components at the cell membrane. IL-6 and IGF-I, but not TNF $\alpha$ , hampered GSK3 activity indicating a different regulation by external signals. Treatment of MM cell lines with the GSK3 inhibitors resulted in growth arrest. Surprisingly, in a IL-6-dependent MM cell line, GSK3 inhibition caused opposite effects depending on whether the cells were cultured with or without IL-6: rescue from apoptosis (- IL-6) or an increase of apoptosis (+IL-6). This could be due to a differential  $\beta$  catenin and NF- $\kappa$ B activity found in the two conditions. Furthermore, GSK3 inhibition caused increased survival of MM cells and enhanced NF- $\kappa$ B and  $\beta$ -catenin-dependent gene transcription when MM cells were cultured with bone marrow stromal cells. Importantly, GSK3 inhibition significantly lowered the extent of bortezomib-induced apoptosis. These results suggest that in MM cells GSK3 is differently regulated downstream from growth signals, playing a peculiar role in cell survival. Further more GSK3 critically modulates the interactions within the surrounding microenvironment and sensitivity to proteasome inhibitors.

**RNOS-induced chemokine inactivation in cancer**

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At the tumor site, the reactions of NO with oxygen (O<sub>2</sub>) or oxygen-related reactive intermediates yield numerous reactive nitrogen as well as oxygen species (RNOS). One of the most intensively studied reaction is the one between NO and superoxide anions yielding peroxynitrite (ONOO<sup>-</sup>), which is a potent oxidant. In the past, we provided data showing that RNOS are involved in tumor-induced immunosuppression and we speculated that RNOS might block T lymphocyte infiltration in the tumor. We decided to study the role of RNS in tumor-induced immunosuppression. Typically, TILs are unable to reach the core of the tumor mass, and they concentrate at the border of the neoplastic lesion. We speculated that RNS modify chemokine biology and keep TILs distant from the tumor. Chemokines are small cytokines with selective chemoattractant properties, coordinating the homeostatic circulation of leukocytes as well as their movement to sites of inflammation or injury. Dysregulated expression of chemokines and their receptors is involved in the development of many human diseases, including autoimmune and chronic inflammatory diseases as well as immunodeficiency and cancer. We found that the chemoattractants CXCL12, CCL21 and CCL2 lose their ability to recruit T lymphocytes if exposed to peroxynitrite. However, the modified chemokine CCL2 retains its capacity of recruiting myeloid-derived suppressor cells. These data suggest that the modified chemokines may modify the tumor microenvironment and favor immunosuppression. All these data prompt us to analyze whether chemokines present in tumors are susceptible to RNS-induced modifications. We thank Tullio Pozzan and Francesco Pagano for support.

# POSTER ABSTRACTS

(Underlined: speaker, **Bold**: Principal Investigator)



## 1. Identification of bacterial factors able to promote a Th17 response in gastric mucosa of *Helicobacter pylori*-infected patients

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*Helicobacter pylori* is a spiral-shaped Gram negative bacterium that colonizes the human gastric mucosa and chronically infects more than half of the human population. *H. pylori* infection is characterized by a marked infiltration of neutrophils, macrophages, and lymphocytes in the gastric mucosa. During *H. pylori* infection, there is a pronounced specific acquired immune response, characterized by generation of antibodies, and by differentiation and activation of effector T cells. *Helicobacter* infection includes both a Th1 and Th2 component. The level of inflammation increases the disease risk but it does not seem to influence the kind of disease, which, in contrast, is thought to be largely influenced by the pattern of gastric inflammation. Emerging experimental evidence demonstrates that *H. pylori* inflammation also triggers a Th17 immune response, which is characterized by IL17 production. IL17 family members play an active role in inflammatory and autoimmune diseases, and in cancer. There is also evidence that the expansion and survival of Th17 cells require additional factors, such as IL23. This suggests that the IL23/IL17 pathway is an important driving force on ongoing gastric inflammation in *H. pylori*-infected patients. The aim of our project is to identify the antigens of *Helicobacter pylori* involved in the development of the Th17 subset and to elucidate the IL23/IL17 axis in inflammation.

## 2. Structural studies of new factors involved in the stomach colonization by *H. pylori*: the HP1028 protein and the cholesterol- $\alpha$ -glucosyltransferase HP0421

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*Helicobacter pylori* is a gram-negative bacterial pathogen specialized in the colonization of the human stomach. It establishes a life-long chronic infection in more than half of the human population: most infected people are asymptomatic, but, sometimes, *H. pylori* causes gastritis, stomach and duodenal ulcers, adenocarcinomas and stomach lymphomas. Recently, new pathogenicity factors have been identified. Among them, HP1028 and HP0421: the former is a protein of unknown function, whilst the second is a cholesterol- $\alpha$ -glucosyltransferase, an enzyme that promotes immune evasion. HP1028 has been cloned, expressed in *E.coli* in high yield and purified for crystallization trials. Since it doesn't present any sequence similarity with a protein of known structure, in order to obtain approximate initial phases, a derivative containing seleno-methionines has been expressed. To increase the number of methionines in the amino acid sequence, a mutant L129M has been prepared. Crystals of both native and derivative forms have been grown and crystal optimization is in progress. HP0421 protein has been cloned in two different constructs and expressed in *E.coli* cells. Since most of the protein can't bind strongly the affinity Ni<sup>2+</sup> resin, it has been purified and refolded in vitro. Despite the low yield, the enzyme can be produced in a sufficient amount and crystallization trials are in progress.

### 3. A structural insight into CK2 inhibition

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The acidophilic Ser/Thr protein kinase CK2 displays some unique properties such as high pleiotropicity and constitutive activity. CK2 is involved in many fundamental aspects of the normal cell life, for instance it promotes cell survival and enhances the tumour phenotype under special circumstances. This makes CK2 an appealing target for the development of inhibitors with pharmacological potential. Here we present an overview of our recent studies on inhibitors directed to the CK2 ATP-binding site whose distinctive features are highlighted by the ability to use both ATP and GTP as co-substrates and by its low susceptibility to staurosporine inhibition. We discuss the effects of the binding of different chemical families of fairly selective inhibitors with potency in the nanomolar or low micromolar range. An important common energetic contribution to the binding is due to the hydrophobic interaction with the apolar surface region of the CK2 binding cleft. The analysis of the known CK2 crystal structures reveals the presence of some highly conserved water molecules in this region. These waters reside near Lys68, in an area with a positive electrostatic potential that is able to attract and orient negatively charged ligands. The presence of this positive region and of two unique bulky residues, Ile66 and Ile174, responsible for the reduced dimension of the CK2 active site, play a critical role in determining ligand orientation and binding selectivity.

### 4. HP-NAP, a key factor in the *Helicobacter pylori* chronic inflammation

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The *Helicobacter pylori* neutrophil-activating protein (HP-NAP) is a virulence factor of *H. pylori* that stimulates in neutrophils high production of oxygen radicals and adhesion to endothelial cells. In this study we also report that the exposure of monocytes to HP-NAP resulted in a significant increase in cell viability; while culture monocytes undergo spontaneous apoptosis after 48 h, HP-NAP-treated cells survived up to 7 days. Accordingly HP-NAP prevents the cleavage of poly-ADP-ribose polymerase (PARP) in monocytes. PARP is a molecular target of caspase 3, which is involved in apoptotic events, thus the presence of PARP fragments is a clear evidence that an apoptotic process occurs. Furthermore HP-NAP protects monocytes from apoptosis induced also by starvation conditions. These data suggest that the capability of HP-NAP to prevent monocytes apoptosis could be involved in the chronicization of *H. pylori* sustained inflammation. Thus, HP-NAP, influencing the phenotype of the immune response and also the life-span of the cells involved in the inflammation, might create an appropriate environment, in the gastric mucosa, for the *H. pylori*-associated inflammation.

## 5. Functional properties of mutants of the nervous tissue specific plasma membrane Ca<sup>2+</sup> pump isoforms 2 (PMCA2)

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The specific isoform of the plasma membrane Ca<sup>2+</sup> ATPase present in the nervous system is the PMCA2. Its genetics dysfunction causes hearing loss and ataxia, reflecting the high level of expression of PMCA2 in the inner ear, but PMCA2 null mice also show alterations in the cerebellum (increased number of Purkinje cells, reduced number of granule cells, thinner molecular layer) We have functionally characterized 2 PMCA2 mutants. One point mutation was located in the ATP binding domain of the pump, the other in its 6th transmembrane domain. Both mutations impaired the ability of the pump to maintain the physiological concentration of calcium in the native outer hair cell and in model cells expressing the mutant pump. Specifically, the mutations decrease the baseline, non activated activity of the pump.

## 6. Trichoplein, a novel protein at the crossroad between mitochondria and intermediate filaments

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Trichoplein is a novel protein that interacts in vitro with the intermediate filament keratin and contains a trichohyalin/plectin homology domain (TPHD). It has been recently described as a tumour suppressor gene frequently deleted in bladder and prostate cancers. Fractionation experiments indicated that a large fraction of trichoplein is retrieved on mitochondria.

We therefore explored the possibility that trichoplein participates in mitochondrial dynamics and morphology. Fusion to GFP of different fragments of trichoplein showed that the first 111 aa are sufficient for a punctuate distribution that partially overlaps with mitochondria. The subcellular fractionation of murine liver homogenates indicated that trichoplein is exclusively localized in mitochondria-associated membranes (MAM) and that keratin 8 is almost completely accumulated in this fraction as well. Levels of trichoplein influence mitochondrial morphology, as its overexpression causes fragmentation of the mitochondrial network, which is dependent of Drp-1, a protein that regulates mitochondrial fission. Since mitochondrial fragmentation is commonly associated with apoptosis, we are investigating a possible role of trichoplein in the death cascade. Preliminary results show that levels of trichoplein correlate with spontaneous apoptosis.

Our results indicate that the crosstalk between intermediate filaments and mitochondria can be crucial to determine morphology of the organelle and its participation in the apoptotic cascade.

## 7. Microbioreactors for controlling cellular environments: design principles for stem cell applications

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Novel cell culture technologies developed in recent years mimic the in vivo cellular microenvironments with an increasing fidelity through improved control and the provision of cascades of multiple regulatory factors. Miniaturization of the culture systems is an important step towards accurate control of the cultured cells and tissues. Some of the most interesting outcomes come from the optimization and accurate use of microfluidic platforms. Small transport distances are key for enabling fast-responses to environmental stimuli in studies involving spatial and temporal gradients of factors. We discuss the utilization of microbioreactors for controlling cellular environments in studies of factors that regulate stem cells behavior; to this end, we accurately describe the operating requirements under steady-state and dynamic conditions and the related control of the mass transport and hydrodynamic shear. We have designed simple and practical systems with the size of a microscope slide that couple microfluidic platforms with arrays of microbioreactors and arrayed cultures. Such systems allow quantitative studies on biological samples under well defined and repeatable conditions offering the possibility to accurately modulate transients in space and time to recreate precise stimulation patterns and deliver particular signals. Case studies will be presented with regards to human embryonic stem cells (hESC), hESC-derived cardiomyocytes and analysis of the Wnt3a- $\beta$  catenin pathway.

## 8. FoxO3 activity is modulated by post-translational modifications

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FoxO proteins are transcription factors that control cell cycle progression, DNA repair, muscle atrophy, stress resistance and apoptosis. These divergent functions are carefully regulated by post-translational modifications including phosphorylation, ubiquitination and acetylation. FoxO acetylation is mediated by acetyl-transferase CREB-binding protein (CBP)/p300 which are modulating FoxO transcription by interfering FoxO binding to target DNA sequence. To verify the consequence of acetylation on FoxO3 transcriptional activity in adult skeletal muscle, we generated FoxO3 mutants in which the lysines residues of the DNA binding domain and of the nuclear localization sequence are replaced by arginine (KR) to block acetylation. In a second round of experiments we mutated the lysines into glutamine (KQ) to mimic a constitutive acetylation. We performed the luciferase assay using as readout of FoxO activity a FoxO-sensor constituted by six repeated FoxO binding elements driving a luciferase gene. Results confirm that acetylation inhibits FoxO transcription activity. Next we tested whether these mutants can affect a more complex condition like the atrophy program. Indeed acetylation reduced FoxO-dependent muscle atrophy by causing re-localization of FoxO proteins to the cytoplasm.

**9. Coordinated control of connexin 26 and connexin 30 at the regulatory and functional level in the inner ear**

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# S.O. and G.D.P. contributed equally to this work

Connexin 26 (Cx26) and connexin 30 (Cx30) are encoded by two genes (GJB2 and GJB6, respectively) that are found within 50 kb in the same complex deafness locus, DFNB1. Immunocytochemistry and quantitative PCR (qPCR) analysis of Cx30 knock-out (KO) mouse cultures revealed that Cx26 is downregulated both at protein, and at mRNA level in non-sensory cells located between outer hair cells (OHC) and stria vascularis (SV). To explore connexin coregulation we manipulated gene expression using the bovine adeno-associated virus (BAAV). Overexpression of Cx30 in the Cx30 KO mouse by transduction with BAAV restored Cx26 expression, permitted the formation of functional gap junction (GJ) channels, and rescued propagating Ca<sup>2+</sup> signals. Ablation of Cx26 by transduction of Cx26loxP/loxP cultures with a Cre recombinase vector caused concurrent downregulation of Cx30 and impaired intercellular communication. The coordinated regulation of Cx26 and Cx30 expression appears to occur as a result of signaling through PLC and NF-κB pathway, as activation of IP3-mediated Ca<sup>2+</sup> responses by stimulation of P2Y receptors for 20 min with 20 nM ATP increased the levels of Cx26 transcripts in Cx30 KO cultures. This effect was inhibited by expressing a stable form of the IκB repressor protein that prevents the activation/translocation of NFκB. Thus our data reveal a Ca<sup>2+</sup> dependent control in the expression of inner ear connexins implicated in hereditary deafness, as well as insight into the hitherto unexplained observation that some deafness associated DFNB1 alleles are characterized by heritable reduction of both GJB2 and GJB6 expression.

**10. Defining novel molecules to rescue immunity against prostate cancer: molecular and biological basis for new therapies**

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Prostate cancer (PCa) is the second leading cause of malignancy-related mortality in males in the Western world. The available treatments for PCa have demonstrated weak curative efficacy. Immunotherapy may provide a valid alternative therapy but the success of this approach depends on the ability of CTL to kill tumor cells. However, if the tumor environment exerts a suppressive action on antigen-specific TIL, immunotherapy will achieve little, if any, success. Thus, it is paramount to understand modulation of TIL responses by the tumor environment. To analyze the role of the prostate tumor environment we have started a study which allowed us to demonstrate that human PCa are infiltrated by terminally differentiated CTL that are completely unresponsive. The steady-state regulation of the dormant state is dependent on the enhanced intratumoral metabolism of L-Arg, because the addition of ARG- and NOS-specific inhibitors was sufficient to activate them and recover their functions. These results identify a mechanism by which PCa induces in situ immunosuppression and suggest novel strategies for the tumor immunotherapy. The major goal of this project is to develop new drugs to treat PCa patients. To achieve this goal we have developed novel small molecules. We found that molecule (AT38) could normalize the immune status of tumor-bearing hosts and restore mice lymphocyte responsiveness, in terms of proliferation and effector functions in both *in vitro* and *in vivo* assays. Moreover, we observed that the newly synthesized NO-releasing COX-inhibitor restore TIL responsiveness in human PCa *in vitro*.

**11. Mitochondrial fusion is an early and protective step of autophagy**

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Autophagy is a catabolic process that allows the recycling of components of the cells. For many years autophagy has been seen as an unselective process, but recent observations indicate that under some circumstances specific organelles like mitochondria are selectively engulfed by autophagosomes. We wished to address the role of mitochondrial shape changes in the control of autophagy. By analysing mitochondrial morphology after inducing autophagy by starvation, we observed a paradoxical mitochondrial elongation. To address whether mitochondrial fusion was responsible for the observed phenotype, we analysed mitochondrial morphology in wt MEFs as well as MEFs knock out for the mitochondrial fusion proteins (Mfn1<sup>-/-</sup>, Mfn2<sup>-/-</sup>, Mfn1<sup>-/-</sup>Mfn2<sup>-/-</sup> and Opa1<sup>-/-</sup> MEFs). We found that mitochondrial fusion proteins are essential for the observed phenotype. Moreover, we found that mitochondrial fusion rate is increased in cells that were subjected to starvation. Our results suggest that fusion of mitochondria is at least one of the mechanisms responsible for the observed mitochondrial elongation. Interestingly, the ablation of the pro-fusion genes that are required for mitochondrial elongation during autophagy results in starvation-induced death, suggesting that these morphological changes protect cells from death induced by limited substrate supply. Our results indicate that mitochondrial shape changes play an important role in the regulation of the fate of cells undergoing autophagy.

**12. Calcium and mitochondrial dysfunction in a cellular model of Huntington's disease**

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Disregulation of the Ca<sup>2+</sup> homeostasis and of the mitochondrial Ca<sup>2+</sup> handling, which have been described and claimed to cause the apoptotic death of striatal neurons in Huntington's disease (HD), have been studied in STHdhQ111 cells, precursors of striatal neurons from a mouse model of HD. Ca<sup>2+</sup> transients induced by ATP and bradykinin (BK) were disturbed due to altered transcription of the purinergic and BK receptors. The transcription of the components of the phosphatidyl-inositol (PI) cycle and of the InsP3-mediated pathway was altered, delaying the production of InsP3 in mutant cells. The mitochondrial Ca<sup>2+</sup> handling was compromised in mutant cells, but only when they were stressed by the inhibition of complex II of the respiratory chain. The impaired ability to handle Ca<sup>2+</sup> loads by mitochondria of mutant cells was prevented by both cyclosporin A and its non-immunosuppressive analog Debio25.

**13. Suspended cell-culture bioreactor for human stem cell expansion**

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A bioreactor was designed to perform stem cell cultures under dynamic conditions as alternative method of cell expansion. The traditional static culture is a highly heterogeneous environment with concentration gradients formed because of cell consumption and production of nutrients, oxygen, and growth factors. The effect of stirring is that of homogenizing the culture environment, and the bioreactor developed is a stirred 6-well system that allows parallel experiments to be performed. It is suitable for small culture volumes (about 10 ml) and prolonged periods of time, also under hypoxic conditions. The geometry of the stirrer was designed to minimize shear stress (<10 dyn/cm<sup>2</sup>) on cell membrane. Computational simulations of oxygen concentration gradients in culture were performed, which were supported by the experimental determination of oxygen solubility in different culture media (DMEM, IMDM, with 10% FBS). The bioreactor was used to culture human CD34+ cord blood hematopoietic stem cells for up to 2 weeks in both normal and hypoxic (pO<sub>2</sub>=0.05 atm) conditions. This dynamic culture system promotes cell proliferation and preserves CD34 expression, associated with hematopoietic stemness. The colony-forming assay in methylcellulose shows a multilineage potential of the cells after expansion in the bioreactor. This innovative device is promising for expanding hematopoietic stem cells from cord blood before transplantations.

**14. Host genetics regulates liver fibrosis progression in patients with chronic hepatitis C (CHC)**

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Outcome of liver fibrosis progression is highly variable and multifactorial. Recently, a Cirrhosis Risk Score (CRS) based on 7 host single nucleotide polymorphisms (SNPs) has been proposed as genetic marker for development of cirrhosis in CHC. Aim of our study was to assess the role of CRS in predicting fibrosis progression in CHC. CRS was measured in genomic DNA using the Luminex® 200™ System. We investigated 274 untreated patients with CHC having no or minimal fibrosis at first biopsy and at least 60 months FU. All patients had a 2° liver biopsy taken during FU to define fibrosis progression. During this period 34.8% of the cases showed no progression, 65.2% progression by at least 1 METAVIR stage, 40.7% by at least 2 METAVIR stage and 15.1 % by >2 METAVIR stage. Mean CRS was significantly higher (p=0.001) in patients with fibrosis progression compared to those without progression. When patients were categorised by CRS levels (low: <0.50, intermediate: 0.50-0.70, high: >0.70) the relative risk for fibrosis progression increased in parallel with increasing CRS values. This was particularly evident in male patients (p=0.001) and in patients having F0 in the initial biopsy (p=0.0001) (not significant in females). Host genetics defined by CRS predicts fibrosis progression in patients with initially mild chronic hepatitis C. CRS might become a useful parameter for prognostic evaluation and treatment decision.

**15. cAMP in olfactory sensory neurons: spatial distribution and temporal dynamics**

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A unique aspect in the topographical organization of the olfactory system is the dual role played by the olfactory receptor (OR) of the olfactory sensory neurons (OSN): transduction of chemical signals and axonal convergence to the target glomeruli in the olfactory bulb. To corroborate this hypothesis the OR protein is expressed not only on the cilia, but also on the axon termini-growth cone. The intracellular signalling triggered by the OR activation in the cilia involves cAMP and calcium. The molecular mechanism underpinning the role of the OR at the growth cone remains unknown. The key question whether the OR on the growth cone is a functional receptor and which signalling cascade is coupled to it remains to be elucidated. To address this question we studied in vitro and in situ the spatio temporal dynamics of cAMP in OSN, taking advantage of genetically encoded sensor for cAMP based on FRET. We demonstrated that the machinery required for the synthesis and the hydrolysis of cAMP is present and functional also at the growth cone. Selective odor stimulation of the OR of the growth cone causes local increases of cAMP and calcium through CNG channels and is followed by the nuclear translocation of the YFP-PKA catalytic subunit. At the nuclear level the cAMP-PKA signal can regulate transcription of genes by CREB phosphorylation. Taken together, these data suggest that the cAMP produced upon the selective activation of the OR on the growth cone can direct OSN axonal targeting acting locally end at the nuclear level regulating the transcription of genes coding for axon guidance molecules. This model has the potentiality to link the identity of the OR with the expression of specific axon guidance molecules, interacting together in the formation of sensory map.

**16. Cortactin expression is tightly connected to B-cell Chronic Lymphocytic Leukemia spreading**

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B-cell Chronic Lymphocytic Leukemia (B-CLL) is the most common leukemia in adults and is characterized by the accumulation of clonal CD5+ B lymphocytes due to uncontrolled growth and resistance to apoptosis. We previously demonstrated that Src kinase Lyn displays anomalous properties in leukemic cells when compared to normal B lymphocytes. Our attention is now focused on Cortactin, a protein originally identified as a substrate for Src kinases. Cortactin is an ubiquitous actin-binding protein overexpressed in several human tumors. By western blotting analysis, we demonstrated that Cortactin is also overexpressed in B-CLL cells compared to control cells ( $0.83 \pm 0.99$  vs  $0.11 \pm 0.21$ , respectively;  $p < 0.05$ ). Moreover, the overexpression of Cortactin is associated with the increase of B-CLL spreading. Infact, by the analysis of chemotaxis activity of leukemic cells, we showed that Cortactin overexpression correlates with the increased response of leukemic chemokine. Furthermore, by zymography assay, we correlated cells to CXCL12/SDF1 $\alpha$  tumoral cell release of MMP-9 to Cortactin expression levels: patients without MMP-9 expression presented low cortactin level ( $0.046 \pm 0.020$ ), while patients characterized by MMP-9 strong expression showed high cortactin level ( $2.43 \pm 0.04$ ;  $p < 0.05$ ). These results suggest that Cortactin is involved in CLL aggressiveness and that this protein could represent an alternative target for the development of new therapeutic strategies.

**17. Autophagy is required for the control of muscle mass**

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The ubiquitin-proteasome and autophagy-lysosome pathways are the two major routes for protein and organelle clearance in eukaryotic cells. Autophagy is an evolutionary conserved mechanism that allows cell survival during starvation. Autophagy deficient mice die soon after birth during the critical starvation period when transplacental nutrient supply is suddenly interrupted. However, the contribution of autophagy to homeostasis of organelle and proteins in skeletal muscles and its role in muscle wasting are not yet understood. We have generated a muscle specific autophagy knockout mice. Deletion of Atg7 gene completely blocked autophagosome formation in skeletal muscles but not in heart and other tissues. These mice develop normally and postnatal growth is unaffected. In adulthood skeletal muscles show signs of degeneration while remaining myofibers are atrophic. Accumulation of abnormal mitochondria, dilated SR, disorganization of sarcomere and aberrant concentric membranous structure have been revealed by electron microscope. The critical genes of muscle atrophy are induced and muscle strength is strongly decreased. Thus maintenance of autophagy flux is important to preserve myofiber integrity. Inhibition or alteration of autophagy can contribute to muscle degeneration in some dystrophies.

**18. Dissecting cGMP signalling in olfactory sensory neurons**

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Cyclic GMP, cGMP, as well as cAMP, is a second messenger in olfactory sensory neurons (OSN) involved in a variety of signal transduction processes, such as adaptation and long term cellular responses. cGMP is produced by two different guanylyl cyclases (GC): soluble and receptor GC, and it is hydrolyzed by several phosphodiesterases, PDEs. The mechanism that control the production and the degradation of cGMP remain largely unknown. To elucidate this mechanisms we are studying the spatio-temporal dynamics of changes in cGMP concentration in OSN. We take advantage of FRET-based sensor for cGMP Cygnet 2.1. Upon pharmacological stimulation of receptor and soluble GC an increase in cGMP is observed throughout the OSN, at the cilia- dendrite level but also at the axon termini growth cone level. Upon physiological stimulation with odors a rise in cGMP is observed in all compartments of olfactory neurons, including the axon termini-growth cone. The kinetic of this cGMP increase is slow and sustained. Odors stimulation in the presence of a soluble GC inhibitor abolish the cGMP increase, while inhibition of CO does not affect odors response. Inhibition of PDEs with IBMX and zaprinast do not produce any increase in cGMP, suggesting the absence of constitutive activity of GC. To reveal a potential interplay between cGMP and cAMP OSN transfected with Cygnet were stimulated with forskolin (an adenylyl cyclase activator). A significant increase of cGMP is observed in all compartments of OSN. We are in the process of dissecting the molecular mechanisms linking cGMP to cAMP signalling.

### 19. Freshly isolated fibers and satellite cells delivered through photopolymerizable Hydrogel improve muscle regeneration

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The ultimate goal in regenerative medicine is to find the best combination between myogenic cells and biocompatible scaffolds. Injection of single, isolated muscle fibers or satellite cells dissociated from their parental myofibers, not expanded in vitro, represent the best source for muscle regeneration. In our work we combined their regenerative potential with an hyaluronic acid-based hydrogel, photopolymerizable with UVA light and injectable in situ. Experiments were performed on C57BL/6J mice. Part of tibialis anterioris (TA) muscle was surgically removed and injected with Hydrogel containing either muscle fibers, dissociated satellite cells or cultured satellite cells derived from C57BL/GFP mice FDB, EDL and soleus muscles. We performed histochemical, immunofluorescence and physiological analyses of the engrafted muscles after 5 weeks. Samples were compared to muscles engrafted only with Hydrogel and sham controls. TA muscles engrafted with fibers and dissociated satellite cells presented increased muscle mass, significantly higher number of GFP+ve fibers and GFP+ve satellite cells inserted under the basal lamina of GFP+ve and GFP-ve myofibers, and a subsequent improvement of muscle force, compared with controls. Hydrogel delivery of muscle fibers is feasible and efficient in the regenerating muscle and could be an interesting approach for clinical application.

### 20. Structural studies of the SulP anion transporters

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The Sulfate Permease (SulP) is a large and ubiquitous family of membrane proteins that are inorganic anion uptake transporters or anion/anion exchange transporters. They show a similar structural organisation: a hydrophobic core with a variable membrane topology and a C-terminal cytoplasmic portion that includes a STAS domain. The STAS domain was identified by sequence analysis of proteins with completely different functions, the SulP transporters and the bacterial antisigma-factor antagonists ASA. Unlike the bacterial ASA, the SulP transporters STAS domains, that are essential for the protein function, are poorly characterized in their structure. In order to study their role in anion transporters, we have selected the STAS from distance-related species: mammalian prestin (rat) and pendrin (human), plant SULTR1.2 (*A. thaliana*) and bacterial Rv1739c (*Mycobacterium tuberculosis*). We have cloned and produced more than ten recombinant STAS variants, in milligram amounts of purified material. These proteins were characterised in solution by analytical Gel Filtration chromatography, Dynamic Light Scattering and Circular Dichroism, and important information on the aggregation propensities, relevant for both NMR and crystallography, have been obtained. We are also trying to express some full-length SulP transporters with cell-free systems based on *E. coli* extracts, an emerging and promising technique for the large scale production of functional membrane proteins.

**21. FOXO3 signals back and forth from mitochondria in a feed-forward loop leading to muscular atrophy**

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Mitochondria are crucial organelles in the production of energy and in the control of signalling cascades. A shaping machinery of pro-fusion and fission proteins controls their morphology and subcellular location. In muscle this results in the orderly pattern of intermyofibrillar and subsarcolemmal mitochondria. Muscular atrophy is a genetically controlled process involving the activation of the autophagy-lysosome and the ubiquitin proteasome systems. Whether and how mitochondria are involved in muscular atrophy is unknown. Here we show that mitochondrial fragmentation is a primary subroutine of the autophagy-lysosome system in atrophying muscles. Activation of FoxO3 triggers mitochondrial remodelling. This process is dependent on the expression of the atypical Bcl-2 family member Bnip3 and on the activation of the core fission machinery, whose inhibition reduces FoxO3-mediated muscle loss. Induction of mitochondrial fragmentation is per se sufficient to induce muscle wasting in adult animals, by triggering organellar dysfunction, AMPK activation and a feed –forward AMPK/FoxO3 loop. Blockage of AMPK or of FoxO3 restores muscle size in myofibers with fragmented mitochondria. Thus, disruption of the mitochondrial network is an essential amplificatory loop of the muscular atrophy program.

**22. A novel ancient myosin in mammalian skeletal muscle**

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We have analyzed the expression of MYH15 at the gene and protein level in mammalian skeletal and cardiac muscle. Quantitative PCR showed that MYH15 mRNA is not detectable in heart and is present at trace levels in slow (SOL) and fast (TA) skeletal muscles, while it is very abundant in extraocular (EO) muscles of both mouse and rat. Next, we prepared polyclonal antibodies against a portion of the N-terminal region of the MyH15 myosin, which corresponds to a sequence very divergent from other sarcomeric MYHs. When tested in immunoblot against rat EO, fast (TA and EDL), slow (SOL), embryonic (E20), neonatal (P2) and cardiac muscle, antibodies gave a positive reaction only with EO muscles. By immunofluorescence, MYH15 was found to be selectively expressed in the in the orbital layer of EO muscles. In addition, it is also present in the polar but not in the equatorial region of intrafusal fibers of muscle spindles. The mammalian MyH15 gene is the ortholog of the chicken and frog ventricular MYH gene (vMYH). Therefore, in order to evaluate a possible evolutionary implication of the MYH15 gene, we performed quantitative PCR on chicken EO, slow (ALD) and fast (PLD) and ventricle, and in *Xenopus* muscles (ventricle and gastrocnemius). vMYH is highly expressed in ventricle both in frog and chicken, and, although at lower levels, also in chicken EO muscles. In conclusion, we demonstrated the presence in the EO orbital layer of a novel myosin encoded by the gene MyH15. Our preliminary comparative studies suggest that the amphibian cardiac-specific vMYH gene is expressed both in cardiac and skeletal muscle in birds, and finally became a gene exclusively expressed in EO muscle of mammals. We are now planning to purify MYH15 to determine its physiological properties.

**23. Apoptosis regulation by the mitochondrial chaperone TRAP-1/HSP-75**

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TRAP1 is a mitochondrial chaperone also known as heat shock protein 75, which is overexpressed in several tumor cell types. Here we analyze whether mitochondrial TRAP1 elicits cytoprotective functions in a model of human osteosarcoma, SAOS-2 cells, either wild-type or in which TRAP1 expression was knocked down by RNA interference. Cells were exposed to different kinds of pro-apoptotic stimuli: chemotherapeutics, oxidative stress or death ligands, and several apoptotic parameters were measured in order to dissect whether and how TRAP1 impacts on these stress-induced transduction pathways. TRAP1 displays a general antiapoptotic role in all the examined conditions, whereas TRAP1 interference increases cell sensitivity to death. Serine phosphorylation and mitochondrial localization are required for TRAP1 cytoprotective function. In fact, a deletion mutant lacking the mitochondrial import sequence is not phosphorylated and is unable to counteract apoptosis induction in all conditions. Preliminary data show that TRAP1 interacts with Bcl-2 family proteins and is involved in the regulation of the mitochondrial permeability transition pore opening. Altogether, these results suggest that TRAP1 acts as a key anti-apoptotic molecule in mitochondria of neoplastic cells.

**24. Structural studies of proteins involved in three different pathways of *Helicobacter pylori***

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*Helicobacter pylori* is a gram-negative, ureolytic organism which colonizes the stomach of about half of the human population. Its infection is associated with a spectrum of gastric pathologies, ranging from mild gastritis to peptic ulcers and gastric cancer. A strong predictor of a severe disease outcome is the infection with a bacterial strain harboring the cytotoxin associated gene pathogenicity island (cag PAI), a 40kb stretch of DNA that encodes homologues of several components of a type IV secretion system. This project is aimed at the structural and functional characterization of *H. pylori* proteins involved in pathogenicity, or relevant for colonization and persistence of the bacterial infection. In particular, studies on proteins of the following three systems are under way: CagL, a protein belonging to the cagPAI; HP0797, a flagellar sheath adhesin; HP1286, a protein involved in the NAD de novo biosynthesis. Crystals of HP1286 enzyme have been obtained and diffraction data measured at the ESRF synchrotron. Crystals belong to the P212121space group, with one dimer per asymmetric unit. The structure has been solved by molecular replacement and the refinement is under way. Small crystals of the other two proteins have been grown and their optimization is in progress. We expect that the structural knowledge of these proteins, essential for the survival of the bacterium, are useful in designing new inhibitors, potentially useful against the pathogen.

**25. TLA1/DR3 expression correlates with sarcoidosis activation state**

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Sarcoidosis is an immunomediated multisystemic disorder of unknown causes. A TNF-like cytokine, TL1A, and its death domain receptor DR3, have been shown to play a pivotal role as co-initiators and amplifiers of the dysregulated Th1 response in ulcerative colitis and Crohn's disease. The role of TL1A/DR3 interaction in Th1-mediated responses prompted us to investigate these molecules in sarcoidosis. T cells and AMs, from bronchoalveolar lavage (BAL) of 22 patients with active sarcoidosis and 11 patients with inactive sarcoidosis, were evaluated by flow cytometry, western blotting and molecular analysis, to reveal TLA1 and DR3 expression. BAL cell recovery resulted increased in active sarcoidosis patients with respect to inactive sarcoidosis subjects (285,705.0±43,000.0 cells/ml and 127,670.0±37,000.0, respectively; p<0.001). TL1A expression showed a significant increase in active sarcoidosis (20.3% ±1.4 of total CD4+ T cells) with respect to inactive disease (9.4% ±1.7; p<0.01). The results of TL1A gene expression support the phenotypic and proteic data. Even AMs bore TL1A protein. A significantly greater percentage of AMs from BAL of active sarcoidosis expressed TL1A with respect to AMs from patients with the inactive form (15.2% ±0.8 and 7.3%±1.3, p<0.01). Interestingly, DR3 expression paralleled TL1A surfacing, both on T cells and AMs, showing a positive correlation with the activity of the disease. The results support that TL1A/DR3 axis are involved in sarcoid process.

**26. CK2-dependent phosphorylation of actin in acute myelocytic leukemia cells induced to differentiation by retinoic acid**

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Acute promyelocytic leukemia (APL) are a subtype of acute myelogenous leukemia characterized by the translocation t(15;17), which generates PML-RAR $\alpha$  fusion protein; this is an oncogenic molecule that causes retinoic acid (RA)-target promoters silencing. RA, used at pharmacological doses, is able to revert this repression restoring myeloid differentiation. CK2 blockade, achieved by pharmacological inhibition or knock down with specific siRNA, turned out to prevent the differentiation induced by RA in AML cell lines. The aim of this study is to understand the role of CK2 in RA-induced differentiation of these cells. We found that, despite the expression and the catalytic activity of CK2 are unchanged in response to the RA treatment, either in the cytosol or in the nucleus, many proteins, whose phosphorylation change in response to RA, are phosphorylated by CK2. We identified  $\beta$ -actin as a CK2 target in cells treated with RA, and we demonstrated that it is an in vitro substrate of CK2. We also found that actin levels increase in response to RA, both in the cytosol and in the nucleus; however, when CK2 is inhibited, nuclear actin level is lower, suggesting that CK2-dependent phosphorylation is required for actin nuclear localization.

**27. Isoform specific phosphorylation of the N-terminal domain of p53 by protein kinase CK1**

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The tumor suppressor p53 protein has been identified as key signal integrator molecule. Alterations in its phosphorylation status can abolish its function resulting in uncontrolled cell growth. The N-term transactivation domain of p53 doesn't show evident specificity determinants for non-primed phosphorylation by CK1. However, several CK1 isoforms have been shown to phosphorylate some important residues in this region in vitro and in cultured cells. These observations prompted us to assay a series of peptides encompassing residues 1-28 of hp53 protein as substrates for different CK1 isoforms. Phosphorylation assays with radiolabelled ATP show that only the CK1 delta phosphorylates p53 peptides, albeit with low efficiency ( $K_m \approx 2\text{mM}$ ). In the peptide model, S15 alone accounts for about 80% of the whole radioactivity incorporated. In the case of full length hp53 protein, both CK1 alpha and delta are able to phosphorylate p53 with  $K_m$  in the low nM range. In this case the phosphorylation pattern is more complex. Kinetic values suggest the existence of a remote docking site in the full length p53 that seems to be isoform specific. A potential docking site in the CK1 sequence could be located in the exposed basic loop between helices E and F. This hypothesis is supported by the alignment of hCK1 protein sequences and by in silico docking model which underscores the importance of two out of three basic residues within this loop for the interaction with corresponding binding box on p53.

**28. Real-time cAMP dynamics in living hippocampal neurons**

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In the hippocampus, NMDA receptor activation may lead to an increase of cAMP and consequent PKA activation and a large body of evidence indicates that cAMP and the consequent activation of PKA critically regulate synaptic plasticity in the mammalian hippocampus. In a variety of cell systems cAMP has been shown to undergo a tight spatial control and to signal via generation of spatially restricted pools of this second messenger. Such compartmentalization of cAMP is believed to be essential to the generation of appropriate and specific responses to extracellular stimuli. The aim of this work is to study if cAMP compartmentalization may be involved in the regulation of NMDA signaling in primary cultures of hippocampal neurons. For this, we use an optical imaging technique based on FRET, that allows a precise spatial and temporal measurement of cAMP changes in vivo. Our results show that NMDA receptor stimulation induces an increase in cAMP level, both in the soma and in the neurites. In addition, we find that pretreatment with dopamine amplifies, in a time-dependent manner, the rise in cAMP level generated by subsequent NMDA stimulation, in the neurites but not in the soma. Interestingly, activation of other G-protein coupled receptors (e. g. beta-adrenergic or serotonin receptors) or forskolin treatment do not produce the same potentiating effect on the level in cAMP upon subsequent NMDA stimulation as observed with dopamine. These results strongly indicate the existence of distinct cAMP compartments in hippocampal neurons and a differential coupling of NMDA signaling with specific GPCR-activated signaling pathways.

**29. Computational approach to the analysis of structure-function relationship in connexin 26 gap junction channel**

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Connexins are membrane proteins that arrange in hexamers called connexons. Two connexons of opposing cells can dock to form ionic channels. Mutations of connexins are linked to many different disease, such as deafness and Charcot Marie Tooth disease. High resolution determination of the structure of connexins still lacks. At the state of the art the only way to recover informations of the relationship between the structure and the function of the connexins is by computational methods. If, on one hand, in order to model the trans membrane region we can start from an existing alpha carbon model, on the other hand the simulation of the extra cellular domain is complicated by the fact that no hints are given on the position of the backbone of the protein. For this region we will first introduce a coarse grained model, and then discuss the methods to refine it in order to recover full atomistic details.



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